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Award Number: DAMD17-02-1-0029

TITLE: Killing Prostate Cancer Cells and Endothelial Cells with a VEGF-Triggered Cell Death Receptor

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REPORT DATE: June 2005

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. **PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.**

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
01-06-2005	Final	14 JAN 2002 - 13 MAY 2005

4. TITLE AND SUBTITLE

Killing Prostate Cancer Cells and Endothelial Cells with a VEGF-Triggered Cell Death Receptor

5a. CONTRACT NUMBER

5b. GRANT NUMBER
DAMD17-02-1-0029

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of California, San Francisco
San Francisco, CA 94118

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
The goal of this project was to test a novel chimeric cell death receptor (termed R2Fas) that is triggered by vascular endothelial growth factor (VEGF), as a means to kill prostate cancer cells and vascular endothelial cells. The scope of this project involved: (i) creating adenoviral reagents to express the R2Fas receptor in prostate cancer cells and endothelial cells; (ii) determining if the R2Fas receptor kills cells in a VEGF-dependent manner; and (iii) identifying methods for increasing the killing activity of R2Fas. The major findings were: (i) we generated replication-defective adenoviral reagents to express R2Fas as well as control adenoviruses; (ii) we demonstrated that adenoviral-mediated expression of R2Fas in prostate cancer cells that overexpress VEGF activates apoptotic signaling and induces cell death; (iii) we demonstrated that adenoviral-mediated expression of R2Fas in human endothelial cell is non-toxic, but rendered the cells sensitive to killing when treated with VEGF; and (iv) we demonstrated that R2Fas-mediated apoptosis can be potentiated by addition of several pharmacologic agents, including camptothecin, etoposide, taxol, doxorubicin, cisplatin, and BisVIII.

15. SUBJECT TERMS
Prostate Cancer Apoptosis VEGF VEGFR Fas Apoptosis

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
U	U	U	UU	135	**19b. TELEPHONE NUMBER** *(include area code)*

Table of Contents

Page

Introduction…….. 4

Body…….. 5

Key Research Accomplishments…….. 10

Reportable Outcomes…… 11

Conclusion…… 12

References…… 13

Bibliography…… 14

Appendices…… 15

Supporting data…… 15

INTRODUCTION

The purpose of this project was to test a novel chimeric cell death receptor (termed R2Fas) that is triggered by vascular endothelial growth factor (VEGF), as a means to kill prostate cancer cells and vascular endothelial cells in vitro.

The scope of this project involved: (1) creating adenoviral reagents to express the R2Fas receptor in prostate cancer cells and endothelial cells; (2) determining if the R2Fas receptor kills prostate cancer cells and vascular endothelial cells in a VEGF-dependent manner; and (3) identifying methods for increasing the killing activity of R2Fas.

The major findings included: (1) we generated adenoviral reagents to express R2Fas as well as three control adenoviruses; (2) we demonstrated that the adenoviral reagents direct expression of R2Fas or control receptors in DU145 prostate cancer cells and endothelial cells, and we confirmed expression of the receptors by immunoblot; (3) we demonstrated that expression of R2Fas in DU145 cancer cells activates apoptotic signaling and induces apoptosis in a very high percent of cancer cells within 72 hours; (4) we demonstrated that expression of R2Fas in endothelial cells does not induce apoptosis, but does render the cells sensitive to killing by VEGF treatment; and (5) we demonstrated that R2Fas-mediated apoptosis is potentiated by chemotherapeutic and pharmacologic agents.

BODY

Statement of Work

Task 1. Generate replication-defective adenovirus expressing R2Fas and appropriate control adenoviruses.

Task 1A: Subclone cDNAs for R2Fas and control receptors into adenoviral vector.

We used the Adeno-X system from Clontech to generate adenoviruses (AdV). The procedure involves four steps: (i) subclone cDNA for protein of interest into a shuttle vector that contains the CMV promoter; (ii) remove cDNA with CMV promoter and adjacent sequences from shuttle vector, and ligate into an adenoviral vector; (iii) transfect adenoviral vector into 293 HEK cells; and (iv) recover adenovirus and confirm expression of protein of interest in target cells.

To make adenovirus that expresses VEGFR2Fas, the cDNA for VEGFR2Fas (which we had previously constructed) was subcloned into the shuttle vector, and then transferred to the adenoviral vector using standard molecular biology techniques. To make the two control adenoviruses, AdV / LacZ and AdV / CG-VEGFR2Fas, the cDNAs for LacZ and CG-VEGFR2Fas were similarly subcloned into the shuttle and adenoviral vectors. LacZ is the beta-galactosidase gene and is a widely used control protein. CG-VEGFR2Fas is identical to VEGFR2Fas except that it has a C-to-G point mutation in the Fas "death domain" that abolishes Fas apoptotic activity. CG-VEGFR2Fas is the optimal control protein to compare with VEGFR2Fas; when cells are killed by expression of VEGFR2Fas but not by expression of CG-VEGFR2Fas, non-specific killing mechanisms can be ruled out. Both VEGFR2Fas and CG-VEGFR2Fas have a hemagglutinin (HA) epitope tag on the carboxyl tail which we have used to detect expression of the receptors by Western blot.

Adenoviral plasmids with VEGFR2Fas, CG-VEGFR2Fas or LacZ sequences were grown up in DH5α bacteria and the plasmids were purified using Qiagen alkaline lysis maxiprep reagents.

Task 1B: Transfect adenoviral cDNA into 293A cells and recover adenovirus.

Adenoviral plasmids with VEGFR2Fas, CG-VEGFR2Fas or LacZ sequences were transfected into 293 cells by calcium phosphate-mediated transfection. Initially we could not generate significant amounts of AdV / VEGFR2Fas, and we suspected this might occur because the 293 cells were expressing and being killed by VEGFR2Fas before they were able to make the adenovirus. Therefore we revised the protocol to include the caspase inhibitor ZVAD (40 μM) in the 293 medium, which resolved the problem. After 7 – 10 days of culture, typical viral plaques were observed in the 293 cells, indicating adenoviral production. Adenovirus was recovered by freeze/thaw lysis of the 293 cells, and amplified by reinfection and recovery from 293 cells. The viral titer of AdV / VEGFR2Fas was determined to be 6.6 X 10^9 viral particles/mL using an immunohistochemical assay kit from Clontech. The titer of AdV / CG-VEGFR2Fas was 2.2 X 10^9 viral particles/mL, and the titer of AdV / LacZ was 6.3 X 10^9 viral particles/mL.

Task 2. Use adenoviruses to express R2Fas or control viruses in prostate cancer cell lines and primary microvascular endothelial cells.

Task 2A: Transfect human prostate cancer cell lines and human microvascular endothelial cells in vitro with AdV/R2Fas or control adenoviruses.

Task 2B: Confirm that R2Fas protein is expressed in transfected cells by immunoblot techniques.

To confirm that the AdV / R2Fas and AdV / CG-R2Fas viruses did in fact direct expression of the receptors, we initially infected porcine aortic endothelial (PAE) cells and examined cell lysates by Western blot for evidence of receptor expression. Both adenoviruses successfully directed expression of their respective receptors, which were detected at their predicted size of ~140 kD.

We then confirmed that AdV / VEGFR2Fas and AdV / CG-VEGFR2Fas directed expression of their respective receptors in DU145 prostate cancer cells and HUVEC cells. DU145 cancer cells and HUVEC cells were infected with either AdV/R2Fas or AdV/CG-R2Fas at a multiplicity-of-infection (MOI, pfu/cell) of 40. Cells were lysed 48 hours after infection and immunoblotted with antibody against the hemagglutinin (HA) epitope tag present on the carboxyl tail of the receptors. As seen in Figure 1, R2Fas and the control receptor CG-R2Fas were expressed in both DU145 cells and HUVEC cells at similar expression levels. Infection of PC3 prostate cancer cells with AdV / R2Fas produced R2Fas expression levels similar to DU145 cells.

Task 2C: For each prostate cancer cell line and endothelial cell type, characterize expression of VEGF, expression of apoptosis signaling or inhibiting proteins downstream of Fas, and sensitivity to endogenous Fas-mediated apoptosis.

We examined VEGF expression in DU145 cancer cells compared to normal human prostate epithelial cells (Figure 2). Cell lysates were immunoblotted with antibody against VEGF. As seen in Figure 2, DU145 cells over-express VEGF, which confirms published reports. Normal prostate epithelial cells do not have detectable VEGF expression.

We examined the expression levels of apoptosis signaling proteins in DU145 and PC-3 prostate cancer cells (Figure 3). Cell lysates were immunoblotted with antibodies against caspases-8, caspase-3, caspase-9, FADD, Bid, or VEGF. As seen in Figure 3, the expression level of these four proteins was similar in the two cancer cell lines. The equivalent expression of VEGF indicates that PC-3 cells, like DU145 cells, over-express VEGF compared to normal prostate epithelial cells. Both DU145 and PC3 cells were sensitive to stimulation with activating Fas ab CH-11.

Task 3. Determine response of prostate cancer cell lines to expression of R2Fas.

Task 3A: Beginning with prostate cancer cell lines that express high levels of VEGF and are sensitive to Fas-mediated apoptosis, determine if expression of R2Fas stimulates apoptosis, using standard apoptosis assays.

Using AdV/R2Fas virus, we demonstrated that expression of R2Fas in DU145 prostate cancer cells stimulated cleavage/activation of caspase-8, caspase-3, caspase-9, and PARP (Figures 1, 4, 5, and 6). Activation of these apoptotic signals occurred in a time-dependent manner (Figure 5) and a titer-dependent manner (Figure 6). The inactive control receptor CG-R2Fas was expressed at even higher levels than R2Fas but did not activate caspase signaling (Figure 4). We then determined that expression of R2Fas directly stimulated apoptosis in

DU145 cells. In this experiment DU145 cells were infected with AdV/R2Fas at various MOI (2.5 pfu/cell up to 20 pfu/cell), or with the control virus AdV/CG-R2Fas at MOI=20, or left uninfected. Cells were photographed daily. As seen in Figure 7, cells infected with the control virus remained normal and were not distinguishable from uninfected cells. In contrast, cells infected with AdV/R2Fas demonstrated cell fragmentation and cell death, beginning at 48 hours after infection. R2Fas expression induced cell death in a time-dependent and MOI-dependent manner, with virtually all cells killed by an MOI=20 at 72 hours. An MOI as low as 2.5 induced apoptosis in the majority of cells by 106 hours. These results demonstrate that DU145 prostate cancer cells can be killed by adenoviral-mediated expression of R2Fas.

To confirm that R2Fas-mediated apoptosis occurs via activation of caspases, we demonstrated that the pancaspase inhibitor ZVAD blocks R2Fas-mediated apoptosis (Figure 8). In this experiment DU145 cells were infected with AdV/R2Fas at an MOI of 10 or 20, in the presence or absence of ZVAD (40 μM). Cells were photographed and lysed at 48 hours after infection. Lysates were immunoblotted with antibody against cleaved/activated caspase-3. As seen in Figure 8 (top panel), caspase-3 activation induced by R2Fas expression was completely blocked by ZVAD. The lower panel of Figure 8 demonstrates that ZVAD blocked cell death as well, confirming that cell death is mediated via caspase activation.

To prove that R2Fas-mediated apoptosis in DU145 cells is due to VEGF over-expression (and not simply due to expression of a Fas domain-containing transgene), we initially attempted to use RNAi to knock down VEGF expression levels in DU145 cells. However, we were unable to significantly change VEGF protein levels even with prolonged and repeated transfection of anti-VEGF RNAi reagents. Therefore we took the opposite approach and demonstrated that further increasing VEGF expression in DU145 cells potentiated R2Fas-mediated apoptosis (Figures 9-11). DU145 cells were infected with AdV/R2Fas (MOI=10) plus either AdV/VEGF (MOI=10) or AdV/LacZ (MOI=10). Cells were lysed 32 hours after infection, and immunoblotting was performed. As seen in Figure 9, R2Fas expression was similar in cells co-infected with VEGF or LacZ (top panel), and VEGF expression was increased in cells infected with AdV/VEGF (middle panel). The bottom panel demonstrates that increased expression of VEGF stimulated increased cleavage/activation of caspase-3. Figure 10 shows a similar experiment in which DU145 cells were infected with AdV/R2Fas + AdV/VEGF, or AdV/R2Fas + AdV/LacZ. Additional controls were cells infected with AdV/LacZ or AdV/VEGF. As seen in Figure 10, cells co-expressing R2Fas and VEGF apoptosed at a significantly higher level than cells expressing R2Fas alone.

In a further experiment, we compared cell death in DU145 cells co-infected with AdV/R2Fas and AdV/VEGF, but in this experiment we varied the order of infection (Figure 11). The first group was infected with both viruses simultaneously. The second group was infected with AdV/R2Fas on day 1, and AdV/VEGF or AdV/LacZ on day 2. The third group was infected with AdV/VEGF or AdV/LacZ on day 1, and AdV/R2Fas on day 2. When examined at 72 hours, all cells that were co-infected with AdV/R2Fas + AdV/VEGF showed increased cell death compared to cells infected with AdV/R2Fas + AdV/LacZ, and the effect was most pronounced in cells infected with AdV/VEGF simultaneous with or after AdV/VEGF. These experiments demonstrate that increased expression of VEGF in DU145 cells potentiated R2Fas-mediated apoptosis. These results rule out the possibility that R2Fas-mediated apoptosis is due to non-specific activation of Fas domain-mediated apoptosis.

We examined the response of PC-3 cells to R2Fas expression (Figure 12). PC-3 cells were infected with AdV/R2Fas or AdV/CG-R2Fas control virus at an MOI=30 or 60 and

photographed at 56 and 128 hours. Cells expressing R2Fas were decreased in number compared to uninfected cells or cells expressing the control receptor CG-R2Fas (Figure 12). We were not able to express R2Fas in LNCaP prostate cancer cells despite many efforts. These cells may not express sufficient levels of the surface protein needed for adenoviral infection.

We examined the response of normal human prostate epithelial cells to R2Fas expression, and hypothesized that these cells would be resistant to killing by R2Fas expression because they do not express VEGF (see Figure 2). Cells were infected with AdV/R2Fas or the control virus AdV/CG-R2Fas at an MOI of 10 or 20. As seen in Figure 13, both receptors were expressed in normal human prostate epithelial cells. Notably, these cells do not undergo apoptosis when R2Fas is expressed (Figure 14). This result is important because it suggests that R2Fas-mediated apoptosis is restricted to cells that overexpress VEGF.

Task 3B: If cells are not immediately killed by expression of VEGFR2Fas, determine if they are made sensitive to killing by exogenous VEGF.

Task 3B: Examine the response of other prostate cancer cell lines that express lower levels of VEGF or demonstrate resistance to Fas pathway-mediated apoptosis, to identify which factors may influence response to VEGFR2Fas.

Task 4A: Confirm that expression of R2Fas in the absence of VEGF does not kill endothelial cells.

We found in preliminary experiments that adenoviral-mediated expression of R2Fas in porcine aortic endothelial cells did not stimulate apoptosis. We extended these results to HUVEC cells (Figure 15). HUVEC cells were infected with AdV/R2Fas or the control AdV/CG-R2Fas at an MOI=40. Expression of the receptors is seen in the top panel of Figure 1. Expression of R2Fas had no apparent effect on the growth or morphology of HUVEC cells, and specifically did not induce apoptosis (third panel, Figure 15). Expression of R2Fas in HUVEC did produce slight cleavage/activation of caspase-3 (Figure 1, middle panel), but no activation of caspase-9 (Figure 1, lower panel). These results demonstrated that HUVEC cells, which do not express VEGF, can express R2Fas at high levels without toxicity <u>if VEGF is not present</u>.

Task 4B: Determine if expression of R2Fas renders endothelial cells sensitive to killing by VEGF.

Our hypothesis is that expression of R2Fas in endothelial cells will cause them to die by apoptosis <u>when treated with VEGF</u>. We found that HUVEC cells expressing R2Fas did in fact undergo apoptosis when treated with VEGF (Figure 15, right panel). In this experiment, HUVEC cells were infected with AdV/R2Fas at an MOI=40. One hundred hours after infection cells expressing R2Fas appeared normal. Cells were then treated with VEGF-165 (1 nM for 24 hours). As seen in the right panel, VEGF treatment induced cell death and fragmentation. This demonstrates that expression of R2Fas renders endothelial cells killable by VEGF treatment.

A likely scenario for a preclinical test of R2Fas as an anti-angiogenic therapy would involve injection of AdV/R2Fas into a tumor. In this setting, R2Fas expression in endothelial cells would occur in a tissue microenvironment already containing VEGF secreted by the tumor. This is different from the experiments we have done in vitro, in which AdV/R2Fas infection and subsequent R2Fas expression occur in the absence of VEGF, and VEGF is then added later. To better approximate the in vivo situation, we asked if AdV/R2Fas expression would still induce

apoptosis in endothelial cells if VEGF was already present in the cell culture medium (Figure 16). In this experiment porcine aortic endothelial cells were infected with AdV/R2Fas at an MOI=20 in medium containing escalating levels of VEGF-165, from 0 nM to 1 nM. We found that expression of R2Fas induced apoptosis when VEGF was present at 1 nM. This result more closely approximates an in vivo setting, and indicates that R2Fas expression can stimulate endothelial apoptosis when VEGF is continuously present.

Task 5. Determine if pharmacologic agents and co-expression of apoptosis effector proteins potentiate R2Fas-mediated apoptosis.

Task 5A:Treat prostate cancer cells and endothelial cells expressing R2Fas with IFNγ, TNFα, cisplatin, actinomycin D, camptothecin or BIS VIII to determine if these agents potentiate the apoptotic activity of R2Fas.

One way to enhance the apoptotic activity of R2Fas is to add pharmacologic agents that may potentiate Fas-mediates apoptotic signaling. We screened six agents on DU145 cells to determine the highest concentration of each drug that did not induce overt cell toxicity. We then combined that drug concentration with AdV/R2Fas infection to determine if the drug potentiated R2Fas-mediated apoptosis. We specifically chose drug concentrations that did not kill DU145 cells on their own so that we would be able to detect enhanced R2Fas-mediated apoptosis. The drug concentrations we used were:

BisVIII	1 μM
Camptothecin	0.05 μM
Etoposide	0.5 μM
Taxol	0.5 nM
Doxorubicin	1.0 μg/mL
Cisplatin	1.0 μg/mL

DU145 cells were pretreated for 12 hours with each of the drugs at the indicated concentrations, and then infected with AdV/R2Fas at an MOI=10. (This relatively low MOI was chosen so that potentiation by the drugs could be detected. In preliminary experiments, a higher MOI induced apoptosis so rapidly that the drug effects were difficult to determine). As seen in Figure 17, all six agents were non-toxic at the concentrations used (top photos). All six agents potentiated R2Fas-mediated apoptosis compared to cells that received R2Fas and vehicle (bottom photos). The extent of apoptosis potentiation was quite remarkable, with virtually complete cell death in cells expressing R2Fas and treated with camptothecin or doxorubicin. These findings are important because they suggest that combining non-cytotoxic drug treatment with R2Fas expression will greatly enhance R2Fas apoptotic activity. In PC3 cells, these agents potentiated the activity of R2Fas but to a lower extent than DU145 cells.

Task 5B: Use adenovirus-mediated gene transfer to co-express R2Fas with downstream effector caspases caspase-8 or caspase-3 in prostate cancer cells and endothelial cells. Determine if co-expression makes cells more sensitive to killing by VEGF.

We attempted to generate replication-defective adenoviruses overexpressing caspase-8 or caspase-3 but were not successful. The reason is not clear but may be due to autoactivation of these apoptosis signaling pathways when overexpressed in the adenoviral packaging cells.

KEY RESEARCH ACCOMPLISHMENTS

a. Created replication-defective adenoviruses that express the R2Fas chimeric death receptor, and control adenoviruses that express the inactive control receptor CG-R2Fas or LacZ.
b. Demonstrated adenoviral-directed expression of R2Fas or control receptor CG-R2Fas in DU145 and PC3 prostate cancer cells and in human endothelial cells.
c. Demonstrated expression of apoptotic signaling proteins in DU145 and PC3 prostate cancer cells.
d. Demonstrated that expression of the R2Fas chimeric death receptor in DU145 and PC3 cancer cells activates apoptotic signaling in a time- and titer-dependent manner.
e. Demonstrated that expression of the R2Fas chimeric death receptor, but not the inactive control receptor CG-R2Fas, stimulates apoptosis in DU145 prostate cancer cells.
f. Demonstrated that R2Fas-mediated apoptosis is potentiated by treatment of cells with: BisVIII, camptothecin, etoposide, taxol, doxorubicin, and cisplatin.

REPORTABLE OUTCOMES

a. The replication-defective adenoviruses expressing the R2Fas chimeric death receptor and the inactive control receptor CG-R2Fas will be shared with other interested investigators.
b. An abstract and poster describing this work was presented at the American Association for Cancer Research - NIH - EORTC International Conference on Molecular Targets and Cancer Therapeutics, November 2003 in Boston.
c. Our work was highlighted in a press release and press conference from the AACR Conference in November 2003.
d. An abstract and poster describing this work was presented at the Keystone Conference on Angiogenesis: Novel Basic Science Insights and Human Therapy, January 2004 in Santa Fe.
e. An abstract and poster describing this work was presented at the American Association for Cancer Research Annual Meeting, April 2005 in Anaheim.
f. A patent titled "VEGF-activated Fas Ligands" was filed by the University of California on 8/15/2006, International Application Number: PCT/US2006/031991
g. A manuscript describing this work is in preparation.
h. Grants funded based on work supported by this award:
    DOD Breast Cancer Research Program, Concept Award, awarded May 2004
    Prostate Cancer Foundation grant, awarded January 2006
    Prostate Cancer Foundation grant, awarded January 2007
    Tobacco-Related Disease Research Program, Univ. of Cal. grant, awarded July 2007

CONCLUSIONS

In summary, we have generated novel replication-defective adenoviral reagents that direct expression of the VEGF-triggered chimeric death receptor R2Fas, and the inactive control receptor CG-R2Fas. We have used these adenoviral reagents to express the R2Fas chimeric death receptor and the CG-R2Fas inactive control receptor in DU145 and PC3 prostate cancer cells and human endothelial cells, and we have demonstrated that expression of R2Fas activates caspase signaling. Most importantly, we have demonstrated that expression of the R2Fas chimeric death receptor in DU145 cells stimulates extensive killing, which can be further potentiated by combined treatment with existing chemotherapeutic agents. This may be the first demonstration that a growth factor secreted by a cancer cell can be forced to act as a death factor by activating an engineered receptor. We have further found that expression of the R2Fas receptor in endothelial cells does not induce apoptosis, but does render the cells sensitive to killing by treatment with VEGF. These findings suggest that expression of the R2Fas chimeric death receptor in prostate cancer cells and tumor blood vessels may be a feasible and novel form of combined tumor and antiangiogenesis therapy.

REFERENCES

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Bibliography

Abstracts:

Title: Killing cancer cells and endothelial cells with a VEGF-triggered cell death receptor
Authors: Timothy Quinn, Carlos San Mateo, and Adrian Padron
Presented at: American Association for Cancer Research-NIH-EORTC International Conference on Molecular Targets and Cancer Therapeutics, November 2003 in Boston.

Vascular endothelial growth factor (VEGF-A) is a dimeric angiogenic factor that is overexpressed by many cancer cells. VEGF produced by tumors is a major mediator of tumor angiogenesis. Consequently, numerous approaches to inhibit VEGF expression or activity are under investigation as anti-angiogenic cancer therapies, including neutralizing antibodies, soluble receptors, ligand traps, small molecule kinase inhibitors, and RNA-targeted agents. While some agents have recently been shown to have efficacy in clinical trials, it is too early to know if anti-VEGF therapies will prove to be highly effective, free of resistance, and without significant side effects.

Given the central role of VEGF in tumor angiogenesis, we have investigated an alternative approach that is not based on inhibition of VEGF activity, but instead converts VEGF from an angiogenic growth and survival factor into a death factor. In essence our goal is to turn tumor-derived VEGF into a weapon against the tumor itself and/or its blood vessels.

We created a chimeric receptor (termed VEGFR2Fas) by fusing the extracellular, VEGF-binding domain and the transmembrane domain of VEGFR-2 (also known as Flk-1/KDR) to the cytoplasmic "death domain" of the Fas apoptosis receptor (CD95/Apo-1). As a control, we also generated an inactive receptor with no apoptotic activity (CG-VEGFR2Fas) by incorporating a previously described inactivating point mutation into the Fas death domain (lprcg mutation).

Using retroviral or adenoviral gene transfer techniques we expressed VEGFR2Fas or the control CG-VEGFR2Fas receptor in endothelial cells and cancer cell lines in vitro. When expressed in porcine aortic endothelial (PAE) cells, VEGFR2Fas had no apoptotic effect in the absence of VEGF. When VEGF was added, cells were rapidly killed by activation of caspase-mediated apoptotic signaling. Cells expressing the control CG-VEGFR2Fas receptor were unaffected by exposure to VEGF. These results demonstrate that VEGFR2Fas is a VEGF-triggered cell death receptor. Further investigation demonstrated that VEGF stimulated formation of hetero-oligomeric receptor complexes composed of VEGFR2Fas and Neuropilin-1 (Np-1), a non-kinase receptor for VEGF. Engagement of Np-1 into a complex with VEGFR2Fas significantly potentiated the apoptotic activity of VEGFR2Fas, which was demonstrated using a peptide that blocked binding of VEGF to Np-1, and by comparing the relative apoptotic activities of different VEGF isoforms that do or do not bind to Np-1.

To determine if expression of VEGFR2Fas induced apoptosis in cancer cells that overexpress VEGF, we used adenovirus to transiently express VEGFR2Fas or the control CG-VEGFR2Fas in DU145 human prostate cancer cells. DU145 cells infected with AdV/VEGFR2Fas (MOI ~25-75) began to express VEGFR2Fas by 24 hours and underwent caspase-mediated apoptosis beginning at 48 hours. Expression of the control CG-VEGFR2Fas receptor had no effect.

Our results demonstrate that VEGF can be transformed from a growth factor into a cell death factor by engaging the artificial chimeric death factor VEGFR2Fas along with the Np-1 receptor.

Further investigation will identify how this effect can be optimized and will determine the feasibility of using this approach in vivo to directly kill tumor cells and/or their blood vessels.

Title: Conversion of VEGF from a Growth Factor into a Death Factor
Authors: Timothy Quinn, Carlos San Mateo, Scott Soifer, and Adrian Padron
Presented at: Keystone Conference on Angiogenesis: Novel Basic Science Insights and Human Therapy, January 2004 in Santa Fe

Vascular endothelial growth factor (VEGF-A) is a dimeric angiogenic factor that is overexpressed by many cancer cells and is a major mediator of tumor angiogenesis. Consequently, numerous approaches to inhibit VEGF expression or activity are under investigation as anti-angiogenic cancer therapies, including neutralizing antibodies, soluble receptors, ligand traps, small molecule kinase inhibitors, and RNA-targeted agents. It is not yet known if anti-VEGF therapies will prove to be highly effective, free of resistance, and without significant side effects.

Given the central role of VEGF in tumor angiogenesis, we have investigated an alternative approach that is based not on inhibition of VEGF activity, but instead converts VEGF from a growth and survival factor into a death factor. In essence our goal is to turn tumor-derived VEGF into a weapon against the tumor itself and/or its blood vessels.

We created a chimeric receptor (VEGFR2Fas) by fusing the extracellular, VEGF-binding domain and the transmembrane domain of VEGFR-2 to the cytoplasmic "death domain" of the Fas apoptosis receptor (CD95/Apo-1). As a control, we generated a receptor with no apoptotic activity (CG-VEGFR2Fas) by incorporating a previously described inactivating point mutation into the Fas death domain (lprcg mutation).

Using retroviral or adenoviral vectors we expressed VEGFR2Fas or the control CG-VEGFR2Fas receptor in endothelial cells and cancer cell lines in vitro. When expressed in porcine aortic endothelial (PAE) cells, VEGFR2Fas had no apoptotic effect in the absence of VEGF. But when VEGF was added, cells were rapidly killed by activation of caspase-mediated apoptotic signaling. Cells expressing the control receptor were unaffected by VEGF. These results demonstrate that VEGFR2Fas is a VEGF-triggered cell death receptor. Further investigation showed that VEGF stimulated formation of hetero-oligomeric receptor complexes composed of VEGFR2Fas and Neuropilin-1 (Np-1), a non-kinase receptor for VEGF. Engagement of Np-1 into a complex with VEGFR2Fas significantly potentiated the apoptotic activity of VEGFR2Fas.

To determine if expression of VEGFR2Fas induced apoptosis in cancer cells that overexpress VEGF, we used adenovirus to transiently express VEGFR2Fas or the control CG-VEGFR2Fas in DU145 human prostate cancer cells. DU145 cells infected with AdV/VEGFR2Fas began to express VEGFR2Fas by 24 hours and underwent caspase-mediated apoptosis beginning at 48 hours. Expression of the control CG-VEGFR2Fas receptor did not stimulate apoptosis.

Our results demonstrate that VEGF can be transformed from a growth factor into a cell death factor by engaging the artificial chimeric death receptor VEGFR2Fas along with the Np-1 receptor. Further investigation will determine the feasibility of using this approach in vivo to directly kill tumor cells that overexpress VEGF and/or their blood vessels.

Supported by grants from American Cancer Society and DOD (DAMD17-02-1-0029).

Title: Killing cancer cells and endothelial cells with a VEGF-triggered cell death receptor
Authors: Timothy Quinn, Carlos San Mateo, and Adrian Padron
Presented at: American Association for Cancer Research Annual Meeting, April 2005 in Anaheim

Vascular endothelial growth factor (VEGF) is an angiogenic factor that is overexpressed by many cancers and is a key mediator of tumor angiogenesis. Consequently, many agents designed to inhibit VEGF are under investigation as anti-angiogenic cancer therapies. It is not yet known if anti-VEGF therapies will prove to be highly effective, free of resistance, and without significant side effects.

We are investigating a completely different approach to targeting VEGF that is not based on inhibiting VEGF, but instead converts VEGF from an angiogenic growth factor into a cell death factor. Our goal is to turn tumor-derived VEGF into a weapon against the tumor itself and its blood vessels.

We created a chimeric receptor (termed R2Fas) by fusing the extracellular domain of VEGFR-2 (Flk-1/KDR) to the cytoplasmic death domain of the Fas apoptosis receptor (CD95/Apo-1). As a control, we generated a receptor with no apoptotic activity (CG-R2Fas) by incorporating an inactivating point mutation into the Fas death domain. We hypothesized that expression of R2Fas in endothelial cells would make VEGF act as an endothelial death factor, and expression of R2Fas in VEGF-overexpressing cancer cell lines would induce apoptosis.

We used retroviral and adenoviral vectors to express R2Fas or the control receptor in endothelial and cancer cell lines in vitro, and performed viability and apoptosis assays (cell counts, caspase activation, oligonucleosome generation, and LDH release). When expressed in porcine aortic endothelial or HUVEC cells, R2Fas had no effect in the absence of VEGF. When VEGF was added, cells were rapidly killed by activation of caspase-mediated apoptotic signaling. Cells expressing the control receptor were unaffected by VEGF, demonstrating that R2Fas is a VEGF-triggered cell death receptor. To determine if R2Fas expression induced apoptosis in human cancer cells that overexpress VEGF, we used U87MG glioblastoma cells and DU145 prostate cancer cells. In both cell lines R2Fas expression induced rapid apoptosis in a time- and adenoviral titer-dependent manner. Control experiments confirmed that apoptosis was due to activation of R2Fas by overexpressed VEGF: (1) no killing by the CG-R2Fas control receptor; (2) inhibition of R2Fas-induced apoptosis by ZVAD, VEGF RNAi, or neutralizing VEGF antibody; and (3) no killing by a chimeric Fas receptor with the Fms extracellular domain. R2Fas-induced apoptosis was potentiated by chemotherapeutic agents including etoposide, cisplatin, and doxorubicin.

Further investigation showed that VEGF stimulated formation of receptor complexes composed of R2Fas and Neuropilin-1 (Np-1), a non-kinase receptor for VEGF. Engagement of Np-1 into a complex with R2Fas significantly potentiated the apoptotic activity of R2Fas, perhaps by inducing R2Fas clustering.

Our results suggest that targeted expression of R2Fas in tumor endothelium and/or tumor cells may yield a new approach to cancer therapy. Supported by DOD (DAMD17-02-1-0029).

Personnel:
Timothy P. Quinn, M.D.

APPENDICES

1. Press release from the AACR conference in November 203 highlighting our work.
2. Patent application

SUPPORTING DATA

Figures are after appendices at end.

AACR-NCI-EORTC International Conference
Molecular Targets and Cancer Therapeutics:
Discovery, Biology, and Clinical Applications
November 17-21, 2003 • Hynes Center, Boston, MA

EMBARGOED FOR RELEASE
NOVEMBER 20, 2003 2:00 P.M. EST

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Scientists Report on Promising New Approaches to Induce Cancer Cell Suicide

BOSTON (November 20, 2003) – In cancer, the normal process by which cells die is defective. Researchers are reporting today on three new approaches to activate cancer cells' ability to commit suicide. In one set of studies, scientists employed a series of molecular and gene therapy tricks to convert an angiogenic protein used by tumors to promote their growth into a cancer cell killer. In separate study, scientists used a fragment of genetic material to disable a protein used by many tumor types as an "off switch" to prevent cell death, triggering cancer cell suicide. A third study focused on a novel inhibitor to improve responses to central nervous system tumor therapy. The findings were presented today at the International Conference on Molecular Targets and Cancer Therapeutics organized by the American Association for Cancer Research (AACR), National Cancer Institute (NCI) and European Organisation for Research and Treatment of Cancer (EORTC) in Boston.

Antisense inhibition of survivin expression as a cancer therapeutic: Abstract 324
Scientists have inhibited cancer in mice using a synthetic fragment of genetic material to block production of a protein that many tumor types use to survive. Researchers at Eli Lilly and Company reported the studies of the compound, LY2181308, which is directed against a molecular target called survivin.

Survivin belongs to a family of proteins, called Inhibitor of Apoptosis Proteins, which play a key role in the regulation of apoptosis and cell division. The protein is expressed in a majority of human cancers but not in normal adult tissues, making it a potential target for cancer therapies. LY2181308 is an anti-sense oligonucleotide that potently downregulated survivin expression in human cancer cells derived from lung, colon, breast, prostate, ovary, cervix, skin and brain.

"We believe that the use of antisense molecules against targets such as survivin are a viable option for the treatment and management of cancer patients, either as a single agent or in combination with chemotherapeutic agents," said Bharvin Patel, Ph.D., of Eli Lilly, and lead investigator of the study.

In the mouse studies, the researchers tested LY2181308 against survivin in xenograft tumor model using human melanoma cells implanted under the skin. Intravenous administration of the survivin antisense molecule LY2181308 to tumor-bearing nude mice significantly inhibited tumor growth compared to animals treated with saline or mis-match control oligonucleotide. Furthermore, they demonstrated that this anti-tumor activity was associated with significant inhibition of survivin expression in these xenograft tumors. Other studies are currently assessing the activity of LY2181308 and chemotherapeutic agents in the cancer model.

Killing cancer cells and endothelial cells with a VEGF-triggered cell death receptor: Abstract 407

Scientists today presented new research on inducing cancer cell suicide. Tumors stimulate new blood vessel growth in order to acquire oxygen and nutrients, a process called tumor angiogenesis, by secreting an angiogenic growth factor called VEGF. Researchers from the University of California, San Francisco today presented a novel treatment approach using the tumor's own weapon against itself by forcing VEGF to act as a cell death factor instead of a growth factor.

VEGF normally works by attaching to the extracellular region of VEGF receptor 2, which activates the intracellular region of the receptor to send growth signals. The research team created an artificial VEGF receptor, called R2Fas, in which the intracellular region of VEGF receptor 2 was replaced with a part of the Fas death receptor, which can trigger a process of cellular suicide termed apoptosis.

Blood vessel cells in culture normally grow when exposed to VEGF. When the R2Fas receptor was expressed in blood vessel cells, the cells were instead rapidly killed by VEGF, showing that VEGF acted as a death factor instead of a growth factor. When the R2Fas receptor was expressed in cancer cells in culture that over-express VEGF, the R2Fas receptor caused the cells to die by apoptosis.

"The ability of the R2Fas receptor to switch the function of VEGF from a growth factor to a death factor may allow a new approach to anti-angiogenesis by simultaneously targeting both the VEGF-producing cancer cells and the tumor blood vessels," said Tim Quinn, M.D., assistant professor at the University of California, San Francisco Pediatrics Department. "Animal studies in which R2Fas will be expressed in tumors and tumor blood vessels will determine the feasibility of this approach."

ZD6474, a vascular endothelial growth factor reception 2 (VEGFR-2) inhibitor, inhibits growth of multiple primary central nervous system tumor types:

Abstract 709

Researchers have shown that a new and experimental drug – ZD6474 -- strongly inhibited the growth of three deadly brain tumors in animals. In the study, conducted at Duke University Medical Center, ZD6474 inhibited the growth of three types of human tumors grown in mice -- glioblastoma, medulloblastoma, and ependymoma – a remarkable finding, given that brain tumors are very distinct in their biologic makeup.

"Despite our best efforts in the laboratory and the clinic, the survival rate for glioblastoma – the most common and lethal brain tumor -- hasn't changed in ten years," said Jeremy Rich, M.D., assistant professor in the Brain Tumor Center at Duke. "This new drug candidate has demonstrated great promise in treating human tumors that were grown in mice, and we feel these results are indicative of how the drug may act in humans."

Mice that received ZD6474 showed a 10- to 25-day delay in growth in all of the tumors compared to control mice. The drug worked by blocking tumor angiogenesis, the process by which tumor cells grow new blood vessels. Specifically, ZD6474 blocked activation of the receptor for vascular endothelial growth factor (VEGF). VEGF is a protein that malignant cells secrete in order to grow and maintain their blood vessels. When the VEGF receptor is not activated, the tumor's blood supply is diminished and the tumor shrinks and slows its spread.

Secondarily, ZD6474 prevented activation of the EGF (epidermal growth factor) receptor, another protein that cells use to grow new blood vessels, to resist dying, and to invade other cells. Additionally, ZD6474 showed promise in treating tumors that had genetically manipulated themselves to resist the effects of toxic chemotherapy.

###

Founded in 1907, the American Association for Cancer Research is a professional society of more than 21,000 laboratory, translational, and clinical scientists engaged in cancer research in the United States and in more than 60 other countries. AACR's mission is to accelerate the prevention and cure of cancer through research, education, communication, and advocacy. This work is carried out through five major peer-reviewed scientific journals and high-quality scientific programs focusing on the latest developments in all areas of cancer research.

The National Cancer Institute, founded in 1971, is the principal United States government agency charged with coordinating the National Cancer Program. It facilitates international cooperation in clinical trials involving U.S. and foreign collaborating institutions.

The European Organisation for Research and Treatment of Cancer was organized in 1962 to conduct, develop, coordinate and stimulate laboratory and clinical research in Europe, and to improve the management of cancer and related problems by increasing the survival and quality of life for patients.

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
22 February 2007 (22.02.2007)

PCT

(10) International Publication Number
WO 2007/022273 A2

(51) International Patent Classification:
C07K 14/705 (2006.01)

(21) International Application Number:
PCT/US2006/031991

(22) International Filing Date: 15 August 2006 (15.08.2006)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/708,723 15 August 2005 (15.08.2005) US

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LV, LY, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

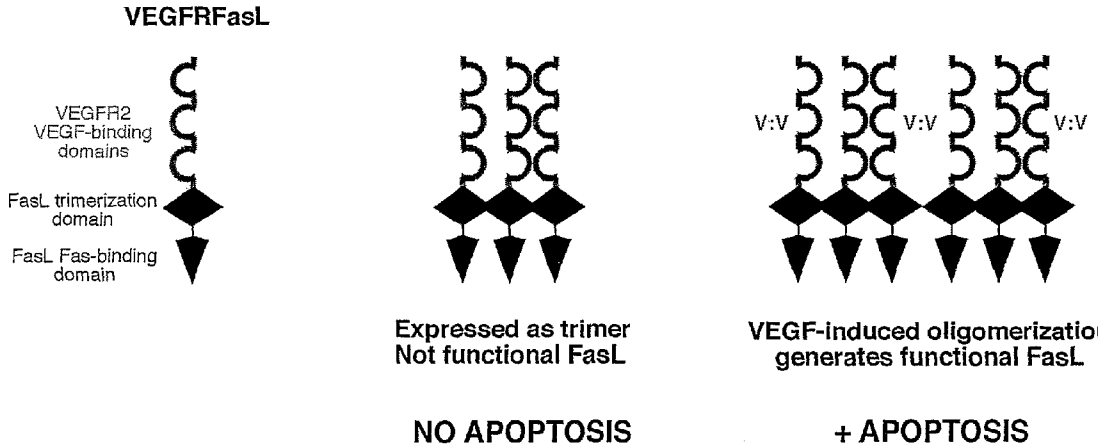
(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Declaration under Rule 4.17:
— *of inventorship (Rule 4.17(iv))*

Published:
— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: VEGF-ACTIVATED FAS LIGANDS



(57) Abstract: The present invention provides fusion proteins comprising an extracellular domain of a VEGF receptor and a death ligand. The fusion proteins bind to VEGF and to death receptors on tumor cells thereby inhibiting VEGF activation of VEGF receptors and inducing apoptosis in the tumor cells. Fusion proteins of the present invention are useful for inducing apoptosis and cytotoxic effects in cells, treating cancer and diseases or disorders related to unregulated angiogenesis and/or vasculogenesis. Thus, this invention further provides methods for treating angiogenesis related diseases using the fusion proteins, polynucleotides encoding the fusion proteins, vectors containing the polynucleotides, pharmaceutical compositions and kits containing the fusion proteins or the polynucleotides encoding the fusion proteins.

WO 2007/022273 A2

VEGF-ACTIVATED FAS LIGANDS

CROSS-REFERENCES TO RELATED APPLICATIONS

[0001] This application claims the benefit of provisional application Ser. No. 60/708,723, filed 08/15/2005, the disclosures of which is incorporated in its entirety herein by reference.

FIELD OF THE INVENTION

[0002] The present invention relates compositions and methods useful for treating diseases and disorders, including cancers, related to unregulated angiogenesis and/or vasculogenesis. More specifically, the present invention provides fusion proteins comprising an extracellular domain of a VEGF receptor and a death ligand useful for treatment of cancers and disorders such as rheumatoid arthritis, macular degeneration and psoriasis.

STATEMENT AS TO RIGHTS TO INVENTIONS MADE UNDER FEDERALLY SPONSORED RESEARCH AND DEVELOPMENT

[0003] This work was supported by grants from the Prostate Cancer Research Program (DAMD17-02-1-0029) and Breast Cancer Research Program, (W81XWH-04-1-0745 BC032859) of the Department of Defense.

BACKGROUND OF THE INVENTION

[0004] Angiogenesis is the process of developing new blood vessels that involves the proliferation, migration and tissue infiltration of capillary endothelial cells from pre-existing blood vessels. Angiogenesis is important in normal physiological processes including embryonic development, follicular growth, and wound healing as well as in pathological conditions involving tumor growth and non-neoplastic diseases involving abnormal neovascularization, including neovascular glaucoma (Folkman and Klagsbrun, *Science* (1987), 235:442-447).

[0005] The link between angiogenesis and cancer is well established. Neovascularization is an important step in the transition from hyperplasia to neoplasia and it must occur for tumors to grow beyond 2-3 mm in diameter and for tumor metastasis (Folkman, Nat Med (1995), 1:27-30; reviewed in Bouck *et al.*, *Adv in Cancer Res* (1996), 69:135-174). A

correlation between microvessel density and severity of disease has been observed in a number of different tumor types including malignant glioma (Plate & Risau, GLIA (1995), 15:339-347), and breast (Horak *et al.*, *Lancet* (1992), 340:1120-124), bladder (Dickinson *et al.*, *Br J Urol* (1994), 74:762-766), colon (Takahashi *et al.*, *Cancer Res* (1995), 55:3964-3968), and endometrial cancer (Kirschner *et al.*, *Am J Obstet Gynecol* (1996), 174:1879-1882).

[0006] Other than cancer, a number of serious diseases are associated with persistent, unregulated angiogenesis. These diseases are dominated by abnormal neovascularization. Included in the diseases in which unregulated angiogenesis is present are endometriosis, ocular disease (e.g., macular degeneration), psoiaris, and rheumatoid arthritis. Arthritis is a serious health care problem. Progressive arthritic conditions in humans cause severe pain, loss of joint mobility and disfigurement, and an overall reduction in the quality of life. In rheumatoid arthritis, the synovium hyperproliferates (aided by new blood vessels) and invades the cartilage which is destroyed.

[0007] Suppression of angiogenesis would inhibit the formation of new vessels and therefore affect tumor growth and generation of metastases. Indeed, it has been estimated that the elimination of a single endothelial cell could inhibit the growth of 100 tumor cells (Thorpe *et al.*, *Breast Cancer Research and Treatment* (1995), 36:237-251). Inhibition of new capillary formation could lessen the joint destruction that occurs in rheumatoid arthritis and halt disease progression.

[0008] So far, several angiogenic factors have been identified (reviewed in Folkman, *Nat Med* (1995), 1:27-30; Hanahan *et al.*, *Cell* (1996), 86:353-364), including the particularly potent vascular endothelial growth factor (VEGF), also known as VPF or vasculotropin (reviewed in Ferrara, *Trends Cardiovasc Med* (1993), 3:244-250; Ferrara and Davis-Smyth, *Endocrine Rev* (1997), 18:4-25). Unlike other angiogenic factors, VEGF acts as an endothelial cell-specific mitogen during angiogenesis (Terman *et al.*, *Biochem Biophys Res Commun* (1992), 187:1579-1586 and Ferrara, *Trends Cardiovasc Med* (1993), 3:244-250). Antibodies raised against VEGF have been shown to suppress tumor growth *in vivo* (Kim *et al.*, *Nature* (1993), 362:841-844), indicating that VEGF antagonists could have therapeutic applications as inhibitors of tumor-induced angiogenesis.

[0009] VEGF is secreted and by a number of human tumor cell lines in culture, including glioma (Tsai *et al.*, *J Neurosurg* (1995), 82:864-867), melanoma (Claffey *et al.*, *Cancer Res*

(1996), 56:172-181), gastric cancer cells (Zhang *et al.*, *World J Gastroenterol* (2002), 8(6):994-8), Kaposi sarcoma, and epidermoid carcinoma cells (Myoken *et al.*, *Proc Natl Acad Sci USA* (1991), 88:5819-5823). More importantly, VEGF transcripts or protein has been identified by in situ hybridization or immunohistochemistry in primary gliomas (Plate, *et al.*, *Lab Invest* (1992), 67:529-534; Plate *et al.*, *Int J Cancer* (1994), 59:520-529), hemangioblastomas (Hatva *et al.*, *Amer J Pathol* (1996), 148:763-775) and breast (Toi *et al.*, *Jpn. J Cancer Res* (1994), 85:1045-1049; Anan *et al.*, *Surgery* (1996), 119:333-339; Yoshiji *et al.*, *Cancer Res* (1996), 56:2013-2016), colon (Brown *et al.*, *Cancer Res* (1993), 53:4727-4735; Takahashi *et al.*, *Cancer Res* (1995), 55:3964-3968) and renal cell tumors (Takahashi *et al.*, *Cancer Res* (1994), 54:4233-4237). In glioblastoma, the message for VEGF is found in cells adjacent to necrotic regions which is consistent with upregulation by hypoxia (Shweiki *et al.*, *Nature* (1992), 359, 843-845; Plate *et al.*, *Lab Invest* (1992), 67:529-534). A marked increase of VEGF mRNA and protein was reported in pituitary tumors (McCabe *et al.*, *J Clin Endocrinol Metab* (2002), 87(9):4238-44) and in melanoma xenografts (Graells *et al.*, *J Invest Dermatol* (2004), 123(6):1151-61). Furthermore, patients with cancer have significantly higher serum VEGF levels than normal volunteers. The highest VEGF concentrations were observed in patients with untreated metastatic cancers.

[0010] VEGF was purified initially from the conditioned media of folliculostellate cells and from a variety of tumor cell lines (Ferrara *et al.*, *Biochem Biophys Res Commun* (1989), 161:851-858; Plouet *et al.*, *EMBO J* (1989), 8:3801-3806). VEGF is a homodimeric glycoprotein consisting of two 23 kD subunits and typically binds as a dimeric polypeptide to its receptors. The human gene encoding VEGF is organized into eight exons, separated by seven introns. Alternative splicing of mRNAs for the VEGF gene results in the generation of five different molecular species, having 121, 145, 165, 189, or 206 amino acid residues in the mature monomer (Tisher *et al.*, *J Biol Chem* (1991), 266:11947-11954; Houck *et al.*, *Mol Endocrinol* (1991), 5:1806-1814. Only $VEGF_{165}$, which lacks the residues encoded by exon 6, is the mature and active form of VEGF. It binds to heparin and cell surface heparin sulfate proteoglycans, and can be expressed as a free or as a cell membrane bound form (Houck *et al.*, 1992). $VEGF_{206}$ and $VEGF_{189}$ are membrane bound forms. Also, recently, a number of VEGF structural homologs have been identified: VEGF-B, VEGF-C, VEGF-D and placenta growth factor (PlGF) (Klagsbrun and D'Amore, *Cytokine Growth Factor Rev* (1996), 7:259-270; reviewed in Ferrara, *J Mol Med* (1999), 77:527-543).

[0011] Two tyrosine kinase receptors have been identified for which VEGF acts as a high affinity ligand: a fims-like tyrosine kinase-1 (Flt-1 or VEGFR-1) and a kinase domain receptor (KDR/Flk-1 or VEGFR-2) (Matthews *et al.*, *Proc Natl Acad Sci USA* (1991), 88:9026-9030; Terman *et al.*, *Biochem Biophys Res Commun* (1992), 187:1579-1586; De Vries *et al.*, *Science* (1992), 255:989-991; Millauer *et al.*, *Cell* (1993), 72:835-846). Although Flt-1 binds VEGF with 50-fold higher affinity than KDR (De Vries *et al.*, *Science* (1992), 255:989-991), most of the VEGF angiogenic properties (mitogenicity, chemotaxis, and induction on morphological changes) are mediated by interaction with KDR (Waltenberger *et al.*, *J Biol Chem* (1994), 269:26988-26995). Therefore, the interaction between VEGF and KDR is the most appropriate to interrupt in order to inhibit angiogenesis.

[0012] VEGF receptors typically are class III receptor-type tyrosine kinases characterized by having several, typically 5 or 7, immunoglobulin-like loops in their amino-terminal extracellular receptor ligand-binding domains (Kaipainen *et al.*, *J Exp Med* (1993),178:2077-2088). The other two regions include a transmembrane region and a carboxy-terminal intracellular catalytic domain interrupted by insertion of hydrophilic interkinase sequences of variable lengths, called the kinase insert domain (Terman *et al.*, *Oncogene* (1991), 6:1677-1683).

[0013] In addition, VEGF binds to a third receptor, neuropilin-1. Neuropilin-1 (NRP-1) was first described as a co-receptor implicated in neuronal guidance that bound members of the semaphorin/collapsin family. NRP-1 is also expressed in endothelial cells and is believed to promote angiogenesis by acting as a co-receptor with VEGFR-2 (Gray *et al.*, *Cancer Res*, (2005), 65(9):3664-70). NRP-1 and VEGFR-2 do not interact directly, but are bridged by one VEGF isoform, $VEGF_{165}$ (Mac Gabhann and Popel, *Am J Physiol Heart Circ Physiol*, (2005), 288(6):H2851-60).

[0014] Thus, VEGF may play a broad role in a range of cancers, including cancers of the colon, rectum, renal cell (kidney), breast, non-small cell lung and ovary. Currently, Avastatin™ (bevacizumab), a therapeutic antibody developed by Genentech designed to inhibit VEGF function and thereby interfering with the blood supply to tumors has been approved as treatment for patients with metastatic cancer of the colon or rectum. Other approaches to block angiogenesis employ monoclonal antibodies specific to VEGF receptors (e.g., U.S. Patent No. 5,955,331), compounds such as indolinone (U.S. Patent No. 6,846,839)

or peptides interacting with VEGF and thus blocking its interaction with its cognate receptor (e.g., U.S. Patent No. 6,559,126).

[0015] However, none of the treatment options currently in clinical trials or known in the prior that block tumor-associated neovascularization by preventing VEGF binding to its cognate receptor on tumor cells, do also attempt to kill the tumor cells. This may not an easy task because, in addition to its major role in angiogenesis, VEGF affects cell survival by interfering with apoptosis (Bairey *et al.*, *Leuk Res* (2004), 28(3):243-8).

[0016] Apoptosis, or programmed cell death, is an important physiological process in multicellular organisms, both during development and for homeostasis. Apoptosis is mediated, at least in part, by a cell surface receptor protein, Fas, which plays an important role in the development and function of the immune system. Malfunction of the Fas system has been shown to cause lymphoproliferative disorders and accelerate autoimmune disorders. (Takahashi *et al.*, *Cell* (1994), 76:969-976).

[0017] Fas is a type I membrane protein with a molecular weight of about 45 kD that belongs to the tumor necrosis factor (TNF) receptor family (Nagata *et al.*, *Science*, 1995), 267:1449). Fas transduces apoptotic signal to the cell as a cell surface antigen. Apoptotic cell death is characterized by nuclear and cytoplasmic shrinkage, membrane blebbing, and degradation of chromosomal DNA in a characteristic pattern, and can be distinguished from necrotic cell death due to acute cellular injury.

[0018] Many tissues and cell lines weakly express Fas, but abundant expression is found in the heart, lung, liver, ovary and thymus (Watanabe-Fukunaga *et al.*, *J Immunol* (1992), 148:1274). Fas transmits a signal for apoptosis or programmed cell death (Thompson, *Science* (1995), 267:1456) when it is triggered by binding of certain antibodies such as APO-1 (Trauth *et al.*, *Science* (1989), 245:301) and anti-Fas (Yonehara *et al.*, *J Exp Med* (1989),169:1747) or the natural ligand for Fas, Fas Ligand (FasL). Fas is also expressed on the surface of tumor cells. For example, the efficiency of the induction of Fas-mediated apoptosis by anti-Fas antibodies, FasL expressing cells or recombinant FasL in tumors has been demonstrated *in vivo* in solid tumors implanted in mice (Timmer *et al.*, *J Pathol* (2002), 196(2):125-34).

[0019] Human, rat, and mouse FasL have been cloned (Takahashi *et al.*, *Internat Immunol* (1994), 6:1567; Suda *et al.*, *Cell* (1993), 75:1169; Lynch *et al.*, *Immunity* (1994),1:131; Takahashi *et al.*, *Cell*(1994), 76:969). Human FasL is highly homologous to rat FasL and

mouse FasL in its extracellular domain, and human FasL is capable of recognizing not only the human Fas but also the mouse Fas, and induces apoptosis. Similarly, rat and mouse FasL are capable of recognizing the human Fas and inducing apoptosis. FasL is a type II membrane protein, i.e, having an extracellular carboxyl-terminal domain and an intracellular amino-terminal domain, belongs to the TNF family of proteins and has a molecular weight of about 40 kD. (Suda *et al.*, *Cell* (1993), 75:1169). The Fas ligand is strongly expressed on activated lymphocytes, in the testis (Suda *et al.*, *Cell* (1993), 75:1169) and the eye (Griffith, *et al.*, *Science* (1995), 270:1189), as well as on some cytotoxic T-lymphocyte (CTL) cell lines (Rouvier *et al.*, *J Exp Med* (1993), 177:195).

[0020] Cells expressing FasL, as well as purified FasL protein (Suda and Nagata, *J Exp Med*(1994), 179:873), are cytotoxic for cells expressing Fas. Thus, FasL transmits a signal for apoptosis by binding to Fas.. Also by analogy with TNF, FasL is believed to function as a trimer and presumably binds one to three Fas molecules at the interface of respective FasL units. Binding of two or more Fas molecules to a FasL trimer presumably causes oligomerization of Fas, which transmits an apoptotic signal to the Fas-expressing cell.

[0021] It would generally be desirable to be able to produce a soluble compound that combines (i) the function of a VEGFR polypeptide, i.e., binding a VEGF polypeptide, (ii) neutralizing VEGF-mediated activation of a VEGFR and thus, preventing tumor-associated neovascularization and (iii) the function of a Fas ligand in its interactions with the Fas receptor, i.e., receptor binding and/or activation of receptor mediated pathways. Such a compound would be useful for killing cancer cells that secrete VEGF and express Fas. However, a significant challenge in the recombinant protein technology has often been the expression of biologically active proteins of a transmembrane protein in the form of a soluble protein. The present invention overcomes these obstacles and meets those and other needs.

BRIEF SUMMARY OF THE INVENTION

[0022] The present invention provides novel fusion proteins that bind to a death receptor. A fusion protein of the present invention comprises (i) a vascular endothelial growth factor receptor (VEGFR) polypeptide that binds a vascular endothelial factor (VEGF) polypeptide and (ii) a death ligand comprising an oligomerization domain and a death receptor recognition moiety, wherein the C-terminus of the VEGFR polypeptide is linked to the N-terminus of the death ligand.

[0023] The VEGFR-death ligand fusion proteins are useful for methods of neutralizing VEGF activation of VEGF receptors. These methods are particularly useful for inducing apoptosis, inducing cytotoxic effects in cells, treating cancer and diseases or disorders related to unregulated angiogenesis and/or vasculogenesis.

[0024] In a preferred embodiment of the invention, the death receptor is Fas and the death ligand is a Fas ligand. Preferably the Fas ligand is a human Fas ligand.

[0025] In another preferred embodiments of the invention, the VEGFR polypeptide of the fusion protein comprises a VEGF binding domain of VEGF receptor-1 (VEGFR-1) or a VEGF binding domain of VEGF receptor-2 (VEGFR-2). VEGFR-1 and VEGFR-2 preferably are human VEGFR-1 and human VEGFR-2. VEGFR-1 and VEGFR-2 can also be from mouse or rat.

[0026] A preferred fusion protein of the invention comprises a murine VEGFR-2 polypeptide and a human Fas ligand. Preferably this fusion protein comprises an amino acid sequence which has at least 80% homology to the amino acid sequence shown in SEQ ID NO:22 or SEQ ID NO:23. In one embodiment of the present invention, the fusion protein comprises the amino acid sequence shown in SEQ ID NO:22 or SEQ ID NO:23.

[0027] In some embodiments, a fusion protein of the invention further comprises an epitope tag. Preferred epitope tags are a FLAG-like tag or an HA tag. Preferably, the epitope tag can be cleaved off.

[0028] Several Fas ligand polypeptides can be linked to a VEGFR polypeptide. In a preferred embodiment, the Fas ligand is selected from the group consisting of (i) a polypeptide comprising the amino acid sequence of SEQ ID NO:11; (ii) a polypeptide comprising the amino acid sequence of SEQ ID NO:12; (iii) a polypeptide comprising the amino acid sequence of SEQ ID NO:13; and (iv) a polypeptide having Fas-binding activity comprising an amino acid sequence wherein one to several amino acid residues have been deleted, substituted, or added in the amino acid sequence of any one of (i) to (iii).

[0029] Several VEGFR-1 polypeptides can be linked to a Fas ligand. In a preferred embodiment, the VEGFR-1 polypeptide is selected from the group consisting of (i) a polypeptide comprising the amino acid sequence of SEQ ID NO:20; (ii) a polypeptide comprising amino acid residues 1 to 747 of SEQ ID NO:19; (iii) a polypeptide comprising amino acid residues 32 to 747 of SEQ ID NO:19; (iv) a polypeptide comprising amino acid

residues 151 to214 of SEQ ID NO:19; (v) a polypeptide comprising amino acid residues 230 to 327 of SEQ ID NO:19; (vi) a polypeptide comprising amino acids 129 to 230 of VEGFR1 (SDTG...NTII as shown in Figure 10; domain 2 (D2)), and (vii) a polypeptide having VEGF binding activity comprising an amino acid sequence wherein one to several amino acid residues have been deleted, substituted, or added in the amino acid sequence of any one of (i) to (vi).

[0030] Several VEGFR-2 polypeptides can be linked to a Fas ligand. In a preferred embodiment, the VEGFR-2 polypeptide is selected from the group consisting of (i) a polypeptide comprising the amino acid sequence of SEQ ID NO:1; (ii) a polypeptide comprising the amino acid sequence of SEQ ID NO:2; (iii) a polypeptide comprising the amino acid sequence of SEQ ID NO:3; (iv) a polypeptide comprising the amino acid sequence of SEQ ID NO:4; (v) a polypeptide comprising the amino acid sequence of SEQ ID NO:5; (vi) a polypeptide comprising the amino acid sequence of SEQ ID NO:6; (iiv) a polypeptide comprising amino acids 141 to 207 of the amino acid sequence of SEQ ID NO:1; (iix) a polypeptide comprising amino acids 224 to 320 of the amino acid sequence of SEQ ID NO:1; and (ix) a polypeptide having VEGF binding activity comprising an amino acid sequence wherein one to several amino acid residues have been deleted, substituted, or added in the amino acid sequence of any one of (i) to (iix).

[0031] In another aspect of the invention, nucleic acids encoding the fusion proteins of the invention are provided. A preferred nucleic acid is shown in SEQ ID NO:14. Further, the invention provides vectors comprising the nucleic acid encoding a fusion protein of the invention. A preferred vector comprises a nucleic acid comprising the nucleotide sequence of SEQ ID NO:14.

[0032] The invention also provides a method of modulating a death receptor-mediated pathway. This method comprises the step of contacting a death receptor expressing cell with a fusion protein comprising (i) a VEGFR polypeptide that binds a VEGF protein; and (ii) a death ligand comprising an oligomerization domain and a death receptor recognition moiety; wherein the VEGFR polypeptide has bound a VEGF protein and wherein the amount of the fusion protein is effective to modulate the death receptor-mediated pathway.

[0033] In a preferred embodiment of the present invention, the Fas-mediated pathway is apoptosis. In this method, the amount of the fusion protein is effective to induce apoptosis.

[0034] Methods of the present invention can be practiced *in vitro* and *in vivo*.

[0035] In a preferred embodiment of the present invention, the death receptor expressing cell is a cancer cell, preferably a cancer cell that overexpresses VEGF. The cancer cell is selected from the group consisting of breast cancer, prostate cancer, colon cancer, lung cancer, glioblastoma, and ovarian cancer.

[0036] In another preferred embodiment of the present invention, the Fas-mediated pathway is modulated in a disease other than cancer. A preferred disease is selected from the group consisting of rheumatoid arthritis, psoriasis, and macular degeneration.

[0037] In a preferred embodiment of the present invention, the method of modulating a death receptor-mediated pathway comprises the step of contacting the death receptor expressing cell with a chemotherapeutic agent. Preferred chemotherapeutic agent are selected from the group consisting of camptothecin, etoposide, bisindolylmaleimide VIII, cisplatin, taxol, doxorubicin, temozolomide, bortezomid, LY294002, and valproic acid.

[0038] This invention also provides pharmaceutical compositions comprising a fusion protein of the present invention and a pharmaceutically acceptable excipient, carrier and/or diluent.

[0039] In another aspect this invention provides a composition comprising a vector comprising a nucleic acid having a nucleotide sequence as shown in SEQ ID NO:14 and a pharmaceutically acceptable excipient, carrier and/or diluent.

BRIEF DESCRIPTION OF THE DRAWINGS

[0040] Figure 1 shows schematic drawings of fusion proteins of the invention as exemplified by a VEGFR-FasL. The figure shows (i) a monomeric fusion protein, (ii) a trimeric fusion protein in the absence of VEGF generating a nonfunctional FasL not capable of inducing significant apoptosis and (iii) a VEGF-induced oligomerization of fusion proteins generating functional FasL capable of inducing apoptosis. V:V indicates binding of two VEGF molecules.

[0041] Figure 2 shows an Alignment of FasL protein sequences from human (H) (GenBank Accession No. P48023), mouse (M) (GenBank Accession No. A53062) and rat (R) (GenBank Accession No. A49266). Identical amino acid residues of mouse and rat FasL to human FasL are indicated by asterisks. Intracellular domain, transmembrane domain, cleavage position, trimerization domain and Receptor binding domains are indicated. The N-terminal border of the FasL-Fas binding domain has not yet been elucidated.

[0042] Figure 3 shows an alignment of extracellular and transmembrane regions of VEGFR-2 protein sequences from human (GenBank Accession No. NP-002244), mouse (GenBank Accession No. P35918) and rat (GenBank Accession No. NP_037914) with human VEGFR-1 (GenBank Accession No. NM_002019). Identical amino acid residues of mouse and rat VEGFR-2 to human VEGFR-2 are indicated by asterisks. Signal sequence, IgG-like domains 1 to 7, and transmembrane domain are indicated for the VEGFR-2 sequences.

[0043] Figure 4 shows an annotated sequence of the extracellular domain of human neuropilin-1 (part of Genbank AAC12921). Domains identified are indicated. The B domain is involved in binding VEGF.

[0044] Figure 5 depicts the construction of a VEGFR/FasL fusion protein encoding nucleic acid, Flk(D1-D3)+FasL(139-281). Details are described in Example 2.

[0045] Figure 6 depicts the construction of a VEGFR/FasL fusion protein encoding nucleic acid, FLAG/FlkhFasL (D1-D3/139-281). Details are described in Example 3.

[0046] Figure 7 shows a cDNA sequence of FlkFasL. The nucleotide sequence for Flk-1 signal sequence and extracellular domain sequence is underlined; the nucleotide sequence of a linker is shown in normal font; and the nucleotide sequence of FasL is shown in bold.

[0047] Figure 8 shows an amino acid sequence of FlkFasL. The amino acid sequence for the Flk-1 signal peptide is shown in italics; the amino acid sequence for the Flk-1 extracellular domain is underlined; the amino acid sequence of a linker is shown in normal font; and the amino acid sequence of FasL is shown in bold.

[0048] Figure 9 shows the nucleic acid sequence of FLAG-tagged R1[D2]FasL. Italics, preprotrypsin leader sequence; underlined, FLAG epitope tag sequence; underlined and bold, VEGFR1 domain 2; standard font, ARGTS encoding linker sequence; bold, FasL trimerization and Fas receptor binding domains. Details are described in Example 3.

[0049] Figure 10 shows the amino acid sequence of R1[D2]FasL using the single letter code for amino acid residues. Details are described in Example 3.

[0050] Figure 11A shows expression of FlkFasL trimers in Cos-7 cells. Cos-7 cells were transfected using a DEAE-dextran protocol with control plasmid pSV/Neo (lane A) or with plasmid pBJ/FlkFasL (lanes B-D). In lane B, cells were transfected with 1 μg plasmid DNA

for 30 minutes; lane C, with 3 μg plasmid DNA for 30 minutes; and lane D, with 3 μg plasmid DNA for 3 hours. Forty-four hours after transfection cells were lysed, lysates were electrophoresed by PAGE, and immunoblotted with antibody against the extracellular domain of VEGFR-2. Figure 11B shows expression of R2FasL by stably transfected CHO cells. The Western blot depicts detection of R2FasL in conditioned medium and after FLAG-tag purification using an anti-VEGFR2 antibody. Details are described in Example 4.

[0051] Figure 12 shows killing of Jurkat cells by FlkFasL in a VEGF-dependent manner. Various amounts of conditioned medium including FlkFasL was added to Jurkat cells. Data are shown as mean number of cells per hemocytometer field ± SEM. For some data points the SEM is smaller than the icon used. Details are described in Example 5.

[0052] Figure 13 shows that killing of Jurkat cells by FlkFasL is dependent on the amount of VEGF. Various amounts of VEGF-165 were added to Jurkat cells. Data are shown as mean number of cells per hemocytometer field ± SEM. For some data points the SEM is smaller than the icon used. Details are described in Example 6.

[0053] Figure 14 shows induction of apoptosis by FlkFasL in a VEGF-dependent manner. Jurkat cells were incubated with control conditioned medium (C) or with conditioned medium including FlkFasL (F) in the indicated volumes of 1 μL or 5 μL. Cells were additionally treated with 2 nM VEGF-165 (V) or without (-) for 60 minutes. Induction of apoptosis was assessed by FACS analysis of Annexin V-positive/propidium-iodine negative cells. Details are described in Example 7.

[0054] Figure 15 shows induction of apoptosis in a human breast cancer cell line. T-47D human breast cancer cells were treated with 25 μL of conditioned medium from Cos-7 cells transfected with control plasmid pSV/Neo (Control CM) or plasmid FlkFasL (FlkFasL CM). In the right panel, cells were additionally treated with 2 nM VEGF-165. Cells were photographed 24 hours after treatment. The center panel shows little cell death induced by FlkFasL. The drastically increased cell death in the right panel shows that FlkFasL apoptotic activity is regulated by VEGF. Details are described in Example 8.

[0055] Figure 16 shows stimulation of cytotoxicity by FlkFasL in a human breast cancer cell line. T-47D human breast cancer cells were treated with 25 μL conditioned medium from Cos-7 cells transfected with control plasmid pSV/Neo (B) or plasmid FlkFasL (C and D). Cells in D were also treated with 2 nM VEGF-165. No conditioned medium was added in lane A. Cells were photographed 24 hours after treatment. Forty-eight hours later

cytotoxic response was assayed by quantifying lactate dehydrogenase (LDH) release into the cell culture supernatant. Details are described in Example 9.

[0056] Figure 17 shows that R2FasL induces cytotoxicity or apoptosis in U87MG human glioblastoma cells and DU145 human prostate cancer cells, but not in U373 human glioblastoma cells. **A.** R2FasL induces cytotoxicity in U87MG cells, which is inhibited by neutralizing antibodies against VEGF (Anti-VEGF Ab) or FasL (Anti-FasL Ab). **B.** R2FasL induces cell death in U87MG glioblastoma cells, which is inhibited by neutralizing antibodies against VEGF (Anti-VEGF Ab) or FasL (Anti-FasL Ab). **C.** R2FasL induces cytotoxicity in DU145 human prostate cancer cells. CM, conditioned medium. **D.** R2FasL does not induce cytotoxicity in U373 glioblastoma cells. Details are described in Example 10.

[0057] Figure 18 depicts the apoptotic activity of R1[D2]FasL. **A.** R1[D2]FasL induces apoptosis in a VEGF-dependent manner. Recombinant human VEGF (rhV165) was added at a concentration of 2 nM. As a control, no VEGF (No V) was added. **B.** R1[D2]FasL is activated by human and mouse VEGF-165; human VEGF-121, human VEGF:PlGF heterodimer, and human PlGF. **C.** The ED_{50} for hVEGF-165 on R1[D2]FasL is approximately 100 pM. Details are described in Example 11.

[0058] Figure 19 depicts potentiation of R2FasL activity by chemotherapeutic agents. **A.** Potentiation of R2FasL apoptotic activity on U87MG glioblastoma cells by BisVIII, camptothecin, and etoposide. **B.** Potentiation of R2FasL cytotoxic activity on U87MG glioblastoma cells by BisVIII, camptothecin, and etoposide. Details are described in Example 12.

[0059] Figure 20A depicts that R2FasL + VEGF does not induce cytotoxicity in human umbilical vein endothelial cells. Figure 20B depicts that R2FasL + VEGF induces cytotoxicity in bovine adrenal cortical endothelial cells (microvascular endothelial cells). Details are described in Example 13.

[0060] Figure 21A depicts that R2FasL is activated by both human and mouse VEGF-165 (hVEGF and mVEGF, respectively). Figure 21B shows that the ED50 of VEGF-165 is between 20 and 200 pM. Details are described in Example 14.

DETAILED DESCRIPTION OF THE INVENTION

I. DEFINITIONS

[0061] Unless defined otherwise, all technical and scientific terms used herein have the meaning commonly understood by a person skilled in the art to which this invention belongs. The following references provide one of skill with a general definition of many of the terms used in this invention: Singleton *et al.*, *Dictionary of Microbiology and Molecular Biology* (2nd ed. 1994); *The Cambridge Dictionary of Science and Technology* (Walker ed., 1988); *The Glossary of Genetics*, 5th Ed., R. Rieger *et al.* (eds.), Springer Verlag (1991); and Hale & Marham, *The Harper Collins Dictionary of Biology* (1991). As used herein, the following terms have the meanings ascribed to them unless specified otherwise.

[0062] The term "amino acid" refers to naturally occurring and synthetic amino acids, as well as amino acid analogs and amino acid mimetics that function similarly to the naturally occurring amino acids. Naturally occurring amino acids are those encoded by the genetic code, as well as those amino acids that are later modified, *e.g.*, hydroxyproline, γ-carboxyglutamate, and O-phosphoserine. "Amino acid analog" refers to a compound that has the same basic chemical structure as a naturally occurring amino acid, *e.g.*, an α carbon that is bound to a hydrogen, a carboxyl group, an amino group, and an R group, *e.g.*, homoserine, norleucine, methionine sulfoxide, methionine methyl sulfonium. Such analogs may have modified R groups (*e.g.*, norleucine) or modified peptide backbones, but retain the same basic chemical structure as a naturally occurring amino acid. "Amino acid mimetic" refers to a chemical compound that has a structure that is different from the general chemical structure of an amino acid, but that functions similarly to a naturally occurring amino acid.

[0063] Amino acids may be referred to herein by either their commonly known three letter symbols or by the one-letter symbols recommended by the IUPAC-IUB Biochemical Nomenclature Commission. Nucleotides, likewise, may be referred to by their commonly accepted single-letter codes.

[0064] "Biological sample" as used herein is a sample of biological tissue or fluid that contains nucleic acids or polypeptides. Such samples are typically from humans, but include tissues isolated from non-human primates, or rodents, *e.g.*, mice, and rats. Biological samples may also include sections of tissues such as biopsy and autopsy samples, frozen sections taken for histological purposes, blood, plasma, serum, sputum, stool, tears, mucus, hair, skin, etc. Biological samples also include explants and primary and/or transformed cell

cultures derived from patient tissues. A "biological sample" also refers to a cell or population of cells or a quantity of tissue or fluid from an animal. Most often, the biological sample has been removed from an animal, but the term "biological sample" can also refer to cells or tissue analyzed *in vivo*, *i.e.*, without removal from the animal. Typically, a "biological sample" will contain cells from the animal, but the term can also refer to noncellular biological material, such as noncellular fractions of blood, saliva, or urine, that can be used to measure cancer-associated polynucleotide or polypeptide levels. Numerous types of biological samples can be used in the present invention, including, but not limited to, a tissue biopsy, a blood sample, a serum sample, or a saliva sample. As used herein, a "tissue biopsy" refers to an amount of tissue removed from an animal, preferably a human, for diagnostic analysis. In a patient with cancer, tissue may be removed from a tumor, allowing the analysis of cells within the tumor. "Tissue biopsy" can refer to any type of biopsy, such as needle biopsy, fine needle biopsy, surgical biopsy, etc.

[0065] "Providing a biological sample" means to obtain a biological sample for use in methods described in this invention. Most often, this will be done by removing a sample of cells from a patient, but can also be accomplished by using previously isolated cells (*e.g.*, isolated by another person, at another time, and/or for another purpose), or by performing the methods of the invention *in vivo*. Archival tissues, having treatment or outcome history, are also useful.

[0066] The phrase "changes in cell growth" refers to any change in cell growth and proliferation characteristics *in vitro* or *in vivo*, such as formation of foci, anchorage independence, semi-solid or soft agar growth, changes in contact inhibition and density limitation of growth, loss of growth factor or serum requirements, changes in cell morphology, gaining or losing immortalization, gaining or losing tumor specific markers, ability to form or suppress tumors when injected into suitable animal hosts, and/or immortalization of the cell. See, *e.g.*, Freshney, *Culture of Animal Cells a Manual of Basic Technique* pp. 231-241 (3rd ed. 1994).

[0067] "Conservatively modified variants" applies to both amino acid and nucleic acid sequences. With respect to particular nucleic acid sequences, conservatively modified variants refers to those nucleic acids which encode identical or essentially identical amino acid sequences, or where the nucleic acid does not encode an amino acid sequence, to essentially identical or associated, *e.g.*, naturally contiguous, sequences. Because of the

degeneracy of the genetic code, a large number of functionally identical nucleic acids encode most proteins. For instance, the codons GCA, GCC, GCG and GCU all encode the amino acid alanine. Thus, at every position where an alanine is specified by a codon, the codon can be altered to another of the corresponding codons described without altering the encoded polypeptide. Such nucleic acid variations are "silent variations," which are one species of conservatively modified variations. Every nucleic acid sequence herein which encodes a polypeptide also describes silent variations of the nucleic acid. One of skill will recognize that in certain contexts each codon in a nucleic acid (except AUG, which is ordinarily the only codon for methionine, and TGG, which is ordinarily the only codon for tryptophan) can be modified to yield a functionally identical molecule. Accordingly, an often silent variations of a nucleic acid which encodes a polypeptide is implicit in a described sequence with respect to the expression product, but not with respect to actual probe sequences.

[0068] As to amino acid sequences, one of skill will recognize that individual substitutions, deletions or additions to a nucleic acid, peptide, polypeptide, or protein sequence which alters, adds or deletes a single amino acid or a small percentage of amino acids in the encoded sequence is a "conservatively modified variant" where the alteration results in the substitution of an amino acid with a chemically similar amino acid. Conservative substitution tables providing functionally similar amino acids are well known in the art. Such conservatively modified variants are in addition to and do not exclude polymorphic variants, interspecies homologs, and alleles of the invention .typically conservative substitutions for one another: 1) Alanine (A), Glycine (G); 2) Aspartic acid (D), Glutamic acid (E); 3) Asparagine (N), Glutamine (Q); 4) Arginine (R), Lysine (K); 5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V); 6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W); 7) Serine (S), Threonine (T); and 8) Cysteine (C), Methionine (M) (*see, e.g.*, Creighton, *Proteins* (1984)).

[0069] "Cancer cells," "transformed" cells or "transformation" in tissue culture, refers to spontaneous or induced phenotypic changes that do not necessarily involve the uptake of new genetic material. Although transformation can arise from infection with a transforming virus and incorporation of new genomic DNA, or uptake of exogenous DNA, it can also arise spontaneously or following exposure to a carcinogen, thereby mutating an endogenous gene. Transformation is associated with phenotypic changes, such as immortalization of cells, aberrant growth control, nonmorphological changes, and/or malignancy (*see*, Freshney, *Culture of Animal Cells a Manual of Basic Technique* (3rd ed. 1994)).

[0070] The term "death ligand" refers to a family of mammalian proteins that can bind to a death receptor and upon binding induce cell killing. Exemplary death ligands include, but are not limited to, FasL, tumor necrosis factor (TNF), lymphotoxin (LT) and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). Typically, a death ligand polypeptide comprises an oligomerization domain and a death receptor recognition moiety.

[0071] The term "death receptor" refers to a family of mammalian proteins expressed on the surface of a mammalian cell that can bind a death ligand and upon binding of the death ligand oligomerize and induce cell killing. Typically, a death receptor polypeptide comprises an oligomerization domain and a death ligand recognition moiety. Eight death receptors and death receptor signaling are reviewed by Lavrik *et al.*, *J Cell Sci*, (2005), 118(Pt2):265-7, hereby incorporated by reference in its entirety. Exemplary death receptors include, but are not limited to, tumor necrosis factor receptor 1 (TNFR1; also known as DR1, CD120a, p55 and p60), CD95 (also known as DR2, APO-1 and Fas), DR3 (also known as APO-3, LARD, TRAMP and WSL1), TNF-related apoptosis-inducing ligand receptor 1 (TRAILR1; also known as DR4 and APO-2), TRAILR2 (also known as DR5, KILLER and TRICK2), DR6, ectodysplasin A receptor (EDAR) and nerve growth factor receptor (NGFR). These are distinguished by a cytoplasmic region of ~80 residues termed the death domain (DD). When these receptors are triggered by corresponding ligands, a number of molecules are recruited to the DD and subsequently a signaling cascade is activated. Death ligands also interact with decoy receptors (DcRs) that do not possess DDs and so cannot form signaling complexes. To date, four decoy receptors have been characterized: TRAILR3 (also known as DcR1), TRAILR4 (also known as DcR2), DcR3 and osteoprotegrin (OPG).

[0072] The term "death receptor recognition moiety" refers to a subdomain of a death ligand necessary and sufficient for binding to a death receptor.

[0073] By "determining the functional effect" is meant assaying for a compound that increases or decreases a parameter that is indirectly or directly under the influence of a fusion protein of this invention such as a VEGFR-FasL, *e.g.*, functional, enzymatic, physical and chemical effects. Such functional effects can be measured by any means known to those skilled in the art, *e.g.*, changes in spectroscopic characteristics (*e.g.*, fluorescence, absorbance, refractive index), hydrodynamic (*e.g.*, shape), chromatographic, or solubility properties for the protein, measuring inducible markers or transcriptional activation of the a fusion protein of this invention such as a VEGFR-FasL; measuring binding activity, *e.g.*,

binding to a death receptor, measuring cellular proliferation, measuring apoptosis, or the like. Determination of the functional effect of a compound on cancer can also be performed using assays known to those of skill in the art such as an *in vitro* assays, *e.g.*, cell growth on soft agar; anchorage dependence; contact inhibition and density limitation of growth; cellular proliferation; cellular transformation; growth factor or serum dependence; tumor specific marker levels; invasiveness into Matrigel; tumor growth and metastasis *in vivo*; mRNA and protein expression in cells undergoing metastasis, and other characteristics of cancer cells. The functional effects can be evaluated by many means known to those skilled in the art, *e.g.*, microscopy for quantitative or qualitative measures of alterations in morphological features, measurement of changes in RNA or protein levels, measurement of RNA stability, identification of downstream or reporter gene expression (CAT, luciferase, β-gal, GFP and the like), *e.g.*, *via* chemiluminescence, fluorescence, colorimetric reactions, antibody binding, inducible markers, and ligand binding assays. "Functional effects" include *in vitro*, *in vivo*, and *ex vivo* activities.

[0074] An "effective amount" of a compound for treating a disorder is an amount that is sufficient to ameliorate, or in some manner, reduce a symptom or stop or reverse progression of a condition. Amelioration of a symptom of a particular disorder by administration of a particular pharmaceutical composition refers to any lessening, whether permanent or temporary, lasting or transit that can be associated

[0075] An "expression vector" is a nucleic acid construct, generated recombinantly or synthetically, with a series of specified nucleic acid elements that permit transcription of a particular nucleic acid in a host cell. The expression vector can be part of a plasmid, virus, or nucleic acid fragment. Typically, the expression vector includes a nucleic acid to be transcribed operably linked to a promoter.

[0076] The term "FasL" or "Fas ligand" refers to isolated nucleic acids, polypeptides and polymorphic variants, alleles, mutants, and interspecies homologues thereof and as further described herein, that: (1) have an amino acid sequence that has greater than about 60% amino acid sequence identity, 65%, 70%, 75%, 80%, 85%, 90%, preferably 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% or greater amino acid sequence identity, preferably over a region of at least about 50, 75, 100, 150, 200, 250, or 281 amino acids, to a human FasL sequence shown below; (2) bind to antibodies, *e.g.*, polyclonal antibodies, raised against an immunogen comprising an amino acid sequence shown below, or conservatively modified

variants thereof; (3) bind to a FasL binding protein; (4) compete with a naturally occurring Fas ligand binding to a Fas ligand binding protein; (5) induce apoptosis in cells having a membrane-bound FasL binding protein; (6) specifically hybridize under stringent hybridization conditions to a nucleic acid sequence shown below, or conservatively modified variants thereof; (7) have a nucleic acid sequence that has greater than about 90%, preferably greater than about 96%, 97%, 98%, 99%, or higher nucleotide sequence identity, preferably over a region of at least about 100, 200, 300, 400 or more nucleotides, to SEQ ID NO:16 (human FasL); and/or (8) have at least 25, often 50, 75, 100, 125 or 143 contiguous amino acid residues of SEQ ID NO:8 or SEQ ID NO:11 (human FasL). A FasL polypeptide may have oligomerization and death receptor recognition domains as described herein.

[0077] A FasL polynucleotide or polypeptide sequence is typically from a human, but may be from other mammals, but not limited to, a non-human primate, a rodent, *e.g.*, a rat, mouse, or hamster; a cow, a pig, a horse, a sheep, or other mammal. Therefore, in some embodiments, a FasL polypeptide and a FasL subdomain polypeptide as described herein can comprise a sequence that corresponds to a human FasL sequence. Thus, exemplary FasL are provided herein and are known in the art. For example, GenBank accession numbers for human FasL polypeptide is P48023. The GenBank accession number for mouse FasL polypeptide, for example, is A53062; and for rat FasL, A49266.

[0078] The term "FasL binding protein" refers to a polypeptide to which a FasL binds.

[0079] A FasL "homolog" or VEGFR "homolog" refers to a polypeptide that comprises an amino acid sequence similar to that of FasL or VEGFR but does not necessarily possess a similar or identical function as FasL or VEGFR.

[0080] A FasL "isoform" or VEGFR "isoform" refers to a variant of FasL or VEGFR, respectively, that is encoded by the same gene, but differs in its pI or MW, or both. Such isoforms can differ in their amino acid composition (*e.g.*, as a result of alternative splicing or limited proteolysis) and in addition, or in the alternative, may arise from differential post-translational modification (*e.g.*, glycosylation, acylation or phosphorylation).

[0081] A FasL "ortholog" or VEGFR "ortholog" as used herein refers to a non-human polypeptide that (i) comprises an amino acid sequence similar to that of human FasL or VEGFR and (ii) possess a similar or identical function to that of human FasL or VEGFR.

[0082] A FasL "related" polypeptide as used herein refers to a FasL homolog, a FasL isoform, or a FasL ortholog. A VEGFR "related" polypeptide, as used herein, refers to a VEGFR homolog, a VEGFR isoform, or a VEGFR ortholog.

[0083] A "host cell" is a naturally occurring cell or a transformed cell that contains an expression vector and supports the replication or expression of the expression vector. Host cells may be cultured cells, explants, cells *in vivo*, and the like. Host cells may be prokaryotic cells such as *E. coli*, or eukaryotic cells such as yeast, insect cells, amphibian cells, or mammalian cells such as Cos cells (*e.g.*, Cos-7), CHO, 293, 3T3, HeLa, and the like (*see, e.g.*, American Type Culture Collection).

[0084] The terms "identical" or percent "identity," in the context of two or more nucleic acids or polypeptide sequences, refer to two or more sequences or subsequences that are the same or have a specified percentage of amino acid residues or nucleotides that are the same (*i.e.*, about 60% identity, preferably 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or higher identity over a specified region, when compared and aligned for maximum correspondence over a comparison window or designated region) as measured using a BLAST or BLAST 2.0 sequence comparison algorithms with default parameters described below, or by manual alignment and visual inspection (*see, e.g.*, NCBI web site http://www.ncbi.nlm.nih.gov/BLAST/ or the like). Such sequences are then said to be "substantially identical." This definition also refers to, or may be applied to, the compliment of a test sequence. The definition also includes sequences that have deletions and/or additions, as well as those that have substitutions, as well as naturally occurring, *e.g.*, polymorphic or allelic variants, and man-made variants. As described below, the preferred algorithms can account for gaps and the like. Preferably, identity exists over a region that is at least about 25 amino acids or nucleotides in length, or more preferably over a region that is 50-100 amino acids or nucleotides in length.

[0085] For sequence comparison, typically one sequence acts as a reference sequence, to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are entered into a computer, subsequence coordinates are designated, if necessary, and sequence algorithm program parameters are designated. Preferably, default program parameters can be used, or alternative parameters can be designated. The sequence comparison algorithm then calculates the percent sequence identities for the test sequences relative to the reference sequence, based on the program parameters.

[0086] A "comparison window", as used herein, includes reference to a segment of one of the number of contiguous positions selected from the group consisting typically of from about 20 to about 600, usually about 50 to about 200, more usually about 100 to about 150 in which a sequence may be compared to a reference sequence of the same number of contiguous positions after the two sequences are optimally aligned. Methods of alignment of sequences for comparison are well-known in the art. Optimal alignment of sequences for comparison can be conducted, *e.g.*, by the local homology algorithm of Smith & Waterman, *Adv Appl Math* 2:482 (1981), by the homology alignment algorithm of Needleman & Wunsch, *J Mol Bio.* 48:443 (1970), by the search for similarity method of Pearson & Lipman, *Proc Nat'l Acad Sci USA* 85:2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI), or by manual alignment and visual inspection (see, *e.g.*, *Current Protocols in Molecular Biology* (Ausubel *et al.*, eds. 1995 supplement)).

[0087] Preferred examples of algorithms that are suitable for determining percent sequence identity and sequence similarity include the BLAST and BLAST 2.0 algorithms, which are described in Altschul *et al.*, *Nucl Acids Res* 25:3389-3402 (1977) and Altschul *et al.*, *J Mol Biol* 215:403-410 (1990). BLAST and BLAST 2.0 are used, with the parameters described herein, to determine percent sequence identity for the nucleic acids and proteins of the invention. Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence, which either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold (Altschul *et al.*, *supra*). These initial neighborhood word hits act as seeds for initiating searches to find longer HSPs containing them. The word hits are extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Cumulative scores are calculated using, *e.g.*, for nucleotide sequences, the parameters M (reward score for a pair of matching residues; always > 0) and N (penalty score for mismatching residues; always < 0). For amino acid sequences, a scoring matrix is used to calculate the cumulative score. Extension of the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below,

due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T, and X determine the sensitivity and speed of the alignment. The BLASTN program (for nucleotide sequences) uses as defaults a wordlength (W) of 11, an expectation (E) of 10, M=5, N=-4 and a comparison of both strands. For amino acid sequences, the BLASTP program uses as defaults a wordlength of 3, and expectation (E) of 10, and the BLOSUM62 scoring matrix (*see* Henikoff & Henikoff, *Proc Natl Acad Sci USA* 89:10915 (1989)) alignments (B) of 50, expectation (E) of 10, M=5, N=-4, and a comparison of both strands.

[0088] The BLAST algorithm also performs a statistical analysis of the similarity between two sequences (*see, e.g.*, Karlin & Altschul, *Proc Natl Acad Sci USA* 90:5873-5787 (1993)). One measure of similarity provided by the BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. For example, a nucleic acid is considered similar to a reference sequence if the smallest sum probability in a comparison of the test nucleic acid to the reference nucleic acid is less than about 0.2, more preferably less than about 0.01, and most preferably less than about 0.001. Log values may be large negative numbers, *e.g.*, 5, 10, 20, 30, 40, 40, 70, 90, 110, 150, 170, etc.

[0089] An indication that two nucleic acid sequences or polypeptides are substantially identical is that the polypeptide encoded by the first nucleic acid is immunologically cross reactive with the antibodies raised against the polypeptide encoded by the second nucleic acid, as described below. Thus, a polypeptide is typically substantially identical to a second polypeptide, *e.g.*, where the two peptides differ only by conservative substitutions. Another indication that two nucleic acid sequences are substantially identical is that the two molecules or their complements hybridize to each other under stringent conditions, as described below. Yet another indication that two nucleic acid sequences are substantially identical is that the same primers can be used to amplify the sequences.

[0090] The terms "inhibit", "inhibiting" or "inhibition" includes any measurable reproducible reduction in the interaction of VEGF and a VEGF receptor, angiogenesis; symptoms of diseases correlated to angiogenesis, or any other activity that VEGF may mediate.

[0091] The terms "isolated," "purified," or "biologically pure" refer to material that is substantially or essentially free from components that normally accompany it as found in its

native state. Purity and homogeneity are typically determined using analytical chemistry techniques such as polyacrylamide gel electrophoresis or high performance liquid chromatography. A protein or nucleic acid that is the predominant species present in a preparation is substantially purified. In particular, an isolated nucleic acid is separated from some open reading frames that naturally flank the gene and encode proteins other than protein encoded by the gene. The term "purified" in some embodiments denotes that a nucleic acid or protein gives rise to essentially one band in an electrophoretic gel. Preferably, it means that the nucleic acid or protein is at least 85% pure, more preferably at least 95% pure, and most preferably at least 99% pure. "Purify" or "purification" in other embodiments means removing at least one contaminant from the composition to be purified. In this sense, purification does not require that the purified compound be homogenous, *e.g.*, 100% pure.

[0092] The term "modulate" as used herein in reference to a death receptor signal transduction refers to the ability of a compound to alter the function of the death receptor in vitro and/or in vivo. A compound preferably activates the activity of the death receptor depending on the concentration of the compound.

[0093] "Nucleic acid" or "oligonucleotide" or "polynucleotide" or grammatical equivalents used herein means at least two nucleotides covalently linked together. Oligonucleotides are typically from about 5, 6, 7, 8, 9, 10, 12, 15, 25, 30, 40, 50 or more nucleotides in length, up to about 100 nucleotides in length. Nucleic acids and polynucleotides are a polymers of any length, including longer lengths, *e.g.*, 200, 300, 500, 1000, 2000, 3000, 5000, 7000, 10,000, etc. A nucleic acid of the present invention will generally contain phosphodiester bonds, although in some cases, nucleic acid analogs are included that may have alternate backbones, comprising, *e.g.*, phosphoramidate, phosphorothioate, phosphorodithioate, or O-methylphophoroamidite linkages (*see*, Eckstein, *Oligonucleotides and Analogues: A Practical Approach*, Oxford University Press); and peptide nucleic acid backbones and linkages. Other analog nucleic acids include those with positive backbones; non-ionic backbones, and non-ribose backbones, including those described in U.S. Patent Nos. 5,235,033 and 5,034,506, and Chapters 6 and 7, ASC Symposium Series 580, *Carbohydrate Modifications in Antisense Research*, Sanghui & Cook, eds. Nucleic acids containing one or more carbocyclic sugars are also included within one definition of nucleic acids. Modifications of the ribose-phosphate backbone may be done for a variety of reasons, *e.g.* to increase the stability and half-life of such molecules in physiological environments or as probes on a biochip. Mixtures of naturally occurring nucleic acids and analogs can be made;

alternatively, mixtures of different nucleic acid analogs, and mixtures of naturally occurring nucleic acids and analogs may be made.

[0094] A variety of references disclose such nucleic acid analogs, including, for example, phosphoramidate (Beaucage *et al.*, *Tetrahedron* 49(10):1925 (1993) and references therein; Letsinger, *J Org Chem* 35:3800 (1970); Sprinzl *et al.*, *Eur J Biochem* 81:579 (1977); Letsinger *et al.*, *Nucl Acids Res* 14:3487 (1986); Sawai *et al.*, *Chem Lett* 805 (1984), Letsinger *et al.*, *J Am Chem Soc* 110:4470 (1988); and Pauwels *et al.*, *Chemica Scripta* 26:141 91986)), phosphorothioate (Mag *et al.*, *Nucl Acids Res* 19:1437 (1991); and U.S. Patent No. 5,644,048), phosphorodithioate (Briu *et al.*, *J. Am. Chem. Soc.* 111:2321 (1989), O methylphophoroamidite linkages (see, Eckstein, *Oligonucleotides and Analogues: A Practical Approach*, Oxford University Press), and peptide nucleic acid backbones and linkages (*see*, Egholm, *J Am Chem Soc* 114:1895 (1992); Meier *et al.*, *Chem Int Ed Engl* 31:1008 (1992); Nielsen, *Nature* 365:566 (1993); Carlsson *et al.*, *Nature* 380:207 (1996), all of which are incorporated by reference). Other analog nucleic acids include those with positive backbones (Denpcy *et al.*, *Proc Natl Acad Sci USA* 92:6097 (1995); non ionic backbones (U.S. Patent Nos. 5,386,023, 5,637,684, 5,602,240, 5,216,141 and 4,469,863; Kiedrowshi *et al.*, *Angew. Chem Intl Ed English* 30:423 (1991); Letsinger *et al.*, *J Am Chem Soc* 110:4470 (1988); Letsinger *et al.*, *Nucleoside & Nucleotide* 13:1597 (1994); Chapters 2 and 3, ASC Symposium Series 580, "*Carbohydrate Modifications in Antisense Research*", Ed. Y.S. Sanghui and P. Dan Cook; Mesmaeker *et al.*, *Bioorganic & Medicinal Chem. Lett* 4:395 (1994); Jeffs *et al.*, *J Biomolecular NMR* 34:17 (1994); *Tetrahedron Lett* 37:743 (1996)) and non ribose backbones, including those described in U.S. Patent Nos. 5,235,033 and 5,034,506, and Chapters 6 and 7, ASC Symposium Series 580, "*Carbohydrate Modifications in Antisense Research*", Ed. Y.S. Sanghui and P. Dan Cook. Nucleic acids containing one or more carbocyclic sugars are also included within one definition of nucleic acids (*see*, Jenkins *et al.*, *Chem Soc Rev* pp 169 176 (1995)). Several nucleic acid analogs are described in Rawls, C & E News June 2, 1997 page 35. All of these references are hereby expressly incorporated by reference.

[0095] Other analogs include peptide nucleic acids (PNA) which are peptide nucleic acid analogs. These backbones are substantially non-ionic under neutral conditions, in contrast to the highly charged phosphodiester backbone of naturally occurring nucleic acids. This results in two advantages. First, the PNA backbone exhibits improved hybridization kinetics. PNAs have larger changes in the melting temperature (T_m) for mismatched versus perfectly

matched base pairs. DNA and RNA typically exhibit a 2-4°C drop in T_m for an internal mismatch. With the non-ionic PNA backbone, the drop is closer to 7-9°C. Similarly, due to their non-ionic nature, hybridization of the bases attached to these backbones is relatively insensitive to salt concentration. In addition, PNAs are not degraded by cellular enzymes, and thus can be more stable.

[0096] The nucleic acids may be single stranded or double stranded, as specified, or contain portions of both double stranded or single stranded sequence. As will be appreciated by those in the art, the depiction of a single strand also defines the sequence of the complementary strand; thus the sequences described herein also provide the complement of the sequence. The nucleic acid may be DNA, both genomic and cDNA, RNA or a hybrid, where the nucleic acid may contain combinations of deoxyribo- and ribo-nucleotides, and combinations of bases, including uracil, adenine, thymine, cytosine, guanine, inosine, xanthine hypoxanthine, isocytosine, isoguanine, etc. "Transcript" typically refers to a naturally occurring RNA, *e.g.*, a pre-mRNA, hnRNA, or mRNA. As used herein, the term "nucleoside" includes nucleotides and nucleoside and nucleotide analogs, and modified nucleosides such as amino modified nucleosides. In addition, "nucleoside" includes non-naturally occurring analog structures. Thus, *e.g.*, the individual units of a peptide nucleic acid, each containing a base, are referred to herein as a nucleoside.

[0097] A "label" or a "detectable moiety" is a composition detectable by spectroscopic, photochemical, biochemical, immunochemical, chemical, or other physical means. For example, useful labels include ^{32}P, fluorescent dyes, electron-dense reagents, enzymes (*e.g.*, as commonly used in an ELISA), biotin, digoxigenin, or haptens and proteins or other entities which can be made detectable, *e.g.*, by incorporating a radiolabel into the peptide or used to detect antibodies specifically reactive with the peptide. The labels may be incorporated into the breast cancer nucleic acids, proteins and antibodies at any position. Any method known in the art for conjugating the antibody to the label may be employed, including those methods described by Hunter *et al.*, *Nature* 144:945 (1962); David *et al.*, *Biochemistry* 13:1014 (1974); Pain *et al.*, *J Immunol Meth* 40:219 (1981); and Nygren, *J. Histochem. and Cytochem* 30:407 (1982).

[0098] A "labeled nucleic acid probe or oligonucleotide" is one that is bound, either covalently, through a linker or a chemical bond, or noncovalently, through ionic, van der Waals, electrostatic, or hydrogen bonds to a label such that the presence of the probe may be detected by detecting the presence of the label bound to the probe. Alternatively, method

using high affinity interactions may achieve the same results where one of a pair of binding partners binds to the other, *e.g.*, biotin, streptavidin.

[0099] As used herein a "nucleic acid probe or oligonucleotide" is defined as a nucleic acid capable of binding to a target nucleic acid of complementary sequence through one or more types of chemical bonds, usually through complementary base pairing, usually through hydrogen bond formation. As used herein, a probe may include natural (*i.e.*, A, G, C, or T) or modified bases (7-deazaguanosine, inosine, etc.). In addition, the bases in a probe may be joined by a linkage other than a phosphodiester bond, so long as it does not functionally interfere with hybridization. Thus, *e.g.*, probes may be peptide nucleic acids in which the constituent bases are joined by peptide bonds rather than phosphodiester linkages. It will be understood by one of skill in the art that probes may bind target sequences lacking complete complementarity with the probe sequence depending upon the stringency of the hybridization conditions. The probes are preferably directly labeled as with isotopes, chromophores, lumiphores, chromogens, or indirectly labeled such as with biotin to which a streptavidin complex may later bind. By assaying for the presence or absence of the probe, one can detect the presence or absence of the select sequence or subsequence. Diagnosis or prognosis may be based at the genomic level, or at the level of RNA or protein expression.

[0100] The term "recombinant" when used with reference, *e.g.*, to a cell, or nucleic acid, protein, or vector, indicates that the cell, nucleic acid, protein or vector, has been modified by the introduction of a heterologous nucleic acid or protein or the alteration of a native nucleic acid or protein, or that the cell is derived from a cell so modified. Thus, *e.g.*, recombinant cells express genes that are not found within the native (non-recombinant) form of the cell or express native genes that are otherwise abnormally expressed, under expressed or not expressed at all. By the term "recombinant nucleic acid" herein is meant nucleic acid, originally formed *in vitro*, in general, by the manipulation of nucleic acid, *e.g.*, using polymerases and endonucleases, in a form not normally found in nature. In this manner, operably linkage of different sequences is achieved. Thus an isolated nucleic acid, in a linear form, or an expression vector formed *in vitro* by ligating DNA molecules that are not normally joined, are both considered recombinant for the purposes of this invention. It is understood that once a recombinant nucleic acid is made and reintroduced into a host cell or organism, it will replicate non-recombinantly, *i.e.*, using the *in vivo* cellular machinery of the host cell rather than *in vitro* manipulations; however, such nucleic acids, once produced recombinantly, although subsequently replicated non-recombinantly, are still considered

recombinant for the purposes of the invention. Similarly, a "recombinant protein" is a protein made using recombinant techniques, *i.e.*, through the expression of a recombinant nucleic acid as depicted above.

[0101] The term "heterologous" when used with reference to portions of a nucleic acid indicates that the nucleic acid comprises two or more subsequences that are not normally found in the same relationship to each other in nature. For instance, the nucleic acid is typically recombinantly produced, having two or more sequences, *e.g.*, from unrelated genes arranged to make a new functional nucleic acid, *e.g.*, a promoter from one source and a coding region from another source. Similarly, a heterologous protein will often refer to two or more subsequences that are not found in the same relationship to each other in nature (*e.g.*, a fusion protein).

[0102] The terms "polypeptide," "peptide" and "protein" as used refer to a polymer of amino acid residues. The terms also apply to amino acid polymers in which one or more amino acid residue is an artificial chemical mimetic of a corresponding naturally occurring amino acid, as well as to naturally occurring amino acid polymers, those containing modified residues, and non-naturally occurring amino acid polymer. Polypeptides and peptides of the present invention include amino acid polymers having D- and L- isoforms of individual amino acid residues, as well as other amino acid variants. Peptides are distinguished by the number of amino acid residues making up the primary structure of the molecule. For purpose of this invention, typically, peptides are amino acid polymers comprising up to 50 amino acid residues and polypeptides comprise more than 50 amino acid residues.

[0103] A "promoter" is defined as an array of nucleic acid control sequences that direct transcription of a nucleic acid. As used herein, a promoter includes necessary nucleic acid sequences near the start site of transcription, such as, in the case of a polymerase II type promoter, a TATA element. A promoter also optionally includes distal enhancer or repressor elements, which can be located as much as several thousand base pairs from the start site of transcription.

[0104] A "constitutive" promoter is a promoter that is active under most environmental and developmental conditions. An "inducible" promoter is a promoter that is active under environmental or developmental regulation. The term "operably linked" refers to a functional linkage between a nucleic acid expression control sequence (such as a promoter, or array of transcription factor binding sites) and a second nucleic acid sequence, wherein the

expression control sequence directs transcription of the nucleic acid corresponding to the second sequence.

[0105] The term "psoriasis" as used herein refers to a common chronic, squamous dermatosis with polygenic inheritance and a fluctuating course. Methods of diagnosis are well-known to those in the art. It is a chronic skin disorder characterized by hyperproliferation of the epidermis, inflammation and angiogenesis.

[0106] The term "rheumatoid arthritis" as used herein refers to a chronic systemic disease primarily of the joints, usually polyarticular, marked by inflammatory changes in the synovial membranes and articular structures and by muscle atrophy and rarefaction of the bones. Forms of rheumatoid arthritis include, but are not limited to, juvenile, chronic villous, cricoarytenoid, deformans, degenerative, mutilans, and proliferative.

[0107] The phrase "selectively (or specifically) hybridizes to" refers to the binding, duplexing, or hybridizing of a molecule only to a particular nucleotide sequence under stringent hybridization conditions when that sequence is present in a complex mixture (*e.g.*, total cellular or library DNA or RNA).

[0108] The phrase "stringent hybridization conditions" refers to conditions under which a probe will hybridize to its target subsequence, typically in a complex mixture of nucleic acids, but to no other sequences. Stringent conditions are sequence-dependent and will be different in different circumstances. Longer sequences hybridize specifically at higher temperatures. An extensive guide to the hybridization of nucleic acids is found in Tijssen, *Techniques in Biochemistry and Molecular Biology--Hybridization with Nucleic Probes*, "Overview of principles of hybridization and the strategy of nucleic acid assays" (1993). Generally, stringent conditions are selected to be about 5-10°C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength pH. The T_m is the temperature (under defined ionic strength, pH, and nucleic concentration) at which 50% of the probes complementary to the target hybridize to the target sequence at equilibrium (as the target sequences are present in excess, at T_m, 50% of the probes are occupied at equilibrium). Stringent conditions will be those in which the salt concentration is less than about 1.0 M sodium ion, typically about 0.01 to 1.0 M sodium ion concentration (or other salts) at pH 7.0 to 8.3 and the temperature is at least about 30°C for short probes (*e.g.*, 10 to 50 nucleotides) and at least about 60°C for long probes (*e.g.*, greater than 50 nucleotides). Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide.

For selective or specific hybridization, a positive signal is at least two times background, preferably 10 times background hybridization. Exemplary stringent hybridization conditions can be as following: 50% formamide, 5x SSC, and 1% SDS, incubating at 42°C, or, 5x SSC, 1% SDS, incubating at 65°C, with wash in 0.2x SSC, and 0.1% SDS at 65°C. For PCR, a temperature of about 36°C is typical for low stringency amplification, although annealing temperatures may vary between about 32°C and 48°C depending on primer length. For high stringency PCR amplification, a temperature of about 62°C is typical, although high stringency annealing temperatures can range from about 50°C to about 65°C, depending on the primer length and specificity. Typical cycle conditions for both high and low stringency amplifications include a denaturation phase of 90°C - 95°C for 30 sec - 2 min., an annealing phase lasting 30 sec. - 2 min., and an extension phase of about 72°C for 1 - 2 min. Protocols and guidelines for low and high stringency amplification reactions are provided, *e.g.*, in Innis *et al.* (1990) *PCR Protocols, A Guide to Methods and Applications*, Academic Press, Inc. N.Y.).

[0109] Nucleic acids that do not hybridize to each other under stringent conditions are still substantially identical if the polypeptides which they encode are substantially identical. This occurs, *e.g.*, when a copy of a nucleic acid is created using the maximum codon degeneracy permitted by the genetic code. In such cases, the nucleic acids typically hybridize under moderately stringent hybridization conditions. Exemplary "moderately stringent hybridization conditions" include a hybridization in a buffer of 40% formamide, 1 M NaCl, 1% SDS at 37°C, and a wash in 1 x SSC at 45°C. A positive hybridization is at least twice background. Those of ordinary skill will readily recognize that alternative hybridization and wash conditions can be utilized to provide conditions of similar stringency. Additional guidelines for determining hybridization parameters are provided in numerous reference, *e.g.*, and *Current Protocols in Molecular Biology*, ed. Ausubel *et al.*

[0110] As used herein, the terms "treat", "treating", and "treatment" include: (1) preventing a disease, such as cancer, i.e. causing the clinical symptoms of the disease not to develop in a subject that may be predisposed to the disease but does not yet experience any symptoms of the disease; (2) inhibiting the disease, i.e. arresting or reducing the development of the disease or its clinical symptoms; or (3) relieving the disease, i.e. causing regression of the disease or its clinical symptoms. Treatment means any manner in which the symptoms or pathology of a condition, disorder, or disease are ameliorated or otherwise beneficially

altered. Treatment also encompasses any pharmaceutical use of the compositions herein. Preferably, the subject in need of such treatment is a mammal, more preferable a human.

[0111] "Tumor cell" refers to precancerous, cancerous, and normal cells in a tumor.

[0112] "VEGF" refers to any member of the vascular endothelial growth factor family as well as splice variants and isoforms. VEGF can be found as four different splice variants known as $VEGF_{121}$ (or VEGF-121), $VEGF_{165}$ (or VEGF-165), $VEGF_{189}$ (or VEGF-189) and $VEGF_{206}$ (or VEGF-206; the number refers to the number of amino acids in the polypeptide). All four isoforms exist as disulfide-linked homodimers. The secretion patterns of the isoforms are different in various cell types, although $VEGF_{165}$ is the most common isoform observed. A fifth variant, $VEGF_{145}$, was recently found in three human carcinoma cell lines that originated from the female reproductive tract (Poltorak *et al.*, *J Biol Chem* (1997) 272:7157-7158). The five isoforms bind with high affinity to two receptors, Flt-1 and Flk-1/KDR, but they differ in their binding affinity for heparin and extracellular matrix. Recently, three new members of the VEGF family have been identified, VEGF-B, VEGF-C, and VEGF-D (Achen *et al.*, *Proc Natl Acad Sci USA* (1998), 95(2):548-53). Two splice variants of VEGF-B have been found (Olofsson *et al.*, *J Biol Chem* (1996), 271:19310-19317; Olofsson *et al.*, *Proc Natl Acad Sci USA* (1996), 93:2576-2581) that stimulate the growth of endothelial cells.

[0113] The term "VEGF binding activity" refers to an activity of a VEGFR polypeptide to bind a VEGF polypeptide. Determination of binding is performed using the binding assays described herein and known in the art.

[0114] The "level of VEGF mRNA" in a biological sample refers to the amount of mRNA transcribed from a VEGF gene that is present in a cell or a biological sample. The mRNA generally encodes a functional VEGF polypeptide, although mutations may be present that alter or eliminate the function of the encoded polypeptide. A "level of VEGF mRNA" typically is quantified and compared to a level from a control sample or a level expected of a control sample. However, a "level of VEGF mRNA" can also simply be detected, *e.g.*, a subjective, visual detection by a human, with or without comparison.

[0115] The "level of VEGF polypeptide" in a biological sample refers to the amount of a VEGF polypeptide translated from a VEGF mRNA that is present in a cell or a biological sample. The polypeptide may or may not have VEGF polypeptide activity. A "level of VEGF polypeptide" typically is quantified and compared to a level from a control sample or

a level expected of a control sample. However, a "level of VEGF polypeptide" can also simply be detected, *e.g.*, a subjective, visual detection by a human, with or without comparison.

[0116] As used herein, the phrases "VEGF expression is up-regulated" or "VEGF is overexpressed" and grammatical equivalents thereof refer to a VEGF polypeptide or VEGF polynucleotide above a determined reference level. Thus, for example, in accordance with the present invention, a reference level of VEGF polypeptide or VEGF polynucleotide in a normal or healthy subject is identified as a cut-off value, above which there is a significant correlation between the level of VEGF polypeptide or VEGF polynucleotide and a cancer. Typically, VEGF levels in the serum of cancer patients are at least about 2 times, and in certain cancers, such as ovarian cancer, usually at least about 5 times and more usually at least about 10 times higher than a VEGF level in a normal or healthy person (e.g., see, Manenti *et al.*, *Eur J Cancer* (2003), 39:1948-1956). The terms "up-regulated" and "overexpressed" are used interchangeably herein. Methods for determining VEGF levels are known in the art and include, but are not limited to RT-PCR and use of anti-VEGF antibodies.

[0117] "Correlating the amount" means comparing an amount of a substance, molecule or marker (such as VEGF) that has been determined in one sample to an amount of the same substance, molecule or marker determined in another sample. The amount of the same substance, molecule or marker determined in another sample may be specific for a given disease or cancer.

[0118] Synonyms of the term "determining the amount" are contemplated within the scope of the present invention and include, but are not limited to, detecting, measuring, testing or determining, the presence, absence, amount or concentration of a molecule, such as VEGF.

[0119] The terms "VEGFR" or "VEGF receptor" refer to receptors that bind VEGF or VEGF family members, splice variants and isoforms. The VEGF receptor family of tyrosine kinases is characterized by seven immunoglobin-like sequences in the extracellular domain and a split tyrosine kinase domain. VEGFRs include: (i) Flt-1 (fms-like tyrosine kinase), which is also known as VEGFR-1 (Shibuya *et al.*, *Oncogene* (1990), 5:519-524; De Vries *et al.*, *Science* (1992), 255:989-991); (ii) Flk-1 (fetal liver kinase), the mouse RTK (Quinn *et al.*, *Proc Natl Acad Sci USA* (1993), 90:7533-7537; Millauer *et al.*, *Cell* (1993), 72:835-846) and its human homolog, KDR (kinase insert domain-containing receptor; Terman *et al.*, *Biochem*

Biophys Res Comm (1992),187:1579-1586); and (iii) Flt-4, which is expressed on lymphatic endothelium, but not vascular endothelium (Pajusola *et al.*, *Cancer Res* (1992), 52:5738-43).

[0120] The term "VEGFR-FasL" refers to a fusion protein that comprises (i) an amino acid sequence of a VEGFR, a VEGFR fragment, a VEGFR domain, a VEGFR related polypeptide or a fragment of a VEGFR related polypeptide and (ii) an amino acid sequence of a FasL, a FasL fragment, a FasL related polypeptide or a fragment of a FasL related polypeptide. Typically, the amino acid sequence of a VEGFR, a VEGFR fragment, a VEGFR domain, a VEGFR related polypeptide or a fragment of a VEGFR related polypeptide is fused to the N-terminal amino acid sequence of a FasL, a FasL fragment, a FasL related polypeptide or a fragment of a FasL related polypeptide using standard molecular cloning techniques.

[0121] The terms "VEGFR polypeptide" or VEGFR nucleic acid" refer to isolated nucleic acids, polypeptides and polymorphic variants, alleles, mutants, and interspecies homologues thereof and as further described herein, that: (1) have an amino acid sequence that has greater than about 60% amino acid sequence identity, 65%, 70%, 75%, 80%, 85%, 90%, preferably 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% or greater amino acid sequence identity, preferably over a region of at least about 50, 75, 100, 150, 200, 250, 300 or more amino acids, to (i) an extracellular domain of a vascular endothelial growth factor (VEGF) receptor-1 sequence shown below; (ii) an extracellular domain of a vascular endothelial growth factor (VEGF) receptor-2 sequence shown below; or (iii) an extracellular domain of a vascular endothelial growth factor (VEGF) receptor-3 sequence shown below; (2) bind to antibodies, *e.g.*, polyclonal antibodies, raised against an immunogen comprising an amino acid sequence shown below, or conservatively modified variants thereof; (3) bind a VEGF polypeptide; (4) compete with a naturally occurring VEGF receptor-1, VEGF receptor-2 or VEGF receptor-3 protein for binding a VEGF polypeptide; (5) inhibits binding of VEGF to a VEGF receptor; (6) specifically hybridize under stringent hybridization conditions to a nucleic acid sequence shown below, or conservatively modified variants thereof; (7) have a nucleic acid sequence that has greater than about 90%, preferably greater than about 96%, 97%, 98%, 99%, or higher nucleotide sequence identity, preferably over a region of at least about 100, 200, 300, 400, 500, 600, 700, 800, 900, 1,000 or more nucleotides, to SEQ ID NO: 17 or SEQ ID NO:18 (human VEGFR-2); SEQ ID NO:21 (human VEGFR-1); and/or (8) have at least 50, often 75, 100, 115, 150, 175, 200, 250, or 300 contiguous amino acid residues of SEQ ID NO:1 or SEQ ID NO:4 (human VEGFR-2) or of SEQ ID NO:19 or SEQ ID NO:20 (human VEGFR-1) or SEQ ID NO:24 (human neurphilin-1) or SEQ ID NO:25 (human VEGFR-3).

[0122] A VEGFR polynucleotide or polypeptide sequence is typically from a human, but may be from other mammals, but not limited to, a non-human primate, a rodent, *e.g.*, a rat, mouse, or hamster; a cow, a pig, a horse, a sheep, or other mammal. Therefore, in some embodiments, a VEGFR polypeptide and a VEGFR subdomain polypeptide as described herein can comprise a sequence that corresponds to a human VEGFR sequence. Thus, exemplary VEGFR are provided herein and are known in the art. For example, GenBank accession numbers for human VEGFR-2 polypeptide are NP_002244 and P35968. The GenBank accession numbers for mouse VEGFR-2 polypeptide, for example, is P35918; and for rat VEGFR-2, O08775. A human VEGFR-2 cDNA sequence can be found at GenBank NM_002253. GenBank accession numbers for human VEGFR-1 polypeptide are NP_002010, P17948, AAC16449, CAI17096, and CAI14846. The GenBank accession numbers for mouse VEGFR-1 polypeptide, for example, is NP_034358; and for rat VEGFR-1, NP_062179 and P53767. A human VEGFR-1 cDNA sequence can be found at GenBank NM_002019.

II. VEGFR-DEATH LIGAND FUSION PROTEINS

[0123] The present invention provides novel fusion proteins that bind to a death receptor. A fusion protein of the present invention comprises (i) a vascular endothelial growth factor receptor (VEGFR) polypeptide that binds a vascular endothelial factor (VEGF) polypeptide and (ii) a death ligand comprising an oligomerization domain and a death receptor recognition moiety, wherein the C-terminus of the VEGFR polypeptide is linked to the N-terminus of the death ligand.

A. VEGF Receptor Polypeptides

[0124] VEGFR polypeptides useful for making the fusion proteins of the present invention can be obtained from several VEGFRs, such as Flt-1 (fms-like tyrosine kinase; VEGFR-1), Flk-1 (fetal liver kinase; KDR; VEGFR-2) and Flt-4. For the purpose of the present invention, a VEGFR polypeptide that binds a VEGF, typically comprises an extracellular domain or a portion thereof of a VEGFR.

[0125] A preferred VEGFR polypeptide comprises an extracellular domain or a portion of the extracellular domain of VEGFR-1. Preferably, the VEGFR-1 is a human VGEFR-1. An amino acid sequence of a human VEGFR-1 is shown in SEQ ID NO:19.

[0126] Several VEGFR-1 polypeptides or VEGFR-1 subdomain polypeptides can be linked to a Fas ligand to generate a fusion protein of the present invention. In a preferred embodiment, a VEGFR-1 polypeptide or a VEGFR-1 subdomain polypeptide is selected from the group consisting of (i) a polypeptide comprising the amino acid sequence of SEQ ID NO:20; (ii) a polypeptide comprising amino acid residues 1 to 747 of SEQ ID NO:19; (iii) a polypeptide comprising amino acid residues 32 to 747 of SEQ ID NO:19; (iv) a polypeptide comprising amino acids 151 to214 of the amino acid sequence of SEQ ID NO:19; (v) a polypeptide comprising amino acids 230 to 327 of the amino acid sequence of SEQ ID NO:19; (vi) a polypeptide comprising amino acids 129 to 230 of VEGFR1 (SDTG...NTII as shown in Figure 10; domain 2 (D2)), and (vii) a polypeptide having VEGF binding activity comprising an amino acid sequence wherein one to several amino acid residues have been deleted, substituted, or added in the amino acid sequence of any one of (i) to (vi).

[0127] The phrase "one to several amino acid residues have been deleted, substituted, or added in the amino acid sequence" means that typically less than 25, more typically less than 20, even more typically less than 15 and most typically less than 10 amino acid residues have been deleted, substituted, or added in the amino acid sequence.

[0128] Each of the VEGFR-1 subdomain polypeptides comprises at least one IgG-like domain. An IgG-like domain of human VEGFR-1 comprises amino acid residues 1 to 747 of SEQ ID NO:19; amino acid residues 32 to 747 of SEQ ID NO:19; amino acid residues 32 to123 of SEQ ID NO:19; amino acid residues 151 to214 of SEQ ID NO:19; amino acid residues 230 to 327 of SEQ ID NO:19; amino acids residues 335 to4221 of SEQ ID NO:19; amino acid residues 428 to553 of SEQ ID NO:19; amino acid residues 556 to654 of SEQ ID NO:19; or amino acid residues 661 to747 of SEQ ID NO:19.

[0129] Also preferred are other mammalian VEGFR-1 polypeptides, including, but not limited mouse and rat VEGFR-1 polypeptides. Other mammalian VEGFR-1 subdomain polypeptides can be identified by alignment of the mammalian VEGFR-1 sequence to the human VEGFR-1 amino acid sequence.

[0130] Another preferred VEGFR polypeptide comprises an extracellular domain or a portion of the extracellular domain of VEGFR-2. Preferably, the VEGFR-2 is a human VGEFR-2. In a preferred embodiment of the present invention, a fusion protein comprises a VEGFR-2 polypeptide comprising the amino acid sequence of SEQ ID NO:1. This VEGFR-2 polypeptide comprises the signal peptide of human VEGFR-2. In another preferred

embodiment of the present invention, a fusion protein comprises a VEGFR-2 polypeptide comprising the amino acid sequence of SEQ ID NO:4. This VEGFR-2 polypeptide does not comprises a signal peptide.

[0131] Several VEGFR-2 polypeptides or VEGFR-2 subdomain polypeptides can be linked to a Fas ligand to generate a fusion protein of the present invention. In a preferred embodiment, a VEGFR-2 polypeptide or a VEGFR-2 subdomain polypeptide is selected from the group consisting of (i) a polypeptide comprising the amino acid sequence of SEQ ID NO:1; (ii) a polypeptide comprising the amino acid sequence of SEQ ID NO:2; (iii) a polypeptide comprising the amino acid sequence of SEQ ID NO:3; (iv) a polypeptide comprising the amino acid sequence of SEQ ID NO:4; (v) a polypeptide comprising the amino acid sequence of SEQ ID NO:5; (vi) a polypeptide comprising the amino acid sequence of SEQ ID NO:6; (iiv) a polypeptide comprising amino acids 141 to 207 of the amino acid sequence of SEQ ID NO:1; (iix) a polypeptide comprising amino acids 224 to 320 of the amino acid sequence of SEQ ID NO:1; and (ix) a polypeptide having VEGF binding activity comprising an amino acid sequence wherein one to several amino acid residues have been deleted, substituted, or added in the amino acid sequence of any one of (i) to (iix).

[0132] Each of the VEGFR-2 subdomain polypeptides comprises at least one IgG-like domain. In a preferred embodiment of the present invention a VEGFR-death ligand fusion protein comprises an IgG-like domain of human VEGFR-2 comprising amino acid residues 141 to 207 of the amino acid sequence of SEQ ID NO:1. In another embodiment, an IgG-like domain of human VEGFR-2 comprises amino acid residues 224 to 320 of the amino acid sequence of SEQ ID NO:1.

[0133] VEGFR subdomain polypeptides as described herein can be interchanged. Thus, a VEGFR polypeptide may be designed that comprises one or more IgG-like domain of VEGFR-1, preferably human VEGFR-1 and one or more IgG-like domain of human VEGFR-2, preferably human VEGFR-2.

[0134] In certain embodiments of the present invention a VEGFR is a VEGFR homolog, a VEGFR isoform, a VEGFR ortholog, or a VEGFR related polypeptide.

B. Death Ligand

[0135] The present invention provides novel fusion proteins comprising a VEGFR polypeptide as described above and a death ligand that can bind to a death receptor. The

death ligand that is linked to a VEGFR polypeptide comprises an oligomerization domain and a death receptor recognition moiety.

[0136] Death ligands useful for making the fusion proteins of the present invention are reviewed in Lavrik *et al.*, *J Cell Sci* (2005), 118:265-267 (hereby incorporated in its entirety by reference) and amino acid sequences and nucleotide sequences are available in GenBank. A death ligand comprising an oligomerization domain and a death receptor recognition moiety useful for making the fusion proteins of the present invention can be obtained from several death ligands, such as Fas ligand (FasL), Tumor necrosis factor (TNF) or lymphotoxin (LT). For the purpose of the present invention, a death ligand comprising an oligomerization domain and a death receptor recognition moiety e that binds to a death receptor typically comprises an extracellular domain or a portion thereof of a death ligand.

C. FasL

[0137] In a preferred embodiment of the present invention, the fusion protein binds to Fas and the death ligand is a Fas ligand (FasL). Thus, a preferred fusion protein of the present invention, VEGFR-FasL, comprises a Fas ligand comprising an oligomerization domain and a death receptor recognition moiety. A FasL, within a VEGFR-FasL fusion protein of the present invention contains at least a functional domain or determinants necessary and sufficient to bind to the Fas protein and transmit an apoptotic signal. Typically, such FasL determinants contain only a portion of the extracellular domain, however, they retain the binding specificity of an intact FasL and are soluble.

[0138] A preferred FasL polypeptide for use in the present invention comprises an extracellular domain or a portion of the extracellular domain of FasL. Preferably, the FasL is a human FasL. An amino acid sequence of a human FasL polypeptide comprising a trimerization domain and a binding domain for its receptor, Fas, is shown in SEQ ID NO:11. Thus, in a preferred embodiment a fusion protein of the present invention comprises a FasL comprising an amino acid sequence as shown in SEQ ID NO:11.

[0139] In other preferred embodiments, the FasL is a mammalian FasL and includes, but is not limited to, FasL from mouse or rat. Thus, in one embodiment, a fusion protein of the present invention comprises a murine FasL polypeptide as shown in SEQ ID NO:12. In another embodiment, a fusion protein of the present invention comprises a rat FasL polypeptide as shown in SEQ ID NO:13.

[0140] Another FasL useful for the present invention is a polypeptide having Fas binding activity comprising an amino acid sequence wherein one to several amino acid residues have been deleted, substituted, or added in the amino acid sequence of SEQ ID NO:11, SEQ ID NO:12 or SEQ ID NO:13.

[0141] A death ligand comprises two subdomains, an oligomerization domain and a death receptor recognition moiety. Figure 1 schematically shows a FasL trimerization domain (i.e., the oligomerization domain) and the FasL Fas-binding domain (i.e., the death receptor recognition moiety). As described further herein, TNF shows a similar structure.

[0142] In one aspect of the invention, death ligand subdomain as described herein can be interchanged. Thus, for example, a FasL polypeptide may be designed that comprises a FasL Fas binding domain and a TNF trimerization domain or vice versa. TNF domains and the corresponding amino acid sequences are known in the art and corresponding domains can be identified by one of ordinary skill in the art without undue experimentation (see also below).

[0143] In another aspect, chimeric FasL polypeptides can be designed that comprise, for example, a FasL Fas recognition moiety of a human FasL and a FasL trimerization domain from another mammal, such as mouse or rat.

[0144] For use in human patients, preferably the human Fas ligand will be used. FasL from other species may be used for *in vitro* testing or for *in vivo* testing in, for example, mice and rats.

[0145] FasL fusion proteins or FasL variants are described, for example, in U.S. Pat Nos. 6,451,759; 6,544,523; 6,348,334; 6,235,878; 6,046,310; 6,001,962; U.S. Patent Application Nos. 2004/0126859; 2004/0053249; and 2005/0013816, all of which are explicitly incorporated herewith by reference. In certain embodiments of the present invention FasL is a FasL homolog, a FasL isoform, a FasL ortholog, or a FasL related polypeptide.

D. VEGFR-Death Ligand Fusion Proteins

[0146] As explained herein in detail, the VEGFR-death ligand fusion proteins of the present invention are different from the many agents developed or being developed that target VEGF or its receptors. All of those agents, including neutralizing antibodies, soluble VEGF receptors, RNA aptamers, RNAi, ribozymes, antisense, small molecule kinase inhibitors) are designed to inhibit the expression or activity of VEGF or its receptors. Non of those exploits VEGF overexpression to generate apoptotic activity. The present invention, provides

compositions, such as VEGFR-death ligand fusion proteins that do exactly that - exploiting, for example, a tumor's overexpression of VEGF to use the tumor's own weapon as a death factor, i.e., to induce apoptosis on those tumors expressing a death receptor.

[0147] The present invention provides novel VEGFR-death ligand fusion proteins. A preferred embodiment of the invention is diagrammed in Figure 1. Such an embodiment, wherein a VEGFR polypeptide is linked to a FasL, is denoted VEGFR-FasL. In this embodiment the carboxy-terminus of the VEGFR polypeptide is linked to the amino-terminus of FasL. The FasL will preferably be attached to the carboxy-terminus of the VEGFR polypeptide, but may also be attached elsewhere.

[0148] A VEGFR-death ligand fusion protein, and in particular a VEGFR-FasL fusion protein, as exemplified herein by VEGFR-2-FasL as shown in SEQ ID NO: 22 and in SEQ ID NO:23, is a soluble protein. This soluble protein combines a VEGF-binding domain from an extracellular domain of a VEGFR with the trimerization domain and a death receptor recognition moiety of a death ligand.

[0149] A VEGFR-death ligand fusion protein of the present invention, and in particular a VEGFR-FasL fusion protein, is designed such that VEGF dimmers would bring together VEGFR-death ligand trimers into clusters, which could then bind to, cluster, and activate a death receptor, and in particular Fas. Such an embodiment is schematically depicted in Figure 1.

[0150] Preferred VEGFR-death ligand fusion proteins are depicted in Figures 5 to 8. These preferred VEGFR-death ligand fusion proteins comprise the D1, D2 and D3 domains of Flk.

[0151] Another preferred VEGFR-death ligand fusion protein of the present invention is depicted in Figures 9 and 10. This preferred VEGFR-death ligand fusion protein comprises domain 2 (D2) of VEGFR1.

1. Signal Peptides

[0152] The present invention provides novel fusion proteins comprising a VEGFR polypeptide and a death ligand. Full-length fusion proteins including a signal peptide sequence (e.g., see SEQ ID NO:22) and mature full-length fusion proteins without a signal peptide (e.g., SEQ ID NO:23) are useful for practicing the methods of this invention and find use as compositions in the pharmaceutical compositions and kits of this invention.

[0153] Thus, the fusion proteins of the present invention may or may not comprise a signal peptide sequence depending on their intended use and mode of production (see further herein). The signal peptide sequence may be a homologous signal peptide sequence, i.e., a signal peptide which is normally found at the N-terminus of a secreted protein (e.g., see Figure 3). Alternatively, for example, a murine VEGFR signal sequence can replace a human signal VEGFR sequence or vice versa, depending on the expression system used to generate the fusion proteins of this invention.

2. Linker Sequences

[0154] Optionally, VEGFR-FasL comprises a linker. Such a polypeptide linker between the C-terminal VEGFR and the N-terminal FasL is preferably made so as to allow binding of a VEGF polypeptide to the VEGFR polypeptide and dimerization or trimerization of FasL and binding of the VEGFR-FasL to Fas. The linker may contain from 1 to about 100 amino acid residues, preferably 5-50. In a preferred embodiment a linker sequence comprises the 5 amino acid residues as shown in SEQ ID NO: 7. Linker sequences that can be inserted between the VEGFR polypeptide and a death ligand polypeptide are not critical. Other preferred linker sequences comprise Gly linkers or Gly/Ser linkers.

E. Other Growth Factor/Death Ligand Proteins

[0155] The basic principle of the present invention, i.e., converting a growth factor activity of a growth factor, as exemplified herein by VEGF, into a death factor, can be applied to other growth factors as well. In these embodiments, the VEGFR polypeptide would be replaced by a binding domain for another growth factor, for example, platelet derived growth factor (PDGF). Thus, without undue experimentation and with a reasonable expectation of success, an ordinary skill in the art will be able, by following this disclosure and obtaining relevant information pertaining to sequences of growth factors, or growth factor receptors from public data bases, such as GenBank or obtaining cloned DNAs encoding such growth factors or growth factor receptors from the American Type Culture Collection (ATCC).

F. Other Death Receptors/Death Ligand Proteins

[0156] The fusion proteins of the present invention bind to a death receptor, preferably to a death receptor on the surface of a cell expressing the death receptor. Death receptors useful as targets in the methods of the present invention typically belong to the TNF superfamily of receptors and include the p55 and p75 tumor necrosis factor receptor (TNFRs) and Fas (also called FAS/APO1). Tumor Necrosis Factor (TNF-α) and lymphotoxin (TNF-β) bind to both

p55 and p75 and thereby initiate events leading to the death of, for example, a tumor cell expressing p55 or p75.

[0157] Thus, in another aspect of the present invention, a VEGFR-death ligand fusion protein comprises a VEGFR polypeptide as described herein, and a TNF polypeptide comprising an oligomerization domain and a death receptor recognition moiety for binding to TNFR. In another embodiment, a VEGFR-death ligand fusion protein comprises a VEGFR polypeptide and a LT polypeptide comprising an oligomerization domain and a death receptor recognition moiety for binding to TNFR.

G. Nucleic Acid Encoding VEGFR-FasL Fusion Protein

[0158] In another aspect, the invention is directed to recombinant nucleic acids that encode all or part of a VEGFR-FasL fusion protein. In a preferred embodiment of the present invention, a nucleic acid that encodes all or part of a VEGFR-FasL fusion protein comprises the nucleotide sequence shown in Figure 7. Another preferred nucleic acid that encodes all or part of a VEGFR-FasL fusion protein comprises the nucleotide sequence shown in Figure 9. Other preferred nucleic acids encoding all or part of a VEGFR-FASL fusion protein are those shown in Figures 7 and 9 in which the sequences encoding the linker sequence or the FLAG-tag are not included. Such nucleic acids can be generated by one of skill in the art using PCR and appropriately designed PCR primers.

[0159] In general, nucleic acid sequences encoding a VEGFR-death ligand fusion protein and related nucleic acid sequence homologs are cloned from cDNA and genomic DNA libraries by hybridization with a probe, or isolated using amplification techniques with oligonucleotide primers. For example, sequences are typically isolated from mammalian nucleic acid (genomic or cDNA) libraries by hybridizing with a nucleic acid probe.

[0160] Amplification techniques using primers can also be used to amplify and isolate nucleic acids from DNA or RNA. Suitable primers for amplification of specific sequences can be designed using principles well known in the art (*see, e.g.*, Dieffenfach & Dveksler, *PCR Primer: A Laboratory Manual* (1995)). These primers can be used, *e.g.*, to amplify either the full length sequence or a fragment of a VEGFR polypeptide or a death ligand.

[0161] Synthetic oligonucleotides can also be used to construct VEGFR-death ligand encoding genes for use as probes or for expression of protein. This method is performed using a series of overlapping oligonucleotides usually 40-120 bp in length, representing both the sense and nonsense strands of the gene. These DNA fragments are then annealed, ligated

and cloned. Alternatively, amplification techniques can be used with precise primers to amplify a specific subsequence of the nucleic acid. The specific subsequence is then ligated into an expression vector.

[0162] The nucleic acid encoding a VEGFR-death ligand fusion protein is typically cloned into intermediate vectors before transformation into prokaryotic or eukaryotic cells for replication and/or expression. These intermediate vectors are typically prokaryotic vectors, *e.g.*, plasmids, or shuttle vectors.

[0163] Optionally, nucleic acids encoding chimeric proteins comprising VEGFR, death ligands or domains thereof can be made according to standard techniques.

H. Expression of VEGFR-Death Ligand Fusion Proteins

[0164] To obtain high level expression of a VEGFR-death ligand nucleic acid, one typically subclones a VEGFR-death ligand nucleic acid into an expression vector that contains a strong promoter to direct transcription, a transcription/translation terminator, and if for a nucleic acid encoding a protein, a ribosome binding site for translational initiation. Suitable bacterial promoters are well known in the art and described, *e.g.*, in Sambrook & Russell, *Molecular Cloning, A Laboratory Manual* (3rd Ed, 2001), Ausubel *et al.* (eds.), *Current Protocols in Molecular Biology*, John Wiley & Sons, NY (1993); and Kriegler, *Gene Transfer and Expression, A Laboratory Manual*, Stockton Press, NY (1990). Bacterial expression systems for expressing the VEGFR-death ligand protein are available in, *e.g.*, *E. coli*, *Bacillus sp.*, and *Salmonella* (Palva *et al.*, *Gene* 22:229-235 (1983); Mosbach *et al.*, *Nature* 302:543-545 (1983). Kits for such expression systems are commercially available. Eukaryotic expression systems for mammalian cells, yeast, and insect cells are well known in the art and are also commercially available. In one embodiment, the eukaryotic expression vector is an adenoviral vector, an adeno-associated vector, or a retroviral vector.

[0165] The promoter used to direct expression of a heterologous nucleic acid depends on the particular application. The promoter is optionally positioned about the same distance from the heterologous transcription start site as it is from the transcription start site in its natural setting. As is known in the art, however, some variation in this distance can be accommodated without loss of promoter function.

[0166] In addition to the promoter, the expression vector typically contains a transcription unit or expression cassette that contains all the additional elements required for the expression of the VEGFR-death ligand-encoding nucleic acid in host cells. A typical

expression cassette thus contains a promoter operably linked to the nucleic acid sequence encoding a VEGFR-death ligand and signals required for efficient polyadenylation of the transcript, ribosome binding sites, and translation termination. The nucleic acid sequence encoding a VEGFR-death ligand may typically be linked to a cleavable signal peptide sequence to promote secretion of the encoded protein by the transformed cell. Such signal peptides would include, among others, the signal peptides from tissue plasminogen activator, insulin, and neuron growth factor, and juvenile hormone esterase of *Heliothis virescens*. Additional elements of the cassette may include enhancers and, if genomic DNA is used as the structural gene, introns with functional splice donor and acceptor sites.

[0167] In addition to a promoter sequence, the expression cassette should also contain a transcription termination region downstream of the structural gene to provide for efficient termination. The termination region may be obtained from the same gene as the promoter sequence or may be obtained from different genes.

[0168] The particular expression vector used to transport the genetic information into the cell is not particularly critical. Any of the conventional vectors used for expression in eukaryotic or prokaryotic cells may be used. Standard bacterial expression vectors include plasmids such as pBR322 based plasmids, pSKF, pET23D, and fusion expression systems such as GST and LacZ. Epitope tags can also be added to recombinant proteins to provide convenient methods of isolation, *e.g.*, c-myc.

[0169] Expression vectors containing regulatory elements from eukaryotic viruses are typically used in eukaryotic expression vectors, *e.g.*, SV40 vectors, papilloma virus vectors, and vectors derived from Epstein-Barr virus. Other exemplary eukaryotic vectors include pMSG, pAV009/A^+, pMTO10/A^+, pMAMneo-5, baculovirus pDSVE, and any other vector allowing expression of proteins under the direction of the SV40 early promoter, SV40 later promoter, metallothionein promoter, murine mammary tumor virus promoter, Rous sarcoma virus promoter, polyhedrin promoter, or other promoters shown effective for expression in eukaryotic cells.

[0170] Some expression systems have markers that provide gene amplification such as thymidine kinase, hygromycin B phosphotransferase, and dihydrofolate reductase. Alternatively, high yield expression systems not involving gene amplification are also suitable, such as using a baculovirus vector in insect cells, with a VEGFR-death ligand - encoding sequence under the direction of the polyhedrin promoter or other strong baculovirus promoters.

[0171] The elements that are typically included in expression vectors also include a replicon that functions in *E. coli*, a gene encoding antibiotic resistance to permit selection of bacteria that harbor recombinant plasmids, and unique restriction sites in nonessential regions of the plasmid to allow insertion of eukaryotic sequences. The particular antibiotic resistance gene chosen is not critical, any of the many resistance genes known in the art are suitable. The prokaryotic sequences are optionally chosen such that they do not interfere with the replication of the DNA in eukaryotic cells, if necessary.

[0172] Standard transfection methods are used to produce bacterial, mammalian, yeast or insect cell lines that express large quantities of a VEGFR-death ligand protein, which are then purified using standard techniques (*see, e.g.*, Colley *et al., J. Biol. Chem.* 264:17619-17622 (1989); *Guide to Protein Purification*, in *Methods in Enzymology*, vol. 182 (Deutscher, ed., 1990)). Transformation of eukaryotic and prokaryotic cells are performed according to standard techniques (*see, e.g.*, Morrison, *J. Bact.* 132:349-351 (1977); Clark-Curtiss & Curtiss, *Methods in Enzymology* 101:347-362 (Wu *et al.*, eds, 1983).

[0173] Any of the well known procedures for introducing foreign nucleotide sequences into host cells may be used. These include the use of calcium phosphate transfection, polybrene, protoplast fusion, electroporation, liposomes, microinjection, plasma vectors, viral vectors and any of the other well known methods for introducing cloned genomic DNA, cDNA, synthetic DNA or other foreign genetic material into a host cell (*see, e.g.*, Sambrook and Russell., *supra*). It is only necessary that the particular genetic engineering procedure used be capable of successfully introducing at least one gene into the host cell capable of expressing a VEGFR-death ligand. Introduction of a VEGFR-FasL nucleic acid into a cell can be performed by transient transfection or stable transfection (Examples 4 and Figures 11a and 11B).

[0174] After the expression vector is introduced into the cells, the transfected cells are cultured under conditions favoring expression of VEGFR-death ligand, which is recovered from the culture using standard techniques (*see, e.g.*, Scopes, *Protein Purification: Principles and Practice* (1982); U.S. Patent No. 4,673,641; Ausubel *et al.*, *supra*; and Sambrook *et al.*, *supra*). Generation of useful VEGFR-FasL fusion proteins is described in Example 4 and Figures 11A and 11B.

I. Purification of VEGFR-FasL Fusion Protein

[0175] VEGFR-death ligand fusion proteins of the present invention can be purified using methods well known in the art, e.g., Sambrook & Russell, *Molecular Cloning, A Laboratory*

Manual (3rd Ed, 2001); Kriegler, *Gene Transfer and Expression: A Laboratory Manual* (1990); and *Current Protocols in Molecular Biology* (Ausubel *et al.*, eds., 1994-1999).

[0176] VEGFR-death ligand fusion protein of the present invention can thus be purified by conventional protein purification methods. Alternatively, a tag attached to the VEGFR-death ligand fusion protein will be employed for protein purification. Tags are known in the art and include, but are not limited to FLAG- and HA-tags. Typically, coding sequences for these tags are linked to a VEGFR-death ligand coding sequence and are expressed when the transcribed RNA is translated. A preferred tag is a FLAG tag. Preferred embodiments of the present invention comprising a FLAG-tagged VEGFR-death ligand fusion protein are shown in Figures 6 and 11 and described in detail in Examples 3 and 4.

[0177] In a preferred embodiment a cleavable peptide sequence is inserted in between the tag and the VEGFR-death ligand fusion protein. This is particularly advantageous for cleaving off the tag after protein purification.

J. Binding of VEGF to VEGFR-FasL

[0178] VEGFR fusion proteins of the invention bind vascular endothelial growth factor (VEGF). Bioassays to monitor binding of VEGF to the extracellular domain of VEGF receptors are known in the art and are described herein. For example, Achen *et al.* (incorporated hereby by reference) describe a bioassay to monitor the binding a VEGF ligand to the extracellular domain of VEGFR-2 and binding assays with soluble VEGFR extracellular domains. Binding of a VEGF to a VEGFR fusion protein of the present invention can be determined using the assays of Achen *et al.* (*Proc Natl Acad Sci USA* (1998), 95:548-553).

[0179] Binding of VEGF to a VEGFR-FasL fusion protein of the present invention activates the VEGFR-FasL fusion protein. A VEGFR-FasL fusion protein of the present invention may be activated by human VEGF, mouse VEGF (Example 11) or any other mammalian VEGF. In addition, a VEGFR-FasL fusion protein of the present invention may be activated by a VEGF/PlGF heterodimer or by PlGF (Example 11).

K. Binding of VEGFR-FasL to Fas

[0180] VEGFR-FasL fusion proteins of the invention bind to Fas. Bioassays to monitor FasL-Fas binding are known in the art and are described herein. For example, Schneider *et al.* (incorporated hereby by reference) describe cytotoxic assays and in vitro Fas-FasL

binding assays to monitor the binding a FasL to the extracellular domain of Fas. Binding of a VEGFR-FasL of the present invention to Fas can be determined using the assays of Schneider *et al.* (*J Biol Chem* (1997), 272(30):18827-18833).

[0181] In a preferred embodiment, a VEGFR-FasL fusion protein will have a greater effectiveness relative to soluble FasL or its extracellular domain to induce apoptosis or death in Fas-expressing cancer cells.

III. METHOD FOR NEUTRALIZATION OF VEGF ACTIVATION OF VEGF RECEPTORS USING A VEGFR-DEATH LIGAND FUSION PROTEIN

[0182] VEGFR-death ligand fusion proteins of the present invention find use in a variety of ways. For example, a VEGFR-death ligand fusion protein, in particular a VEGFR-FasL fusion protein, can be used as an anti-cancer agent for tumors that overexpress VEGF. A VEGFR-death ligand fusion protein of the present invention can also be used as an anti-angiogenic agent for use in diseases characterized by pathologic angiogenesis, such as cancer, rheumatoid arthritis, or proliferative retinopathy.

[0183] In a preferred embodiment of this invention, a method of neutralizing a VEGF activation of a VEGF receptor on using a VEGFR-death ligand fusion protein is provided. This method comprises the step of contacting a VEGF with a VEGFR-death ligand fusion protein.

[0184] Neutralization of VEGF activation of a VEGF receptor on cells, such as tumor cells, may be performed *in vitro* or *in vivo*. Neutralization of VEGF activation of a VEGF receptor comprises contacting a biological sample comprising a cell expressing a VEGF receptor on its cell surface with a fusion protein of this invention. *In vitro*, the biological sample, can be contacted with the fusion protein, e.g., VEGFR-FasL before, simultaneously with, or after, adding VEGF.

[0185] *In vivo*, a fusion protein of this invention is contacted with a biological fluid, such as blood, or a tumor by administration to a mammal. This in vivo neutralization method is useful for inhibiting or preventing angiogenesis in a mammal associated with pathological conditions such as tumor growth. Thus, the fusion proteins of the present invention, such as VEGFR-FasL are anti-angiogenic and anti-tumor therapeutics.

[0186] This method is effective for treating subjects with tumors and neoplasms, including malignant tumors and neoplasms, such as blastomas, carcinomas or sarcomas, and especially

highly vascular tumors and neoplasms. Some examples of tumors that can be treated with the antibodies and fragments of the invention include epidermoid tumors, squamous tumors, such as head and neck tumors, colorectal tumors, prostate tumors, breast tumors, lung tumors, including small cell and non-small cell lung tumors, pancreatic tumors, thyroid tumors, ovarian tumors, and liver tumors.

IV. METHODS FOR INDUCING APOPTOSIS USING A VEGFR-DEATH LIGAND FUSION PROTEIN

[0187] A VEGFR-death ligand fusion protein of the present invention finds use in a variety of ways. In a preferred embodiment of this invention, a method of reversing the activity of a VEGF from an angiogenic factor into a cell death factor is provided. This method is based on the observation that VEGF is overexpressed by many cancers, and in particular human cancers. Cancers overexpressing VEGF include, but are not limited to, glioma, melanoma, gastric cancer, Kaposi sarcoma, epidermoid carcinoma, hemangioblastoma, breast cancer, colon cancer, renal cell tumors, pituitary tumors, lung cancer and prostate cancer. A preferred cancer is a glioblastoma. Another preferred cancer is prostate cancer.

[0188] The concept of this method is to make the VEGF that is overexpressed by a tumor act as a death factor against the tumor itself or its blood vessels. In essence, to turn the tumor's weapon, overexpressed VEGF, which it needs to sustain growth and metastasize, against the tumor itself. Thus, the compositions of the present invention are useful to induce apoptosis or induce cytotoxic effects in cancer cells.

[0189] This method comprises the step of contacting a VEGF with a VEGFR-death ligand fusion protein of the present invention. This method further comprises the step of contacting a death receptor on the surface of a cell with a VEGFR-death ligand fusion protein of the present invention to which a VEGF polypeptide has bound. Thus, in this embodiment, without binding a VEGF polypeptide, a VEGFR-death ligand fusion protein will not bind to a death receptor.

[0190] The compositions of the present invention are also useful to induce apoptosis or induce cytotoxic effects in cells other than cancer cells. For example, the VEGFR-FasL fusion proteins of the present invention are useful for inducing apoptosis and cytotoxic effects in microvascular endothelial cells, such as adrenal cortical endothelial cells.

[0191] Upon binding of the VEGFR-death ligand fusion protein (having bound a VEGF polypeptide) to a death receptor on a cell, apoptosis, i.e., cell death, is induced. The VEGFR-death ligand fusion protein binds to the death receptor via a death receptor recognition moiety as described herein. For example a FasL which is produced as a trimer, induces apoptosis by binding to, clustering and thereby activating Fas. A VEGFR-FasL fusion protein of the present invention comprising a FasL polypeptide comprising an oligomerization domain and a death receptor recognition moiety induces apoptosis in the same manner. Thus, an important feature is that Fas clustering by FasL or by a VEGFR-FasL fusion protein is required for Fas activation.

[0192] Preferably, a VEGFR-ligand fusion protein of the present invention has no or minimal apoptotic activity in the absence of VEGF. This has been demonstrated in several *in vitro* experiments and is shown exemplary in Figures 11, 13, 14 and 15.

[0193] Preferably, a VEGFR-ligand fusion protein of the present invention has increased apoptotic activity in the absence of VEGF. This has been demonstrated in several *in vitro* experiments and is shown exemplary in Figures 11, 13, 14 and 15. An "increased apoptotic activity" in this context means at least a two-fold stimulation as compared to without VEGF, preferably a three-fold stimulation, more preferably a five-fold

[0194] In a preferred embodiment, the method for inducing apoptosis in a cell wherein VEGF expression is up-regulated and wherein the cell expresses a death receptor comprises the step of exposing the cell to a composition or contacting the cell with a composition comprising a VEGFR-death ligand fusion protein or a polynucleotide encoding a VEGFR-death ligand fusion protein as described herein. In a preferred embodiment, the polynucleotide encodes the VEGFR-death ligand fusion protein of SEQ ID NO:22 or SEQ ID NO:23.

[0195] In another preferred embodiment of the present invention, the polynucleotide comprises SEQ ID NO:14.

[0196] In one aspect of the present invention, a VEGFR-death ligand fusion protein or polynucleotide is used to induce apoptosis *in vitro*, e.g., in a cultured cell line. In another preferred aspect, the VEGFR-death ligand fusion protein or polynucleotide is used to induce apoptosis *in vivo*, i.e., in an animal, preferably a mammal, including human, and preferably in cancer cells.

V. METHOD FOR TREATING A CANCER OVEREXPRESSING VEGF AND EXPRESSING FAS RECEPTOR

A. Measuring of VEGF in Normal Individuals and in Cancer Patients

[0197] Bioassays for measuring or determining VEGF in normal individuals and in cancer patients have been described in the prior art and are useful to determine levels of endogenous VEGF in an individual prior to administering a VEGFR-death ligand fusion protein of the present invention. For example, Cooper *et al.* assessed the clinical relevance of serum VEGF levels in distinguishing patients with ovarian cancer from those with benign adnexal masses and concluded that preoperative VEGF levels may be useful in differentiating benign adnexal masses from malignancy (*Clin Cancer Res*, (2002) 8(10):3193-7).

B. Measuring Fas Receptor Expressed in a Cancer Cell

[0198] Bioassays for measuring or determining Fas receptor expressed in a cancer cell have been described in the prior art and are useful to determine whether a targeted cancer cell expresses Faso e any other death receptor and thus, is susceptible for induction of apoptosis by a VEGFR-death ligand fusion protein of the present invention. Determining whether the cancer in a patient expresses Fas or any other death receptor is desirable prior to administering a VEGFR-death ligand fusion protein of the invention.. For example, the bioassay described by Algeciras-Schimnich *et al.*, *Proc Natl Acad Sci USA*, (2003) 100:11445) can be used.

C. Method For Treating Cancer

[0199] Methods of the present invention comprise treating a cancer cell wherein VEGF is up-regulated and wherein the cancer cell expresses a death receptor. The method typically comprises inducing apoptosis using a VEGFR-death ligand fusion protein of the present invention. A preferred cancer cell is selected from the group consisting of breast cancer, prostate cancer, colon cancer, lung cancer, glioblastoma, and ovarian cancer.

[0200] This method is effective for treating subjects with tumors and neoplasms, including malignant tumors and neoplasms, such as blastomas, carcinomas or sarcomas, and especially highly vascular tumors and neoplasms. Some examples of tumors that can be treated with the antibodies and fragments of the invention include epidermoid tumors, squamous tumors, such as head and neck tumors, colorectal tumors, prostate tumors, breast tumors, lung tumors, including small cell and non-small cell lung tumors, pancreatic tumors, thyroid tumors, ovarian tumors, and liver tumors.

[0201] The present invention provides a method for treatment of a cancer wherein VEGF expression is up-regulated. This method comprises the step of administering to a patient a pharmaceutical composition. Such pharmaceutical compositions comprise, for example, a VEGFR-death ligand fusion protein, a VEGFR-death ligand fusion protein analog, a VEGFR-death ligand fusion protein mimetic, a VEGFR-death ligand fusion protein related polypeptide; or a polynucleotide encoding a VEGFR-death ligand fusion protein, a VEGFR-death ligand fusion protein analog, a VEGFR-death ligand fusion protein mimetic, a VEGFR-death ligand fusion protein related polypeptide. Pharmaceutical compositions of the present invention are administered alone or in combination with one or more additional therapeutic compounds or treatments. Examples of such therapeutic compounds or treatments include, but are not limited to, taxol, cyclophosphamide, tamoxifen, fluoruracil and doxorubicin.

D. Inhibition of Cell Proliferation

[0202] A VEGFR-death ligand fusion protein of the present invention finds use in a variety of ways. In a preferred embodiment of this invention, a method of inhibiting proliferation of a cell that overexpresses VEGF is provided. "Proliferation" refers to the growth of a cell, the reproduction or multiplication of a cell or morbid cysts. The VEGF that is overexpressed can be either a VEGF polypeptide or a VEGF mRNA. This method comprises the step of contacting the cell with an amount of a VEGFR-death ligand fusion protein effective to inhibit proliferation of the cell.

[0203] In a preferred embodiment of the present invention, this method is practiced *in vitro*. As further described herein, methods of the present invention can also be practiced *in vivo*.

VI. METHODS FOR TREATING DISEASES AND DISORDERS RELATED TO UNREGULATED ANGIOGENESIS AND/OR VASCULOGENESIS USING A VEGFR-FASL FUSION PROTEIN

[0204] Preventing or inhibiting angiogenesis is also useful to treat diseases, disorders and non-neoplastic pathologic conditions, related to unregulated angiogenesis and/or vasculogenesis, such as rheumatoid arthritis, neovascular glaucoma, proliferative retinopathy including proliferative diabetic retinopathy, macular degeneration, hemangiomas, angiofibromas, and psoriasis. Compositions of this invention are useful to treat such diseases.

[0205] VEGFR-death ligand fusion proteins of the present invention find use in a variety of ways. In another preferred embodiment of this invention a method of treating a disease

associated with overexpression of VEGF or pathologic angigenesis is provided. This method comprises the step of administering to a subject, preferably to a subject in need of such treatment, an amount of a polypeptide having VEGFR-death ligand fusion protein activity effective for treating the disease. Preferably, the subject is a human.

A. Rheumatoid Arthritis

[0206] In a preferred embodiment of the present invention, the disease treated with a VEGFR-death ligand fusion protein of the present invention or with a polynucleotide encoding a VEGFR-death ligand fusion protein, is rheumatoid arthritis. Rheumatoid arthritis (RA) is an inflammatory joint disease that is characterized by cellular infiltration of synovial fluid by neutrophils, and of the synovial membrane by T lymphocytes and macrophages, hyperproliferation of cells of the synovial membrane which results in formation of a pannus, and destruction of cartilage and bone (Feldman *et al.*, *Ann Rev Immunol* (1996), 14:397-440; Paleolog, *Br J Rheumatol* (1996), 35:917-920). Angiogenesis is thought to have an important role in the pathogenesis of RA (Colville-Nash & Scott, *Annals Rheumatic Diseases* (1992), 51:919-925, and references therein).

[0207] The strongest evidence for a role as a direct angiogenic factor in RA exists for VEGF. VEGF expression is significantly higher in synovial fluid and tissue from RA patients than from patients with other types of arthritis (Fava, et al., J. Exp. Med. (1994) 180:341-346; Koch *et al.*, *J Immunol* (1994) 152:4149-4156). The source of this VEGF appears to be elevated expression in synovial lining cells, subsynovial macrophages, fibroblasts surrounding microvessels, and vascular smooth muscle cells (Fava, et al., J. Exp. Med. (1994), 180:341-346; Koch *et al.*, *J Immunol* (1994), 152:4149-4156; Nagashima *et al.*, *J Rheumatol* (1995), 22:1624-1630). Indirect induction of VEGF by other factors may occur as well.

B. Psoriasis

[0208] In another preferred embodiment of the present invention, the disease treated with a VEGFR-death ligand fusion protein of the present invention or with a polynucleotide encoding a VEGFR-death ligand fusion protein, is psoriasis. Psoriasis is a chronic skin disorder that is characterized by hyperproliferation of the epidermis, inflammation, and angiogenesis. Angiogenesis appears to be crucial in the pathogenesis of psoriasis, and microvascular changes are one of the earliest detectable events in developing psoriatic lesions (for a review see Creamer & Barker, *Clin Exp Dermatol* (1995), 20:6-9). Several reports

have implicated the epidermis as the origin of angiogenic factors (Nishioka & Ryan, *J Invest Dermatol* (1972), 58:33-45; Wolf & Harrison, *J Invest Dermatol* (1973), 59:40-43; Barnhill *et al.*, *Br J Dermatol* (1984), 110:273-281; Malhotra *et al.*, *Lab Invest* (1989), 61:162-165).

[0209] Of the many angiogenic factors identified in skin (Arbiser, *Am Acad Derm* (1996), 34:486-497), VEGF has been the best characterized as a direct inducer of angiogenesis. VEGF is overexpressed in keratinocytes of psoriatic skin, but only minimally expressed in normal epidermis (Detmar *et al.*, *J Exp Med* (1994), 180:1141-1146). VEGF is also overexpressed in other skin diseases such as bullous pemphigoid, dermatitis herpetiformis, and erythema multiforme (Brown *et al.*, *Invest Dermatol* 1995, 104, 744-749), in delayed skin hypersensitivity reactions (Brown *et al.*, *J Immunol* 1995, 154, 2801-2807), and probably after sun exposure, as suggested by the induction of VEGF expression in cultured keratinocytes following exposure to ultraviolet light (Brauchle *et al.*, *J Biol Chem* (1996), 271:21793-21797).

C. Macular Degeneration

[0210] In a preferred embodiment of the present invention, the disease treated with a VEGFR-death ligand fusion protein of the present invention or with a polynucleotide encoding a VEGFR-death ligand fusion protein, is macular degeneration. The release of angiogenic factors from the ischemic retina has been hypothesized to be the central stimulus for retinal neovascularization. Glaucoma, vitreous hemorrhage and retinal detachment, secondary to intraocular neovascularization, accounts for the resultant vision loss in several ocular disorders such as retinopathy of prematurity, age-related macular degeneration, and diabetic retinopathy. The release of angiogenic factors by the ischemic retina to induce new blood vessel growth and increase the oxygen supply to the area turns out to be harmful as the new vessels do not grow with normal architecture. Edema, hemorrhage, vessel tortuosity, and pathological neovascularization subsequently result in retinal detachment and lead to blindness.

[0211] VEGF is constitutively expressed in the vascularized tissues of the normal eye (Adamis *et al.*, *Arch Ophthalmol* (1996), 114:66-71), however, intraocular VEGF gene expression is increased in disease states like diabetic retinopathy (Adamis *et al.*, *Amer J Ophthalmology* (1994), 118:445-450; Malecaze *et al.*, *Arch Ophthalmology* (1994), 112:1476-1482).

VII. COMBINATION THERAPIES

[0212] As detailed herein, the invention provides methods for using VEGFR-death ligand fusion proteins for neutralizing VEGF activation of VEGF receptors. These methods are particularly useful for inducing apoptosis, inducing cytotoxic effects in cells, treating cancer and diseases or disorders related to unregulated angiogenesis and/or vasculogenesis. In a preferred embodiment of the present invention, each of these methods may further comprise administering to a patient a second therapeutic agent, such as a chemotherapeutic agent or radiation therapy.

[0213] Examples of chemotherapeutic agents include, but are not limited to, daunorubicin, daunomycin, dactinomycin, doxorubicin, epirubicin, idarubicin, esorubicin, bleomycin, mafosfamide, ifosfamide, cytosine arabinoside, bis-chloroethylnitrosurea, busulfan, mitomycin C, actinomycin D, mithramycin, prednisone, hydroxyprogesterone, testosterone, tamoxifen, dacarbazine, procarbazine, hexamethylmelamine, pentamethylmelamine, mitoxantrone, amsacrine, chlorambucil, methylcyclohexylnitrosurea, nitrogen mustards, melphalan, cyclophosphamide, 6-mercaptopurine, 6-thioguanine, cytarabine (CA), 5-azacytidine, hydroxyurea, deoxycoformycin, 4-hydroxyperoxycyclophosphor- amide, 5-fluorouracil (5-FU), 5-fluorodeoxyuridine (5-FUdR), methotrexate (MTX), colchicine, taxol, vincristine, vinblastine, etoposide, trimetrexate, teniposide, cisplatin and diethylstilbestrol (DES). See, generally, The Merck Manual of Diagnosis and Therapy, 15th Ed. 1987, pp. 1206-1228, Berkow et al., eds., Rahway, N.J.

[0214] In a preferred embodiment of the present invention, a chemotherapeutic agent is selected from the group consisting of camptothecin, etoposide, bisindolylmaleimide VIII, cisplatin, taxol, doxorubicin, temozolomide, bortezomid, LY294002, and valproic acid.

VIII. ADMINISTERING A VEGFR-DEATH LIGAND FUSION PROTEIN

[0215] In one aspect of the present invention, a nucleic acid molecule that express a VEGFR-death ligand fusion protein, such as a VEGFR-FasL as described in detail herein, may be used to introduce that nucleic acid into a mammalian cell or target tissue. Conventional viral and non-viral based gene transfer methods can be used to introduce nucleic acids encoding VEGFR-death ligand fusion proteins in mammalian cells or target tissues. Non-viral vector delivery systems include DNA plasmids, naked nucleic acid, and nucleic acid complexed with a delivery vehicle such as a liposome. Viral vector delivery

systems include DNA and RNA viruses, which have either episomal or integrated genomes after delivery to the cell. For a review of gene therapy procedures, *see*, Anderson, *Science* 256:808-813 (1992); Nabel & Felgner, *TIBTECH* 11:211-217 (1993); Mitani & Caskey, *TIBTECH* 11:162-166 (1993); Dillon, *TIBTECH* 11:167-175 (1993); Miller, *Nature* 357:455-460 (1992); Van Brunt, *Biotechnology* 6(10):1149-1154 (1988); Vigne, *Restorative Neurology and Neuroscience* 8:35-36 (1995); Kremer & Perricaudet, *British Medical Bulletin* 51(1):31-44 (1995); Haddada *et al.*, in *Current Topics in Microbiology and Immunology* Doerfler and Böhm (eds) (1995); and Yu *et al.*, *Gene Therapy* 1:13-26 (1994).

A. Non-viral Delivery Methods

[0216] Methods of non-viral delivery of nucleic acids encoding engineered polypeptides of the invention include lipofection, microinjection, biolistics, virosomes, liposomes, immunoliposomes, polycation or lipid:nucleic acid conjugates, naked DNA, artificial virions, and agent-enhanced uptake of DNA. Lipofection is described in *e.g.*, US 5,049,386, US 4,946,787; and US 4,897,355) and lipofection reagents are sold commercially (*e.g.*, Transfectam™ and Lipofectin™). Cationic and neutral lipids that are suitable for efficient receptor-recognition lipofection of polynucleotides include those of Felgner, WO 91/17424, WO 91/16024. Delivery can be to cells (*ex vivo* administration) or target tissues (*in vivo* administration).

[0217] The preparation of lipid:nucleic acid complexes, including targeted liposomes such as immunolipid complexes, is well known to one of skill in the art (*see, e.g.*, Crystal, *Science* 270:404-410 (1995); Blaese *et al.*, *Cancer Gene Ther.* 2:291-297 (1995); Behr *et al.*, *Bioconjugate Chem.* 5:382-389 (1994); Remy *et al.*, *Bioconjugate Chem.* 5:647-654 (1994); Gao *et al.*, *Gene Therapy* 2:710-722 (1995); Ahmad *et al.*, *Cancer Res.* 52:4817-4820 (1992); U.S. Pat. Nos. 4,186,183, 4,217,344, 4,235,871, 4,261,975, 4,485,054, 4,501,728, 4,774,085, 4,837,028, and 4,946,787).

B. Viral Delivery Methods

[0218] The use of RNA or DNA viral based systems for the delivery of VEGFR-death ligand fusion protein encoding nucleic acids is known in the art. Conventional viral based systems for include retroviral, lentivirus, adenoviral, adeno-associated and herpes simplex virus vectors for gene transfer.

[0219] In many gene therapy applications, it is desirable that the gene therapy vector be delivered with a high degree of specificity to a particular tissue type, *e.g.*, lung tissue or

breast tissue. A viral vector can be modified to have specificity for a given cell type by expressing a ligand as a fusion protein with a viral coat protein on the viruses outer surface. The ligand is chosen to have affinity for a receptor known to be present on the cell type of interest. For example, Han *et al.*, *Proc Natl. Acad. Sci. U.S.A.* 92:9747-9751 (1995), reported that Moloney murine leukemia virus can be modified to express human heregulin fused to gp70, and the recombinant virus infects certain human breast cancer cells expressing human epidermal growth factor receptor. This principle can be extended to other pairs of virus expressing a ligand fusion protein and target cell expressing a receptor. For example, filamentous phage can be engineered to display antibody fragments (*e.g.*, Fab or Fv) having specific binding affinity for virtually any chosen cellular receptor. Although the above description applies primarily to viral vectors, the same principles can be applied to nonviral vectors. Such vectors can be engineered to contain specific uptake sequences thought to favor uptake by specific target cells.

[0220] Gene therapy vectors can be delivered *in vivo* by administration to an individual patient, typically by systemic administration (*e.g.*, intravenous, intraperitoneal, intramuscular, subdermal, or intracranial infusion) or topical application, as described below. Alternatively, vectors can be delivered to cells ex vivo, such as cells explanted from an individual patient.

[0221] *Ex vivo* cell transfection for diagnostics, research, or for gene therapy (*e.g.*, via re-infusion of the transfected cells into the host organism) is well known to those of skill in the art. In some embodiments, cells are isolated from the subject organism, transfected with VEGFR-death ligand encoding nucleic acids and re-infused back into the subject organism (*e.g.*, patient). Various cell types suitable for ex vivo transfection are well known to those of skill in the art (*see, e.g.*, Freshney *et al.*, *Culture of Animal Cells, A Manual of Basic Technique* (3rd ed. 1994)) and the references cited therein for a discussion of how to isolate and culture cells from patients).

[0222] Vectors (*e.g.*, retroviruses, adenoviruses, liposomes, etc.) containing therapeutic nucleic acids can also be administered directly to the organism for transduction of cells *in vivo*. Alternatively, naked DNA can be administered. Administration is by any of the routes normally used for introducing a molecule into ultimate contact with blood or tissue cells. Suitable methods of administering such nucleic acids are available and well known to those of skill in the art, and, although more than one route can be used to administer a particular composition, a particular route can often provide a more immediate and more effective reaction than another route.

[0223] Pharmaceutically acceptable carriers are determined in part by the particular composition being administered, as well as by the particular method used to administer the composition. Accordingly, there is a wide variety of suitable formulations of pharmaceutical compositions of the present invention, as described below (*see, e.g., Remington's Pharmaceutical Sciences*, 17th ed., 1989).

IX. PHARMACEUTICAL COMPOSITIONS

[0224] The present invention provides pharmaceutical compositions comprising a VEGFR-death ligand fusion protein of the present invention.

[0225] In a preferred embodiment of the present invention, a pharmaceutical composition comprises (i) a fusion protein comprising: (1) a VEGFR polypeptide that binds a VEGF protein; and (2) a Fas ligand comprising an oligomerization domain and a Fas receptor recognition moiety of the extracellular domain of a Fas ligand protein; and(ii) a pharmaceutically acceptable excipient, carrier and/or diluent.

[0226] In another embodiment of the present invention, a composition is provided comprising a vector comprising a nucleic acid encoding a VEGFR-death ligand fusion protein and a pharmaceutically acceptable excipient, carrier and/or diluent. In one embodiment, the nucleic acid encoding a VEGFR-death ligand fusion protein has a nucleotide sequence as shown in SEQ ID NO:14.

[0227] Pharmaceutical compositions are useful for treating cancers overexpressing VEGF and expressing a death receptor, such as Fas. Pharmaceutical compositions are also useful for treating diseases characterized by pathologic angiogenesis as described herein.

A. Administration of Pharmaceutical Compositions

[0228] Pharmaceutical compositions comprising an activator of VEGFR-death ligand fusion protein or a VEGFR-death ligand fusion protein encoding polynucleotide can be administered to a patient for the treatment of cancer, *e.g.*, lung cancer or breast cancer. As described in detail below, the compounds are administered, optionally with pharmaceutically acceptable carriers.

[0229] A VEGFR-death ligand fusion protein or a VEGFR-death ligand fusion protein encoding polynucleotide can be administered to a patient at therapeutically effective doses to prevent, treat, or control cancer. The compounds are administered to a patient in an amount sufficient to elicit an effective therapeutic response in the patient. An effective therapeutic

response is a response that at least partially arrests or slows the symptoms or complications of the disease. An amount adequate to accomplish this is defined as "therapeutically effective dose." The dose will be determined by the efficacy of the particular VEGFR-death ligand employed and the condition of the subject, as well as the body weight or surface area of the area to be treated. The size of the dose also will be determined by the existence, nature, and extent of any adverse effects that accompany the administration of a particular compound or vector in a particular subject.

[0230] Toxicity and therapeutic efficacy of such compounds can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, for example, by determining the LD50 (the dose lethal to 50% of the population) and the ED50 (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and can be expressed as the ratio, LD_{50}/ED_{50}. Compounds that exhibit large therapeutic indices are preferred. While compounds that exhibit toxic side effects can be used, care should be taken to design a delivery system that targets such compounds to the site of affected tissue to minimize potential damage to normal cells and, thereby, reduce side effects.

[0231] The data obtained from, for example, cell culture assays and animal studies can be used to formulate a dosage range for use in humans. The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED_{50} with little or no toxicity. The dosage can vary within this range depending upon the dosage form employed and the route of administration. For any compound used in the methods of the invention, the therapeutically effective dose can be estimated initially from cell culture assays. A dose can be formulated in animal models to achieve a circulating plasma concentration range that includes the IC_{50} (the concentration of the test compound that achieves a half-maximal inhibition of symptoms) as determined in cell culture. Such information can be used to more accurately determine useful doses in humans. Levels in plasma can be measured, for example, by high performance liquid chromatography (HPLC). In general, the dose equivalent of a modulator is from about 1 ng/kg to 10 mg/kg for a typical subject.

[0232] Pharmaceutical compositions for use in the present invention can be formulated by standard techniques using one or more physiologically acceptable carriers or excipients. The compounds and their physiologically acceptable salts and solvates can be formulated for administration by any suitable route, including *via* inhalation, topically, nasally, orally, parenterally (*e.g.*, intravenously, intraperitoneally, intravesically or intrathecally) or rectally.

[0233] For oral administration, the pharmaceutical compositions can take the form of, for example, tablets or capsules prepared by conventional means with pharmaceutically acceptable excipients, including binding agents, for example, pregelatinised maize starch, polyvinylpyrrolidone, or hydroxypropyl methylcellulose; fillers, for example, lactose, microcrystalline cellulose, or calcium hydrogen phosphate; lubricants, for example, magnesium stearate, talc, or silica; disintegrants, for example, potato starch or sodium starch glycolate; or wetting agents, for example, sodium lauryl sulphate. Tablets can be coated by methods well known in the art. Liquid preparations for oral administration can take the form of, for example, solutions, syrups, or suspensions, or they can be presented as a dry product for constitution with water or other suitable vehicle before use. Such liquid preparations can be prepared by conventional means with pharmaceutically acceptable additives, for example, suspending agents, for example, sorbitol syrup, cellulose derivatives, or hydrogenated edible fats; emulsifying agents, for example, lecithin or acacia; non-aqueous vehicles, for example, almond oil, oily esters, ethyl alcohol, or fractionated vegetable oils; and preservatives, for example, methyl or propyl-p-hydroxybenzoates or sorbic acid. The preparations can also contain buffer salts, flavoring, coloring, and/or sweetening agents as appropriate. If desired, preparations for oral administration can be suitably formulated to give controlled release of the active compound.

[0234] For administration by inhalation, the compounds may be conveniently delivered in the form of an aerosol spray presentation from pressurized packs or a nebulizer, with the use of a suitable propellant, for example, dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide, or other suitable gas. In the case of a pressurized aerosol, the dosage unit can be determined by providing a valve to deliver a metered amount. Capsules and cartridges of, for example, gelatin for use in an inhaler or insufflator can be formulated containing a powder mix of the compound and a suitable powder base, for example, lactose or starch.

[0235] The compounds can be formulated for parenteral administration by injection, for example, by bolus injection or continuous infusion. Formulations for injection can be presented in unit dosage form, for example, in ampoules or in multi-dose containers, with an added preservative. The compositions can take such forms as suspensions, solutions, or emulsions in oily or aqueous vehicles, and can contain formulatory agents, for example, suspending, stabilizing, and/or dispersing agents. Alternatively, the active ingredient can be

in powder form for constitution with a suitable vehicle, for example, sterile pyrogen-free water, before use.

[0236] The compounds can also be formulated in rectal compositions, for example, suppositories or retention enemas, for example, containing conventional suppository bases, for example, cocoa butter or other glycerides.

[0237] Furthermore, the compounds can be formulated as a depot preparation. Such long-acting formulations can be administered by implantation (for example, subcutaneously or intramuscularly) or by intramuscular injection. Thus, for example, the compounds can be formulated with suitable polymeric or hydrophobic materials (for example as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt.

[0238] The compositions can, if desired, be presented in a pack or dispenser device that can contain one or more unit dosage forms containing the active ingredient. The pack can, for example, comprise metal or plastic foil, for example, a blister pack. The pack or dispenser device can be accompanied by instructions for administration.

B. Therapeutic Effective Amount And Dosing

[0239] In one embodiment of the present invention, a pharmaceutical composition or medicament is administered to a subject, preferably a human or a non-human animal, at a therapeutically effective dose to prevent, treat, or control a pathological condition or disease as described herein. The pharmaceutical composition or medicament is administered to a subject in an amount sufficient to elicit an effective therapeutic response in the subject. An effective therapeutic response is a response that at least partially arrests or slows the symptoms or complications of the pathological condition, disorder, or disease. An amount adequate to accomplish this is defined as "therapeutically effective dose" also referred to as "therapeutically effective amount."

[0240] The dosage of active agents administered is dependent on the species of warm-blooded animal (mammal), the body weight, age, individual condition, surface area or volume of the area to be treated and on the form of administration. The size of the dose also will be determined by the existence, nature, and extent of any adverse effects that accompany the administration of a particular small molecule compound in a particular subject. A unit dosage for oral administration to a mammal of about 50 to 70 kg may contain between about 5 and 500 mg of the active ingredient. Typically, a dosage of the active compounds of the

present invention, is a dosage that is sufficient to achieve the desired effect. Optimal dosing schedules can be calculated from measurements of agent accumulation in the body of a subject. In general, dosage may be given once or more daily, weekly, or monthly. Persons of ordinary skill in the art can easily determine optimum dosages, dosing methodologies and repetition rates.

[0241] The dosage of active agents administered is also dependent on the nature of the agent. For example, a therapeutically effective amount of protein or polypeptide of the present invention (i.e., an effective dosage) ranges from about 0.001 to 30 mg/kg body weight, preferably about 0.01 to 25 mg/kg body weight, more preferably about 0.1 to 20 mg/kg body weight, and even more preferably about 1 to 10 mg/kg, 2 to 9 mg/kg, 3 to 8 mg/kg, 4 to 7 mg/kg, or 5 to 6 mg/kg body weight. The protein or polypeptide can be administered one time per week for between about 1 to 10 weeks, preferably between 2 to 8 weeks, more preferably between about 3 to 7 weeks, and even more preferably for about 4, 5, or 6 weeks.

[0242] In addition, it is understood that the specific dose level for any particular animal subject will depend upon a variety of factors including the activity of the specific VEGFR-FasL fusion protein employed, the age, body weight, general health, gender, and diet of the subject, the time of administration, the route of administration, the rate of excretion, any drug combination, and the degree of expression or activity to be modulated.

[0243] In one embodiment of the present invention, a pharmaceutical composition or medicament comprising a VEGFR-death ligand fusion protein of the present invention is administered in a daily dose in the range from about 1 mg of each compound per kg of subject weight (1 mg/kg) to about 1g/kg for multiple days. In another embodiment, the daily dose is a dose in the range of about 5 mg/kg to about 500 mg/kg. In yet another embodiment, the daily dose is about 10 mg/kg to about 250 mg/kg. In another embodiment, the daily dose is about 25 mg/kg to about 150 mg/kg. A preferred dose is about 10 mg/kg. The daily dose can be administered once per day or divided into subdoses and administered in multiple doses, e.g., twice, three times, or four times per day. However, as will be appreciated by a skilled artisan, polypeptides and proteins, such as the VEGFR-death ligand fusion proteins of the present invention may be administered in different amounts and at different times. The skilled artisan will also appreciate that certain factors may influence the dosage and timing required to effectively treat a subject, including but not limited to the severity of the disease

or disorder, previous treatments, the general health and/or age of the subject, and other diseases present. Moreover, treatment of a subject with a therapeutically effective amount of a compound can include a single treatment or, preferably, can include a series of treatments.

[0244] To achieve the desired therapeutic effect, VEGFR-death ligand fusion proteins or nucleic acids encoding them may be administered for multiple days at the therapeutically effective daily dose. Thus, therapeutically effective administration of VEGFR-death ligand fusion proteins or nucleic acids encoding them to treat a pathological condition or disease described herein in a subject requires periodic (e.g., daily) administration that continues for a period ranging from three days to two weeks or longer. Typically, VEGFR-death ligand fusion proteins or nucleic acids encoding them will be administered for at least three consecutive days, often for at least five consecutive days, more often for at least ten, and sometimes for 20, 30, 40 or more consecutive days. While consecutive daily doses are a preferred route to achieve a therapeutically effective dose, a therapeutically beneficial effect can be achieved even if the VEGFR-death ligand fusion proteins or nucleic acids encoding them are not administered daily, so long as the administration is repeated frequently enough to maintain a therapeutically effective concentration of the VEGFR-death ligand fusion proteins or nucleic acids encoding them in the subject. For example, one can administer the VEGFR-death ligand fusion proteins or nucleic acids encoding them every other day, every third day, or, if higher dose ranges are employed and tolerated by the subject, once a week.

[0245] Optimum dosages, toxicity, and therapeutic efficacy of VEGFR-death ligand fusion proteins or nucleic acids encoding them may vary depending on the relative potency of individual VEGFR-death ligand fusion proteins or nucleic acids encoding them and can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, for example, by determining the LD_{50} (the dose lethal to 50% of the population) and the ED_{50} (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and can be expressed as the ratio, LD_{50}/ED_{50}. VEGFR-death ligand fusion proteins or nucleic acids encoding them that exhibit large therapeutic indices are preferred. While VEGFR-death ligand fusion proteins or nucleic acids encoding them that exhibit toxic side effects can be used, care should be taken to design a delivery system that targets such agents to the site of affected tissue to minimize potential damage to normal cells and, thereby, reduce side effects.

[0246] The data obtained from, for example, cell culture assays and animal studies can be used to formulate a dosage range for use in humans. The dosage of such VEGFR-death ligand fusion proteins or nucleic acids encoding them lies preferably within a range of circulating concentrations that include the ED_{50} with little or no toxicity. The dosage can vary within this range depending upon the dosage form employed and the route of administration. For any VEGFR-death ligand fusion protein or nucleic acid encoding them used in the methods of the invention, the therapeutically effective dose can be estimated initially from cell culture assays. A dose can be formulated in animal models to achieve a circulating plasma concentration range that includes the IC_{50} (the concentration of the agent that achieves a half-maximal inhibition of symptoms) as determined in cell culture. Such information can be used to more accurately determine useful doses in humans. Levels in plasma can be measured, for example, by high performance liquid chromatography (HPLC). In general, the dose equivalent of agents is from about 1 ng/kg to 100 mg/kg for a typical subject.

[0247] Following successful treatment, it may be desirable to have the subject undergo maintenance therapy to prevent the recurrence of the condition or disease treated.

X. KITS FOR USE IN DIAGNOSTIC, RESEARCH, AND THERAPEUTIC APPLICATIONS

[0248] For use in diagnostic, research, and therapeutic applications suggested above, kits are also provided by the invention. In the diagnostic and research applications such kits may include any or all of the following: assay reagents, buffers, VEGFR-death ligand polypeptides, VEGFR-death ligand specific nucleic acids or antibodies, hybridization probes and/or primers, VEGFR-death ligand expression constructs, small molecule activators of VEGFR-death ligand *etc.* A therapeutic product may include sterile saline or another pharmaceutically acceptable emulsion and suspension base.

[0249] In addition, the kits may include instructional materials containing directions (*i.e.*, protocols) for the practice of the methods of this invention. The instructions may be present in the subject kits in a variety of forms, one or more of which may be present in the kit. While the instructional materials typically comprise written or printed materials they are not limited to such. Any medium capable of storing such instructions and communicating them to an end user is contemplated by this invention. Such media include, but are not limited to electronic storage media (*e.g.*, magnetic discs, tapes, cartridges, chips), optical media (*e.g.*,

CD ROM), and the like. Such media may include addresses to internet sites that provide such instructional materials.

[0250] In a preferred embodiment of the present invention, the kit is a pharmaceutical kit and comprises a pharmaceutical composition comprising (i) a polynucleotide encoding a VEGFR-death ligand polypeptide and (ii) a pharmaceutical acceptable carrier. In another preferred embodiment of the present invention, the kit is a pharmaceutical kit and comprises a pharmaceutical composition comprising (i) a VEGFR-death ligand polypeptide and (ii) a pharmaceutical acceptable carrier. Pharmaceutical kits optionally comprise an instruction stating that the pharmaceutical composition can or should be used for treating a cancer wherein VEGF expression is up-regulated.

[0251] The kits according to the present invention may further comprise a reagent for performing mass spectrometry. Such reagents are well known to those skilled in the art and include, for example, a probe or a chip.

[0252] Additional kit embodiments of the present invention include optional functional components that would allow one of ordinary skill in the art to perform any of the method variations described herein.

[0253] Although the forgoing invention has been described in some detail by way of illustration and example for clarity and understanding, it will be readily apparent to one ordinary skill in the art in light of the teachings of this invention that certain variations, changes, modifications and substitution of equivalents may be made thereto without necessarily departing from the spirit and scope of this invention. As a result, the embodiments described herein are subject to various modifications, changes and the like, with the scope of this invention being determined solely by reference to the claims appended hereto. Those of skill in the art will readily recognize a variety of non-critical parameters that could be changed, altered or modified to yield essentially similar results.

[0254] While each of the elements of the present invention is described herein as containing multiple embodiments, it should be understood that, unless indicated otherwise, each of the embodiments of a given element of the present invention is capable of being used with each of the embodiments of the other elements of the present invention and each such use is intended to form a distinct embodiment of the present invention.

[0255] As can be appreciated from the disclosure above, the present invention has a wide variety of applications. The invention is further illustrated by the following examples, which

are only illustrative and are not intended to limit the definition and scope of the invention in any way.

XI. EXAMPLES

Example 1: Material and Methods

1. General Recombinant DNA Methods

[0256] Unless otherwise indicated, for generating nucleic acids encoding the fusion proteins of the present invention and for expression of the fusion proteins, routine techniques in the field of recombinant genetics were employed. Basic texts disclosing the general methods of use in this invention include Sambrook & Russell, *Molecular Cloning, A Laboratory Manual* (3rd Ed, 2001); Kriegler, *Gene Transfer and Expression: A Laboratory Manual* (1990); and *Current Protocols in Molecular Biology* (Ausubel *et al.*, eds., 1994-1999).

[0257] For nucleic acids, sizes are given in either kilobases (kb) or base pairs (bp). These are estimates derived from agarose or acrylamide gel electrophoresis, from sequenced nucleic acids, or from published DNA sequences. For proteins, sizes are given in kilodaltons (kDa) or amino acid residue numbers. Proteins sizes are estimated from gel electrophoresis, from sequenced proteins, from derived amino acid sequences, or from published protein sequences.

[0258] Oligonucleotides that are not commercially available can be chemically synthesized according to the solid phase phosphoramidite triester method first described by Beaucage & Caruthers, *Tetrahedron Letts.* 22:1859-1862 (1981), using an automated synthesizer, as described in Van Devanter *et al.*, *Nucleic Acids Res.* 12:6159-6168 (1984). Purification of oligonucleotides is by either native acrylamide gel electrophoresis or by anion-exchange HPLC as described in Pearson & Reanier, *J. Chrom.* 255:137-149 (1983).

[0259] The sequence of the cloned genes and synthetic oligonucleotides can be verified after cloning using, *e.g.*, the chain termination method for sequencing double-stranded templates of Wallace *et al.*, *Gene* 16:21-26 (1981).

2. Cell Lines and Tissue Culture

[0260] Cos-7 cells were obtained from the UCSF Cell Culture Facility. Cells were grown in DME-H21 medium supplemented with 10% fetal bovine serum and penicillin/streptomycin, in a tissue culture incubator at 37°C with 5% CO_2.

[0261] T-47D human breast cancer cells were obtained from the UCSF Cell Culture Facility. Cells were grown in RPMI-1640 medium supplemented with insulin (0.2 U/mL), 10% fetal bovine serum, and penicillin/streptomycin, in a tissue culture incubator at 37°C with 5% CO_2.

[0262] Jurkat E6.1 cells were obtained from the UCSF Cell Culture Facility. Cells were grown in RPMI-1640 medium supplemented 10% fetal bovine serum, and penicillin/streptomycin, in a tissue culture incubator at 37°C with 5% CO_2.

[0263] U87MG and U373 are human glioblastoma cell line that were obtained from the American Type Culture Collection (ATCC; Mannassas, VA 20108, USA).. Both are cultured in MEM Eagle's with Earl's BSS medium, supplemented with 10% fetal calf serum and antibiotics. U373 cells are resistant to Fas receptor-mediated apoptosis, while U87MG cells are sensitive (Rieger *et al.*, 1998, *FEBS Lett* 427:124-128; Yount *et al.*, 1999, *Cancer Res* 59:1362-1365).

[0264] DU145 is a human prostate cancer cell line. DU145 was obtained from the ATCC and cultured in MEM Eagle's with Earl's BSS medium, supplemented with 10% fetal calf serum and antibiotics.

[0265] Human umbilical vein endothelial cells (HUVEC) were obtained from Clonetics and cultured in manufacturer's supplied medium EGM-2 supplemented with fetal calf serum, hydrocortisone, hFGF, IGF, and ascorbic acid. Bovine adrenal cortical endothelial cells (microvascular cells) were obtained from Dr. Richard Weiner at University of California at San Francisco. These cells were cultured in DME H-16 with 1 g/L glucose, supplemented with 10% fetal calf serum and antibiotics (Ferrara et al., 1991, Endocrinology 129:896-900).

3. Western Blotting

[0266] Antibody against the extracellular domain of VEGFR-2 was purchased from Becton-Dickinson/Pharmingen (#555307). The rat monoclonal antibody was used for Western blotting at a final concentration of 0.5 micrograms/mL. The secondary antibody was goat anti-rat IgG HRP-conjugated antibody purchased from Santa Cruz Biotechnology (#SC-2065) and used at a final concentration of 0.4 micrograms/mL. Bands were visualized using chemiluminescence (ECL reagents from Amersham).

4. PCR Methods

[0267] PCR was used to amplify a fragment of human FasL encoding amino acids 139-281. The sense primer (purchased from the UCSF Biomedical Core) was: 5'-GGGCTCGAGGGACTAGTGAAAAAAAGGAGCTGAGGAAAGTGGCCCAT-3', which includes a XhoI and SpeI sites on the 5' end. The antisense primer (purchased from the UCSF Biomedical Core) was: 5'-GGGTCTAGATCTTAGAGCTTATATAAGCCGAAAAACGTCTG-3', which includes a BglII site and an XbaI site on the 3' end. The template was pOTB7/hFasL (purchased from ATCC); the DNA polymerase was Pfu (Stratagene); and the dNTPs were from Roche.

[0268] PCR conditions: Template pOTB7/ hFasL: 2.0 micrograms; Primers: 100 pMol; dNTPs: 20 nMol; Pfu: 2.5 Units; First cycle: 95°C for 5'; 95°C. to 35°C. over 10'; 72°C for 5'; Cycles 2-11: 95°C for 45"; 60°C for 45"; 72°C for 60". A 440 bp PCR fragment was purified by agarose gel chromatography, purified with the Gel Extraction kit (Qiagen), and subcloned into the pBJ vector at the XhoI/XbaI restriction sites.

5. Apoptosis Analysis

[0269] Apoptosis and cytotoxicity was assayed by three assays: cell counting, FACS analysis of Annexin V positive cells, and LDH (lactate dehydrogenase) release. For cell counting of Jurkat cells, 100 uL of cell suspension was mixed with an equal volume of Trypan blue stain (0.4%), and kept at room temperature for 2 minutes. Live cells were counted using a hemocytometer in triplicate. Mean number of cells/visual field and SEM were calculated using InStat statistical software. For FACS analysis, the Annexin-V-Fluos kit from Roche was used according to the manufacturer's protocol. Briefly, after treatment with FlkFasL or control conditioned medium ± VEGF-165 (2nM, from Peprotech), Jurkat cells were pelleted and resuspended in 100 µL of Annexin-V-Fluos labeling solution (FITC-Annexin V plus propidium iodide) for 15 minutes at room temperature. FACS analysis was performed on a Becton Dickinson FACSCalibur ™ using CellQuest Software, with gating to distinguish Annexin V-positive (apoptotic) versus negative cells, and propidium iodide-positive (i.e. necrotic) versus negative cells. The percent of the cell population that was apototic (Annexin-V-positive and propidium iodide-negative) was determined. For LDH release assays, the LDH Cytotoxicity Detection kit from Roche was used according to the manufacturer's protocol. Briefly, after 48 hours treatment with FlkFasL or control conditioned medium ± VEGF-165 (2nM), cell culture supernatants were centrifuged 500 g for 3 minutes to pellet cell debris. 25 µL of supernatant in triplicate was mixed with LDH

detection reagent, and LDH activity quantified by measuring absorbance at 492 nM. Mean and SEM was calculated using InStat statistical software.

Example 2: Construction of Nucleic Acids Encoding A Chimeric Mouse/Human VEGFR/FasL Fusion Protein

[0270] The pBJ plasmid encoding FlkFasL, pBJ/Flk(D1-D3)+FasL(139-281), was constructed in a stepwise manner. First, PCR was performed to amplify the human FasL sequence encoding amino acids 139-281 with flanking 5' Xho I/Spe I and 3' Bgl II/Xba I sites. The PCR fragment was digested with Xho I and Xba I, and subcloned into the pBJ mammalian expression vector at Xho I and Xba I sites to create pBJ/hFasL(139-281)(Figure 6). This plasmid was then cut with Xho I, and into it was subcloned the Xho I/Xho I fragment from the plasmid LNCX/Flk(1-3)HA, which included the Flk-1 signal sequence and immunoglobulin-like domains 1-3. The resulting plasmid pBJ/Flk(D1-D3)+FasL(139-281) (Figure 5) contains the FlkFasL nucleotide sequence shown in Figure 7.

[0271] The plasmid pBJ/Flk(D1-D3)+FasL(139-281) encodes the VEGFR-2-FasL fusion protein having an amino acid sequence shown in Figure 8.

Example 3: Construction of Nucleic Acids Encoding Chimeric Mouse/Human FLAG-VEGFR/FasL Fusion Proteins

[0272] To generate a plasmid expressing FlkFasL with a FLAG epitope tag, pFLAG/FlkhFasL (D1-D3/139-281; Figure 6), the pFLAG-CMV-3 vector was purchased from SIGMA. As shown in Figure 6, the pFLAG-CMV-3 vector was cut with NotI and the ends filled in with Klenow enzyme. The plasmid pBJ/FlkFasL (D1-D3 / 139-281) was cut with AvaI and blunted with Mung bean nuclease. The resulting fragment encodes the Flk-1 extracellular domain from amino acid Ala-19, at the end of the signal sequence, to Ser-336 at the end of domain 3. Ligation of the AvaI/Mung fragment into NotI/Klenow-treated pFLAG-CMV-3 produces pFLAG-Flk(D1 – D3), in which the Flk-1 sequence is placed in-frame downstream of the nucleotides encoding the FLAG epitope tag. To complete the assembly of the FlkFasL cDNA in the pFLAG vector, the BspEI/Bgl II fragment from pBJ/FlkFasL (D1-D3 / 139-281) was subcloned in. The final plasmid, pFLAG-FlkFasL, encodes FlkFasL with an N-terminal FLAG epitope tag.

[0273] R1[D2]FasL protein consists of the second domain of human VEGFR1 (amino acids 129 to 230: SDTG...NTII; Figure 10) fused to the trimerization and Fas receptor binding domains of hFasL (amino acids 139 to 281; Figure 10). A five amino acid linker sequence

(ARGTS) is present between the VEGFR1 and FasL domains (Figure 10). The linker sequence and the FasL domains are identical to the originally described R2FasL protein. In addition, at the 3' end there is an in-frame preprotrypsin leader sequence and a FLAG epitope tag, which were present in the pFLAG3 vector (Figure 10).

[0274] Briefly, a plasmid including the R1[D2]FasL nucleic acid sequence was constructed as follows. The cDNA for VEGFR1 domain 2 was amplified from a VEGFR1-containing plasmid (deVries *et al.*, 1992, *Science* 255:989-991) using as 5' primer (5'-CCCGCGGCCGCCAGTGATACAGGTAGACCTTTCG-3') and as 3' primer (5'-GGCCTCGAGCTATGATTGTATTGGTTTGTCG-3'). The resulting PCR fragment was subcloned into NotI and XhoI restriction sites to yield the pFLAG3/R1[D2]FasL plasmid. The nucleic acid sequence of FLAG-tagged r1[D2]FasL is shown in Figure 9.

Example 4: Generation of Conditioned Medium Containing VEGFR/FasL Fusion Proteins

[0275] Cos-7 cells were used to generate conditioned medium containing FlkFasL protein. Cos-7 cells were transfected using a DEAE-dextran protocol modified from Sambrook & Russell, *Molecular Cloning, A Laboratory Manual* (3rd Ed, 2001). Briefly, Cos-7 cells grown in 10 cm plates were washed twice in medium without serum or antibiotics. Three mL of medium without serum or antibiotics were added to each plate, and 3 mL of a DEAE-dextran solution to yield a final concentration of DEAE/dextran = 0.4 mg/mL. Plasmid DNA (1 to 3 μg of either pBJ/FlkFasL or control plasmid pBJ/Neo encoding only the neomycin resistance gene) was added to each plate, and plates were returned to the incubator. After 30 minutes or 3 hours the DEAE-dextran-DNA solution was aspirated and each plate was washed with complete medium with serum. Twenty-four hours later the medium was changed to serum-free medium (Hybridoma-SFM, GIBCO). Conditioned medium was collected from the plates after 72 hours, filtered through a 0.2 μM filter, and stored at 4°C. for use in experiments. To confirm expression of FlkFasL protein, 48 hours after DEAE-dextran transfection Cos-7 cells were lysed with glycerol/Triton X-100 lysis buffer. Lysates were separated by PAGE and immunoblotted with antibody against the extracellular domain of VEGFR-2 (Pharmingen), confirming expression of FlkFasL at molecular weight sizes consistent with monomers, dimers, and trimers (Figure 11A).

[0276] Stably transfected CHO cells secreting FLAG-tagged R2FasL were also generated. Briefly, CHO cells were electroporated with a mixture of pFLAG/R2FasL and the neomycin

resistance-expressing vector pBSR-alpha at a ratio of 10:1. Forty-eight hours later cells were split and grown in complete medium with neomycin (1 mg/mL). Colonies growing in neomycin were selected for subculture and their conditioned media were screened for secretion of R2FasL by Western blotting using an antibody against the extracellular domain of VEGFR2 (Pharmingen). A positive clone was reselected by limiting cell dilution and secretion of R2FasL into the conditioned medium reconfirmed by Western blotting. Affinity chromatography of the conditioned medium using anti-FLAG antibody (Sigma) demonstrated expression of the FLAG-tagged R2FasL protein (Figure 11B).

[0277] To produce R1[D2]FasL protein, the pFLAG3/R1[D2]FasL plasmid was transfected into Cos7 cells using DEAE-dextran mediated transfection. Conditioned medium was collected at 96 hours. R1[D2]FasL was purified from the conditioned medium using anti-FLAG antibody affinity chromatography (M2 gel from Sigma) and eluted with FLAG epitope peptide.

Example 5: Cell Killing by a VEGFR/FasL Fusion Protein is Dose-Dependent

[0278] Jurkat cells were plated in 24-well plates (500,000 cells in 500 µl/well) and were treated with increasing volumes of conditioned medium obtained from Cos-7 cells transfected either with either control plasmid pSV/Neo or plasmid pBJ/FlkFasL. Jurkat cells were additionally treated with VEGF-165 (2 nM, from Peprotech) or without VEGF. Thirty-two hours later viable cells were counted after trypan blue staining. A representative result is shown in Figure 12.

Example 6: Cell Killing by a VEGFR/FasL Fusion Protein is Dependent on the Amount of VEGF

[0279] Jurkat cells were plated in 24-well plates (500,000 cells in 500 µl/well) and were treated with 25 µL/well of conditioned medium obtained from Cos-7 cells transfected either with control plasmid pSV/Neo or plasmid pBJ/FlkFasL. Jurkat cells were additionally treated with varying amounts of VEGF-165. Twenty-four hours later viable cells were counted after trypan blue staining. A representative result is shown in Figure 13.

Example 7: FlkFasL Induces Apoptosis in a VEGF-Dependent Manner

[0280] Jurkat cells were plated in 24-well plates (500,000 cells in 500 µl/well) and were treated with either 1 µL/well or 5 µL/well of conditioned medium obtained from Cos-7 cells transfected either with control plasmid pSV/Neo or plasmid pBJ/FlkFasL. Some wells were

additionally treated with VEGF-165 (2 nM) for 60 minutes. Induction of apoptosis was assessed by FACS analysis of FITC-Annexin V-positive/propidium-iodide negative cells. A representative result is shown in Figure 14.

Example 8: VEGFR/FasL Fusion Protein Induces Apoptosis in Breast Cancer Cells

[0281] T-47D human breast cancer cells were plated in 24-well plates (500,000 cells in 500 µl/well) and grown to confluence. Cells were treated with 25 µL/well of conditioned medium from Cos-7 cells transfected either with control plasmid pSV/Neo or plasmid FlkFasL. Cells were also treated with 2 nM VEGF-165 or without. Cells were photographed 24 hours after treatment. In the presence of endogenous VEGF produced by T-47D cells, FlkFasL induced apoptosis (Figure 13, center). Upon adding exogenous VEGF, a drastic increase in cell death is observed indicating that FlkFasL apoptotic activity is regulated by VEGF. A representative result is shown in Figure 15.

Example 9: VEGFR/FasL Fusion Protein Induces Cytotoxicity in Breast Cancer Cells in a VEGF-Dependent Manner

[0282] T-47D human breast cancer cells were plated in 24-well plates (500,000 cells in 500 µl/well) and grown to confluence. Cells were treated with 25 µL/well of conditioned medium from Cos-7 cells transfected either with control plasmid pSV/Neo or plasmid FlkFasL. Cells were also treated with 2 nM VEGF-165 or without. Forty-eight hours later cytotoxicity was assayed by LDH Cytotoxicity Detection kit (Roche). In the presence of endogenous VEGF produced by T-47D cells, FlkFasL induced cytotoxicity (Figure 14, lane B). Upon adding exogenous VEGF, a drastic increase in cytotoxicity is observed indicating that FlkFasL apoptotic activity is regulated by VEGF (Figure 16, lane D).

[0283] Upon comparing the results shown in Figure 14 (induction of apoptosis in Jurkat cells) to those shown in Figure 16 (stimulation of cytotoxicity in a breast cancer cell) one notes that the base level of apoptosis induction/cytotoxicity stimulation in the presence of a control conditioned medium with or without added VEGF is similarly low (compare Figure 16, lanes A and B to Figure 16). While adding a conditioned mediuym in the absence of VEGF did not increase apoptotic induction in Jurkat cells (Figure 14), a marked increase is observed when breast cancer cells are analyzed under similar conditions (Figure 16, lane C). It is known that breast cancer cells express and secrete endogenous VEGF. Thus, the induction of stimulation of cytotoxic effects in breast cancer cells can be explained by the

endogenous VEGF binding to FlkFasL and subsequent binding of the FlkFasL fusion protein (having bound a VEGF polypeptide) to a Fas on the surface of the breast cancer cells. Thus, Figure 16, lane C describes an apoptotic response to endogenous VEGF that is secreted by a cancer cell and demonstrates the visibility of *in vivo* administration of the fusion proteins of the present invention. Notably, upon adding exogenous VEGF, the cytotoxic effect is even enhanced.

Example 10: R2FasL Induces Cytotoxicity or Apoptosis in U87MG Human Glioblastoma Cells and DU145 Human Prostate Cancer Cells, But Not in U373 Human Glioblastoma Cells

[0284] To investigate if R2FasL induces cytotoxicity in human glioblastoma the following experiment was performed. U87MG human glioblastoma cells were plated in 96-well plates in 100 μL of complete medium with 10% fetal calf serum at 25,000 cells/well, and allowed to grow for 84 hours without change of medium. R2FasL solution (0, 0.01, 0.1, 1, or 10 μL), purified by FLAG epitope tag affinity chromatography, was then added to each well (Figure 17A). In addition, each well received 300 ng of neutralizing anti-VEGF antibody, neutralizing anti-FasL antibody, or control goat Ig (all from R&S Systems). Twenty-four hours later the cells were assayed for cytotoxicity using the LDH release assay (Roche), in which 10 μL of cell supernatant is mixed with 100 μL LDH reaction mixture in a total reaction volume of 200 μL. Cytotoxicity was assayed spectrophotometrically by measuring absorbance at 492 nm. Inhibition of cytotoxicity by neutralizing antibodies against VEGF or FasL demonstrate that both are required for R2FasL-mediated cytotoxicity (Figure 17A).

[0285] To investigate if R2FasL induces apoptosis in human glioblastoma the following experiment was performed. U87MG human glioblastoma cells were plated in 12-well plates in 500 μL of complete medium with 10% fetal calf serum at approximately 287,500 cells/well. Twenty-four hours later conditioned medium from Cos7 cells transfected with either pFLAG/R2FasL or empty pFLAG vector was added (Figure 17B). The concentration of R2FasL was determined separately by quantitative immunoblotting using commercially produced rhsFasL as standard (R&S Systems). After 36 more hours, cells were trypsinized, stained with Trypan blue, and counted on a hemocytometer. Data in Figure 17B are shown as mean ± SEM.

[0286] To investigate if R2FasL induces cytotoxicity in human prostate cancer cells the following experiment was performed. DU145 human prostate cancer cells were plated in 24-

well plates in 500 μL of complete medium with 10% fetal calf serum and allowed to grow for 54 hours without change of medium. Conditioned medium from Cos7 cells transfected with either pFLAG/R2FasL or empty pFLAG vector was added in the indicated volumes. After 34 more hours cytotoxicity was assayed using the LDH release assay as described above. A representative set of data is shown in Figure 17C.

[0287] To investigate the specificity of the R2FasL activity on U87MG glioblastoma cells, the cytotoxic effect of R2FasL on the U373 glioblastoma cell line, was analyzed. U373 cells are known to be resistant to Fas receptor-mediated killing, i.e., U373 cells are essentially a negative control to show that R2FasL is not constitutively toxic. U87MG and U373 human glioblastoma cells were plated in 96-well plates in 100 μL of complete medium with 10% fetal calf serum at 25,000 cells/well, and allowed to grow for 96 hours without change of medium. Conditioned medium from Cos7 cells transfected with pFLAG/R2FasL vector was added in the indicated volumes. After 36 more hours cytotoxicity was assayed using the LDH release assay as described herein (Figure 17D).

Example 11: R1[D2]FasL Induces Apoptosis in a VEGF-Dependent Manner

[0288] To investigate if R1[D2]FasL induces apoptosis in in a VEGF-dependent manner the following experiment was performed. Jurkat E6.1 human T cells were plated in 96-well plates in 100 μL of complete medium with 10% fetal calf serum. Conditioned medium (0.001, 0.01, 0.1, 1, or 10 μL) from Cos7 cells transfected with pFLAG/R1[D2]FasL vector was added in the absence or presence of rhVEGF-165 (final concentration 2 nM). Twenty-two hours later cell viability was assayed using the resazurin assay (AlamarBlue reagent from BioSource). Conversion of AlamarBlue substrate to product by viable cells was determined by adding 20 μL of AlamarBlue to each well and measuring absorbance at 540 nm and 620 nM. Calculation of % conversion of substrate to product was performed according to the manufacturer's protocol. A representative set of data demonstrating that R1[D2]FasL induces apoptosis in a VEGF-dependent manner is shown in Figure 18A.

[0289] To investigate which growth factors activate R1[D2]FasL the following experiment was performed. Jurkat E6.1 human T cells were plated in 96-well plates in 100 μL of complete medium with 10% fetal calf serum. Conditioned medium from Cos7 cells transfected with pFLAG/R1[D2]FasL vector was added (2 μL/well), in the absence or presence of rhVEGF-165 (recombinant human VEGF-165), rmVEGF-164 (recombinant mouse VEGF-164), rhVEGF-121 (recombinant human VEGF-121), rhVEGF-165/rhPlGF-

129 heterodimer, or rhPlGF-129. All growth factors were used at a final concentration of 10 nM, and were from Peprotech or R&D Systems. Nineteen hours later cell viability was assayed using the resazurin assay as described above. Representative data are shown in Figure 18B. The results show that R1[D2]FasL is activated by all growth factors tested in these experiments.

[0290] In a similar experiment, the concentration of the growth factors tested was varied. Briefly, Jurkat E6.1 human T cells were plated in 96-well plates in 100 µL of complete medium with 10% fetal calf serum. Conditioned medium from Cos7 cells transfected with either pFLAG/R2FasL vector or empty pFLAG vector was added (1 µL/well), in the absence or presence of rhVEGF-165 or rmVEGF-165 at the final concentrations of 0.001, 0.01, 0.1, 1, and 10 nM. Eighteen hours later cell viability was assayed using the resazurin assay as described herein. The data shown in Figure 21A demonstrate that human and mouse VEGF-165 similarly activate R2FasL.

[0291] To determine the ED50 for hVEGF-165 on R1[D2]FasL the following experiment was performed. Jurkat E6.1 human T cells were plated in 96-well plates in 100 µL of complete medium with 10% fetal calf serum. Conditioned medium from Cos7 cells transfected with pFLAG/R1[D2]FasL vector was added (2 µL/well), in the absence or presence of rhVEGF-165 at 0, 0.01, 0.1,1, 10, 100, 1,000, or 10,000 nM. Nineteen hours later cell viability was assayed using the resazurin assay as described above. The ED50 for hVEGF-165 on R1[D2]FasL was found to be approximately 100 pM (Figure 18C).

[0292] In a similar experiment the ED50 for hVEGF-165 on R2FasL was determined. Briefly, Jurkat E6.1 human T cells were plated in 96-well plates in 100 µL of complete medium with 10% fetal calf serum. Conditioned medium from Cos7 cells transfected with pFLAG/R2FasL vector was added in the volumes indicated, in the absence or presence of rhVEGF-165 at the final concentrations of 0, 0.02, 0.02, 0.2 and 2 nM. Eighteen hours later cell viability was assayed using the resazurin assay. The ED50 for rhVEGF-165 was between 20 pM and 200 pM (Figure 21B).

Example 12: R2FasL Activity is Potentiated by Chemotherapeutic Agents

[0293] To determine if the activity of R2FasL can be potentiated by chemotherapeutic agents, the following experiments were performed. U87MG human glioblastoma cells were plated in 96-well plates in 100 µL of complete medium with 10% fetal calf serum at 25,000 cells/well. Forty-eight hours later cells were treated with either bisindolylmaleimide VIII

(Bis VIII, final concentration 1 μM), camptothecin (final concentration 20 nM), etoposide (final concentration 5 μM), or DMSO vehicle (1 μL/well). Bis VIII, camptothecin, and etoposide (all from Biomol) were dissolved in DMSO and added at 1 μL/well. After 72 hours of drug exposure, cells were treated with FLAG antibody affinity-purified R2FasL solution (10 μL/well), and incubated for an additional 40 hours. Cell viability and cytotoxicity were assayed using the resazurin assay and the LDG release assay, respectively, as described above. The result shown in Figures 19A and 19B demonstrates that the activity of R2FasL is potentiated by the chemotherapeutic agents tested in these experiments.

Example 13: R2FasL Does Not Induce Cytotoxicity in Macrovascular Endothelial Cells, But Does Have Activity in Microvascular Endothelial Cells

[0294] Microvascular and macrovascular To determine if R2FasL induces cytotoxicity in macrovascularendothelial cells may express different populations of death factor receptors and growth factor receptors. R2FasL may be differentially active in tumor endothelial cells (microvascular) and less toxic to normal macrovascular endotherlial cells, such as arteries and veins. To determine if R2FasL induces cytotoxicity in macrovascular endothelial cells, the following experiments were performed. Human umbilical vein endothelial cells (macrovascular endothelial cells; HUVEC, passage 4, from Clonetics) were plated in 96-well plates in 100 μL of the supplied medium with 10% fetal calf serum but without VEGF. Twenty-four hours later serum starvation was begun (to sensitize cells to death signals) by withdrawing serum. After fifteen hours of serum starvation cells were treated Cos7 cell conditioned medium containing R2FasL (10 μL/well) in the absence or presence of rhVEGF-165 (final concentration 2 nM). After 22 hours of treatment with R2FasL ± rhVEGF cell viability was assayed using the resazurin assay as described herein. No effect of R2FasL+VEGF was seen on these macrovascular endothelial cells (Figure 20A).

[0295] To determine if R2FasL induces cytotoxicity in microvascular endothelial cells, the following experiments were performed. Bovine adrenal cortical endothelial cells (microvascular endothelial cells) were plated in 96-well plates in 100 μL of complete medium with 1% fetal calf serum. Twenty-two hours later conditioned medium from Cos7 cells transfected with either pFLAG/R2FasL or empty pFLAG vector was added (2 μL/well), in the absence or presence of rhVEGF-165 (final concentration 2 nM). Twenty-four hours later cytotoxicity was assayed using the LDH release assay as described above. Figure 20B shows that R2FasL and VEGF induce cytotoxicity in adrenal cortical endothelial cells. Thus,

R2FasL does not have activity on macrovascular endothelial cells (e.g., HUVEC), but does have activity on microvascular endothelial cells.

Example 14: Testing VEGFR-FasL *in Vivo*

[0296] The activity of a fusion protein of the present invention can also be tested in vivo, for example, in an adjuvant arthritis model. The term "adjuvant arthritis model" is used herein to refer to rats, preferably Wistar-Lewis or other rat strains commonly known to those skilled in the art, in which disease was induced by injecting 0.1 mL Freund's adjuvant into the base of the tail. This adjuvant arthritis model is only one example of an animal model that can be used to test the compounds of the invention. For a review of the three most common animal models, see Oliver & Brahn (1996) J. Rheumatol. 23:56-60, hereby enclosed herein by reference in its entirety, including any drawings, figures, or tables.

[0297] A number of animal models have been developed to investigate the function of VEGF in tumor angiogenesis. For example, rat C6 glioma and human U87MG glioblastoma cells secrete VEGF and grow subcutaneously in athymic mice (Saleh *et al.*, *Cancer Res* (1996) 56:393-401; Cheng *et al.*, *Proc. Natl. Acad. Sci. USA* (1996), 93:8502-8507). The introduction of antisense constructs to VEGF mRNA into these cell lines reduces their *in vivo* growth, as well as the degree of neovascularization. Monoclonal antibodies against VEGF inhibit the subcutaneous growth of human rhabdomyosarcoma, glioblastoma, leiomyosarcoma (Kim *et al.*, *Nature* (1993), 362:841-844) and fibrosarcoma (Asano *et al.*, *Cancer Res* (1995), 55:5296-5301) in athymic mice. Metastasis of fibrosarcoma (Asano *et al.*, *Cancer Res* (1995), 55:5296-5301) and colon cancer tumors (Warren *et al.*, *J Clin Invest* (1995), 95:1789-1797) was also blocked by anti-VEGF antibodies. Thus, these animal models will be useful for testing the *in vivo* activities of the fusion proteins of the present invention.

[0298] Although the foregoing invention has been described in some detail by way of illustration and example for clarity and understanding, it will be readily apparent to one of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit and scope of the appended claims.

[0299] All publications and patent applications cited in this specification are herein incorporated by reference as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference.

WHAT IS CLAIMED IS:

1. A fusion protein which binds to a death receptor, the fusion protein comprising:

(i) a vascular endothelial growth factor receptor (VEGFR) polypeptide that binds a vascular endothelial growth factor (VEGF) polypeptide; and

(ii) a death ligand comprising an oligomerization domain and a death receptor recognition moiety,

wherein the C-terminus of the VEGFR polypeptide is linked to the N-terminus of the death ligand.

2. The fusion protein of claim 1, wherein the death receptor is Fas and the death ligand is a Fas ligand.

3. The fusion protein of claim 1, wherein the VEGFR polypeptide comprises a VEGF binding domain of VEGF receptor-1 (VEGFR-1).

4. The fusion protein of claim 1, wherein the VEGFR polypeptide comprises a VEGF binding domain of VEGF receptor-2 (VEGFR-2).

5. The fusion protein of claim 3, wherein the VEGF receptor-1 is a human VEGF receptor-1.

6. The fusion protein of claim 4, wherein the VEGF receptor-2 is a human VEGF receptor-2.

7. The fusion protein of claim 2, wherein the Fas ligand is a human Fas ligand.

8. The fusion protein of claim 4, wherein the VEGFR-2 is a murine VEGFR-2 and the death ligand comprises a human Fas ligand.

9. The fusion protein of claim 8, wherein the fusion protein comprises an amino acid sequence, which has at least 80% homology to the amino acid sequence shown in SEQ ID NO:22 or SEQ ID NO:23.

10. The fusion protein of claim 8, wherein the fusion protein comprises the amino acid sequence shown in SEQ ID NO:22 or SEQ ID NO:23.

11. The fusion protein of claim 1, further comprising:

(iii) an epitope tag.

12. The fusion protein of claim 11, wherein the epitope tag comprises a FLAG-like tag or an HA tag.

13. The fusion protein of claim 12, wherein the epitope tag can be cleaved off.

14. The fusion protein of claim 2, wherein the Fas ligand is selected from the group consisting of:

(i) a polypeptide comprising the amino acid sequence of SEQ ID NO:11;

(ii) a polypeptide comprising the amino acid sequence of SEQ ID NO:12;

(iii) a polypeptide comprising the amino acid sequence of SEQ ID NO:13; and

(iv) a polypeptide having Fas-binding activity comprising an amino acid sequence wherein one to several amino acid residues have been deleted, substituted, or added in the amino acid sequence of any one of (i) to (iii).

15. The fusion protein of claim 3, wherein the VEGF R-1 is selected from the group consisting of:

(i) a polypeptide comprising the amino acid sequence of SEQ ID NO:20;

(ii) a polypeptide comprising amino acid residues 1 to 747 of SEQ ID NO:19;

(iii) a polypeptide comprising amino acid residues 32 to 747 of SEQ ID NO:19;

(iv) a polypeptide comprising amino acid residues 151 to214 of SEQ ID NO:19;

(v) a polypeptide comprising amino acid residues 230 to 327 of SEQ ID NO:19; and

(vi) a polypeptide having VEGF binding activity comprising an amino acid sequence wherein one to several amino acid residues have been deleted, substituted, or added in the amino acid sequence of any one of (i) to (v).

16. The fusion protein of claim 4, wherein the VEGFR-2 polypeptide is selected from the group consisting of:

(i) a polypeptide comprising the amino acid sequence of SEQ ID NO:1;

(ii) a polypeptide comprising the amino acid sequence of SEQ ID NO:2;

(iii) a polypeptide comprising the amino acid sequence of SEQ ID NO:3;

(iv) a polypeptide comprising the amino acid sequence of SEQ ID NO:4;

(v) a polypeptide comprising the amino acid sequence of SEQ ID NO:5;

(vi) a polypeptide comprising the amino acid sequence of SEQ ID NO:6;

(iiv) a polypeptide comprising amino acid residues 141 to 207 of SEQ ID NO:1;

(iix) a polypeptide comprising amino acid residues 224 to 320 of SEQ ID NO:1; and

(ix) a polypeptide having VEGF binding activity comprising an amino acid sequence wherein one to several amino acid residues have been deleted, substituted, or added in the amino acid sequence of any one of (i) to (iix).

17. A nucleic acid comprising the nucleotide sequence shown in SEQ ID NO:14.

18. A vector comprising the nucleic acid of claim 17.

19. A method of modulating a death receptor-mediated pathway comprising the step of:

contacting a death receptor expressing cell with a fusion protein comprising:

(i) a VEGFR polypeptide that binds a VEGF protein;

(ii) a death ligand comprising an oligomerization domain and a death receptor recognition moiety;

wherein the VEGFR polypeptide has bound a VEGF protein and wherein the amount of the fusion protein is effective to modulate the death receptor-mediated pathway.

20. The method of claim 19, wherein the death receptor is Fas and the death ligand is a Fas ligand.

21. The method of claim 20, wherein the Fas-mediated pathway is apoptosis.

22. The method of claim 21, wherein the amount of the fusion protein is effective to induce apoptosis.

23. The method of claim 19, which is practiced *in vitro*.

24. The method of claim 19, which is practiced *in vivo*.

25. The method of claim 19, wherein the cell is a cancer cell.

26. The method of claim 25, wherein the cancer cell overexpresses VEGF.

27. The method of claim 25, wherein the cancer cell is selected from the group consisting of breast cancer cell, prostate cancer cell, colon cancer cell, lung cancer cell, glioblastoma cell, and ovarian cancer cell.

28. The method of claim19, wherein the Fas-mediated pathway is modulated in a disease selected from the group consisting of rheumatoid arthritis, psoriasis, and macular degeneration.

29. The method of claim 19, further comprising the step of contacting the death receptor expressing cell with a chemotherapeutic agent.

30. The method of claim 29, wherein the chemotherapeutic agent is selected from the group consisting of camptothecin, etoposide, bisindolylmaleimide VIII, cisplatin, taxol, doxorubicin, temozolomide, bortezomid, LY294002, and valproic acid.

31. A pharmaceutical composition comprising:

(i) a fusion protein comprising:

(1) a VEGFR polypeptide that binds a VEGF protein;

(2) a Fas ligand comprising an oligomerization domain and a Fas receptor recognition moiety of the extracellular domain of a Fas ligand protein; and

(ii) a pharmaceutically acceptable excipient, carrier and/or diluent.

32. The pharmaceutical composition of claim 31, wherein the Fas ligand is a human Fas ligand.

33. The pharmaceutical composition of claim 31, wherein the VEGFR polypeptide comprises a VEGF binding domain of a VEGF receptor.

34. A composition comprising:

(i) a vector comprising a nucleic acid having a nucleotide sequence as shown in SEQ ID NO:14; and

(ii) a pharmaceutically acceptable excipient, carrier and/or diluent.

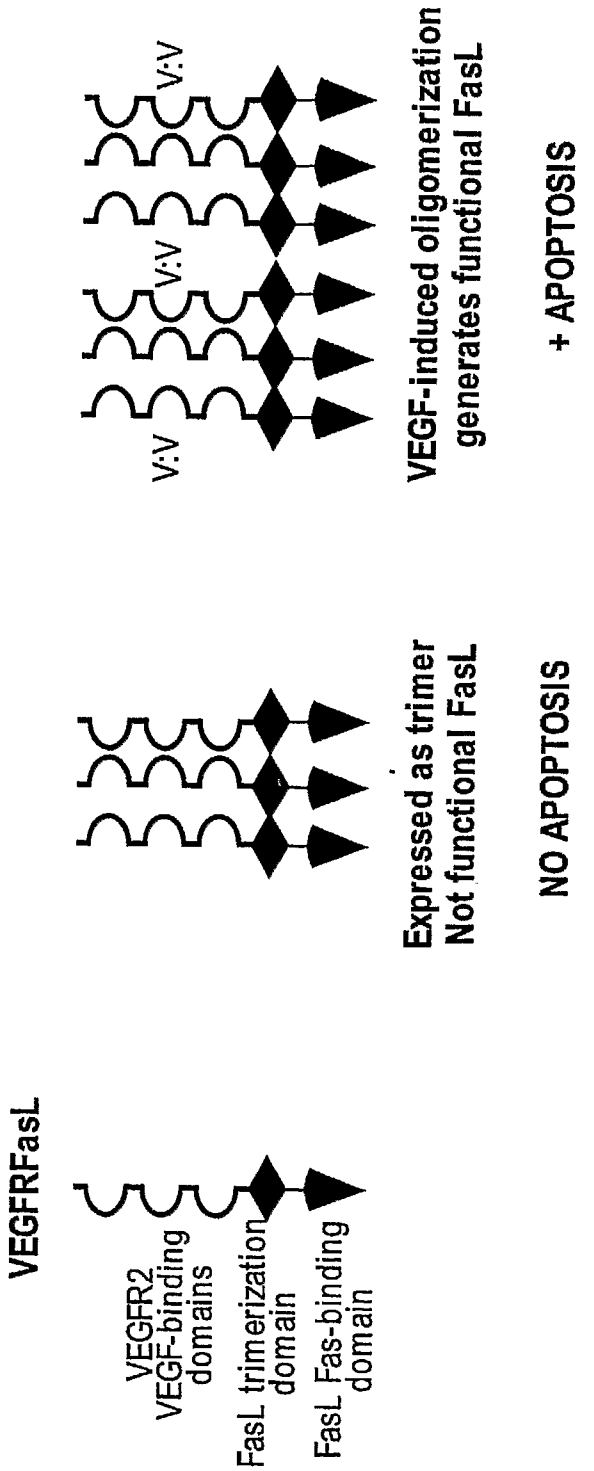

VEGFRFasL
VEGFR2 VEGF-binding domains
FasL trimerization domain
FasL Fas-binding domain
Expressed as trimer Not functional FasL
NO APOPTOSIS
V:V
V:V
V:V
VEGF-induced oligomerization generates functional FasL
+ APOPTOSIS

FIG. 1

```
    [INTRACELLULAR DOMAIN••••••••••••••••••••••••••••••••••••••••
H 1 MQQPFNYPYPQIYWVDSSASSPWAPPGTVLPCPTSVPRRPGQRRPPPPPPPPLPPPPPP
M 1 ****M***C***F******T*S*****S*F***SCG**G*D**********VSPL***SQ
R 1 ****V***C**********T*******S*FS**S*G**G***********SPL***SQ

    ••••••INTRACELLULAR][•••TRANSMEMBRANE••••]
61  PPLPPLPLPPLKKRGNHSTGLCLLVMFFMVLVALVGLGLGMFQLFHLQKELAELRESTSQ
61  *-**LP**T****K-D*N*N*W*P*V**********M****Y**************F*N*
61  **-*LP**S****K-D-NIE*W*P*I**********M****Y**************F*NH

 CLEAVAGE ><          [••••••••••••TRIMERIZATION DOMAIN••••••••••
121 MHTASSLEKQIGHPSPPPEKKELRKVAHLTGKSNSRSMPLEWEDTYGIVLLSGVKYKKGG
119 SLKV**F****AN**T*S****P*S******NPH***I*********TA*I*********
118 SLRV**F****AN**T*S*T*KP*S******NPR***I*********TA*I*********

    ••]
181 LVINETGLYFVYSKVYFRGQSCNNLPLSHKVYMRNSKYPQDLVMMEGKMMSYCTTGQMWA
179 ************************Q**N***********E***L**E*RLN******I**
178 *****A******************SQ**********F***G***L**E*KLN******I**

                •••••••••••••RECEPTOR BINDING]
241 RSSYLGAVFNLTSADHLYVNVSELSLVNFEESQTFFGLYKL
239 H*******************I*Q***I*****K********
238 H***********V*******I*Q***I*****K********
```

FIG. 2

```
          [ SIGNAL SEQUENCE ]                                  [••••••••••••••
Human     1 MQSKVLLAVALWLCVETRAASVGLPSVSLDLPRLSIQKDILTIKANTTLQITCRGQRDLD
Mouse     1 *E**A*******F***********GDF*HP*K**T*******L***************
Rat       1 *E*RA*******F***********GD**HP*K**T*******L***************
hR1 1   MVSYWDTGV*LC**LS*LLLTGS*S*S---K*KD*E**LKGTQHIMQ*GQ**HLQ***EAAHK

      ••••••••••IgG-like domain 1•••••••••••••••••••••••••••••••]
61    WLWPNNQSGSEQRVEVTECSDG----LFCKTLTIPKVIGNDTGAYKCFY------RETDLASVIYVYV
61    *****A*RD**E**L****GG*--DSI********R*V*********S*------*DV*I**TV****
61    *****TPRD**E**L****G*S----I******V*R*V**********------*D**VS*IV****
61    *SL*EMV*KESE*LSI*KSAC*RNGKQ**S***LNTAQA*H**F*S*K*LAVPTSKKKETE*A**IFI

                                [•••••IgG-like domain 2 binds VEGF••••
119   QDYRSPFIASVSDQHGVVYITENKNKTVVIPCLGSISNLNVSLCARYPEKRFVPDGNRIS
121   R***************I**************R*************************
119   **H**********E**I**************R*************************
129   S*TGR**VEMY*EIPEIIHM**GRE--L****RVTSP*IT*T*-KKF*LDTLI***K**I

      ••••••••••••••••••••••••••]                  [••••••••••••••
179   WDSKKGFTIPSYMISYAGMVFCEAKINDESYQSIMYIVVVVGYRIYDVVLSPSHGIELSV
181   ***EI***L*********************T*****************I***P*E****A
179   ***E************************T********L************P*E****A
186   ***R***I*SNATYKEI*LLT***TV*GHL*KTNYLTHRQTNTI*DVQIST*-RPVK*LR

      ••••••••••••••••IgG-like domain 3 binds VEGF•••••••••••••••
239   GEKLVLNCTARTELNVGIDFNWEYPSSKHQHKKLVNRDLKTQSGSEMKKFLSTLTIDGVT
241   ****************L**T*HS*P**SH***I****V*PFP*TVA*M*******ES**
239   ****************L**S*QF*********I****V*SLP*TVA*M********S**
245   *HT*******T*P**TRVQMT*S**DE*NKRASVRR*I--D**N*HANI*Y*V****KMQ

      ••••••••••••••••••••]          [•••••••••••••••••••••••••••••
299   RSDQGLYTCAASSGLMTKKNSTFVRVHEKPFVAFGSGMESLVEATVGER-VRIPAKYLGYP
301   K****E***V****R*I*R*R******T***I******K********SQ-****V***S**
299   K****E***T*Y********K******T***I******K********SQ-****V***S**
303   NK*K*****RVR**PSF*SVN*S*HIYD*A*ITVKHRKQQVL*TVA*K*SY*LSM*VKAF*

      •••IgG-like domain 4 not required for VEGF binding•••••••]
359   PPEIKWYKNGIPL--ESNHTIKAGHVLTIMEVSERDTGNYTVILTNPISKEKQSHVVSLVVY
361   A*D****R**R*I--***Y*MIV*DE******T***A***********M*****M*****N
359   A*D****R**R*I--***Y*MIV*DE**********A***********M*****M*****N
364   S**VV*L*D*L*ATEK*ARYLTR*YS*I*KD*T*E*A****IL*SIKQ*NVFKNLTAT*I*N

        [•••IgG-like domain 5 not required for VEGF binding ••••••
419   VPPQIGEKSLISPVDS--YQYGTTQTLTCTVYAIPPPHHIHWYWQLEEECANEPSQAVSVTN
421   ********A****M**--*****M*********N**L***Q*******A*SYR*G*-----*S
419   ********A****M**--*****M*********N**L***Q*******A*SYR***-----**
426   *K***Y**AVS*FP*PAL*PL*SR*I****A*G**Q*T-*K*F*H---P*NHNH*E*RCDFC
```

FIG. 3

```
     ••••••
```

```
    [••Signal sequence••]     [••••••••••••••••••••••••••••••••
1   MERGLPLLCAVLALVLAPAGAFRNDKCGDTIKIESPGYLTSPGYPHSYHPSEKCEWLIQA

    ••••••••••A Domain-Not required for VEGF Binding••••••••••••
61  PDPYQRIMINFNPHFDLEDRDCKYDYVEVFDGENENGHFRGKFCGKIAPPPVVSSGPFLF

    ••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••
121 IKFVSDYETHGAGFSIRYEIFKRGPECSQNYTTPSGVIKSPGFPEKYPNSLECTYIVFAP

    ••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••
181 KMSEIILEFESFDLEPDSNPPGGMFCRYDRLEIWDGFPDVGPHIGRYCGQKTPGRIRSSS

    ••••••••••••••••••••••]                [••••••••••••••••••••
241 GILSMVFYTDSAIAKEGFSANYSVLQSSVSEDFKCMEALGMESGEIHSDQITASSQYSTN

    ••••••••••••••••••B DOMAIN BINDS VEGF••••••••••••••••••••••••
301 WSAERSRLNYPENGWTPGEDSYREWIQVDLGLLRFVTAVGTQGAISKETKKKYYVKTYKI

    ••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••
361 DVSSNGEDWITIKEGNKPVLFQGNTNPTDVVVAVFPKPLITRFVRIKPATWETGISMRFE

    ••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••
421 VYGCKITDYPCSGMLGMVSGLISDSQITSSNQGDRNWMPENIRLVTSRSGWALPPAPHSY

    ••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••
481 INEWLQIDLGEEKIVRGIIIQGGKHRENKVFMRKFKIGYSNNGSDWKMIMDDSKRKAKSF

    ••••••••••••••••••••••••••••••••••••••]
541 EGNNNYDTPELRTFPALSTRFIRIYPERATHGGLGLRMELLGCEVEAPTAGPTTPNGNLV

                                         [••••••••••••••••
601 DECDDDQANCHSGTGDDFQLTGGTTVLATEKPTVIDSTIQSEFPTYGFNCEFGWGSHKTF

    ••••••C/MAM DOMAIN NOT REQUIRED FOR VEGF BINDING••••••••••••
661 CHWEHDNHVQLKWSVLTSKTGPIQDHTGDGNFIYSQADENQKGKVARLVSPVVYSQNSAH

    ••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••
721 CMTFWYHMSGSHVGTLRVKLRYQKPEEYHQLVWMAIGHQGDHWKEGRVLLHKSLKLYQVI

    ••••••••••••••••••••••••••••••]
781 FEGEIGKGNLGGIAVDDISINNHISQEDCAKPADLDKKNPEIKIDETGSTPGYEGEGEGD

                        [•••TRANSMEMBRANE•••••]
841 KNISRKPGNVLKTLDPILITIIAMSALGVLLGAVCGVVLYCACWHNGMSERNLSALENYN
```

FIG. 4

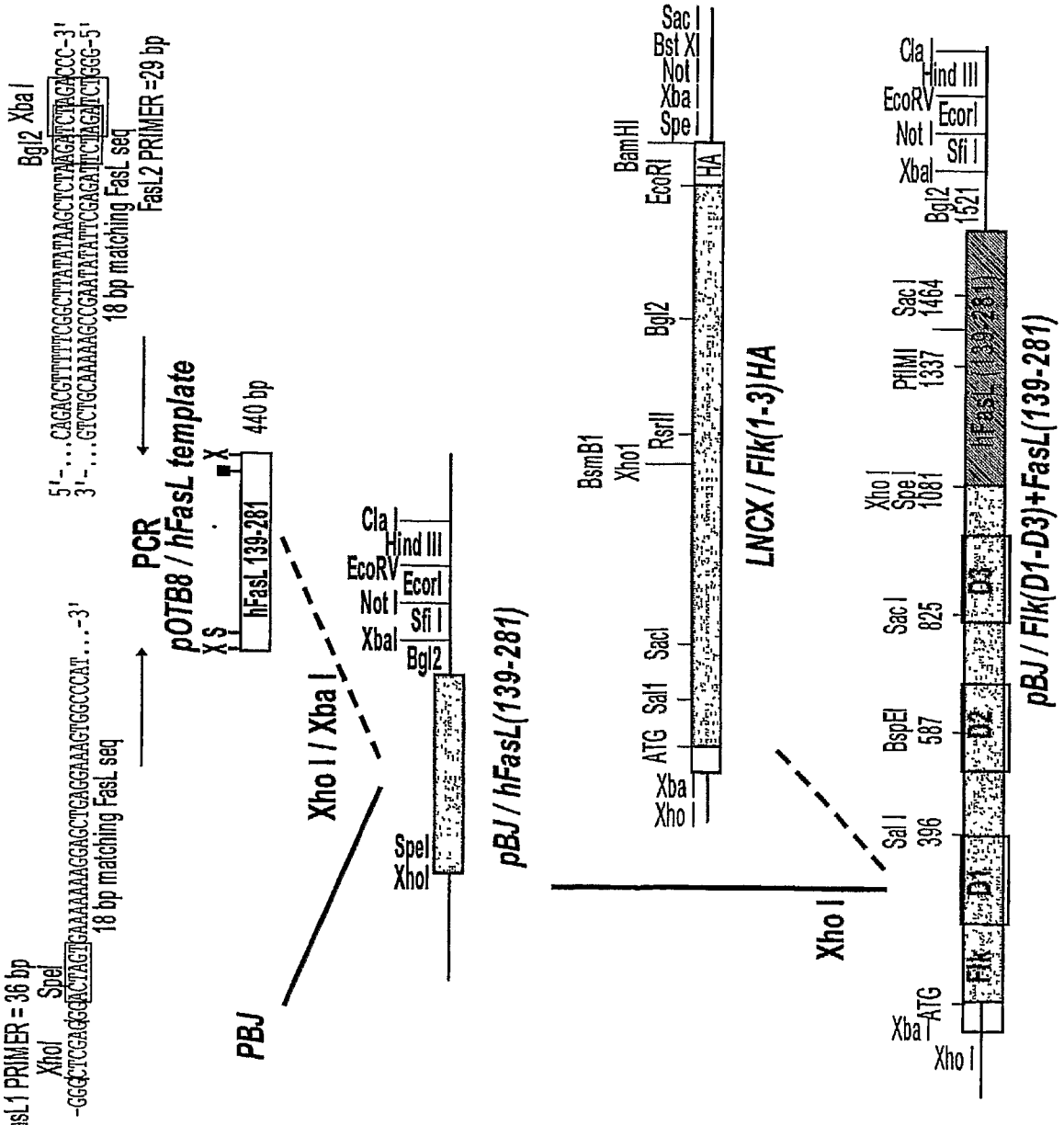

FasL1 PRIMER = 36 bp
XhoI
SpeI
18 bp matching FasL seq
PCR
pOTB8 / hFasL template
hFasL 139-281
440 bp
Bgl2 XbaI
FasL2 PRIMER =29 bp
18 bp matching FasL seq
PBJ
Xho I / Xba I
Cla
Hind III
EcoRV
EcoRI
Not I
XbaI
Sfi I
Bgl2
SpeI
XhoI
pBJ / hFasL(139-281)
BsmBI
Xho1
RsrII
Bgl2
BamHI
EcoRI
HA
Sac I
Bst XI
Not I
Xba I
Spe I
LNCX / Flk(1-3)HA
ATG
Sal1
SacI
Xba
Xho
Xho I
Sal I
396
BspEI
587
Sac I
825
Xho I
Spe I
1081
PflMI
1337
Sac I
1464
Bgl2
1521
Cla
Hind III
EcoRV
EcoRI
Not I
Sfi I
XbaI
pBJ / Flk(D1-D3)+FasL(139-281)
Xba
ATG
Xho I

FIG. 5

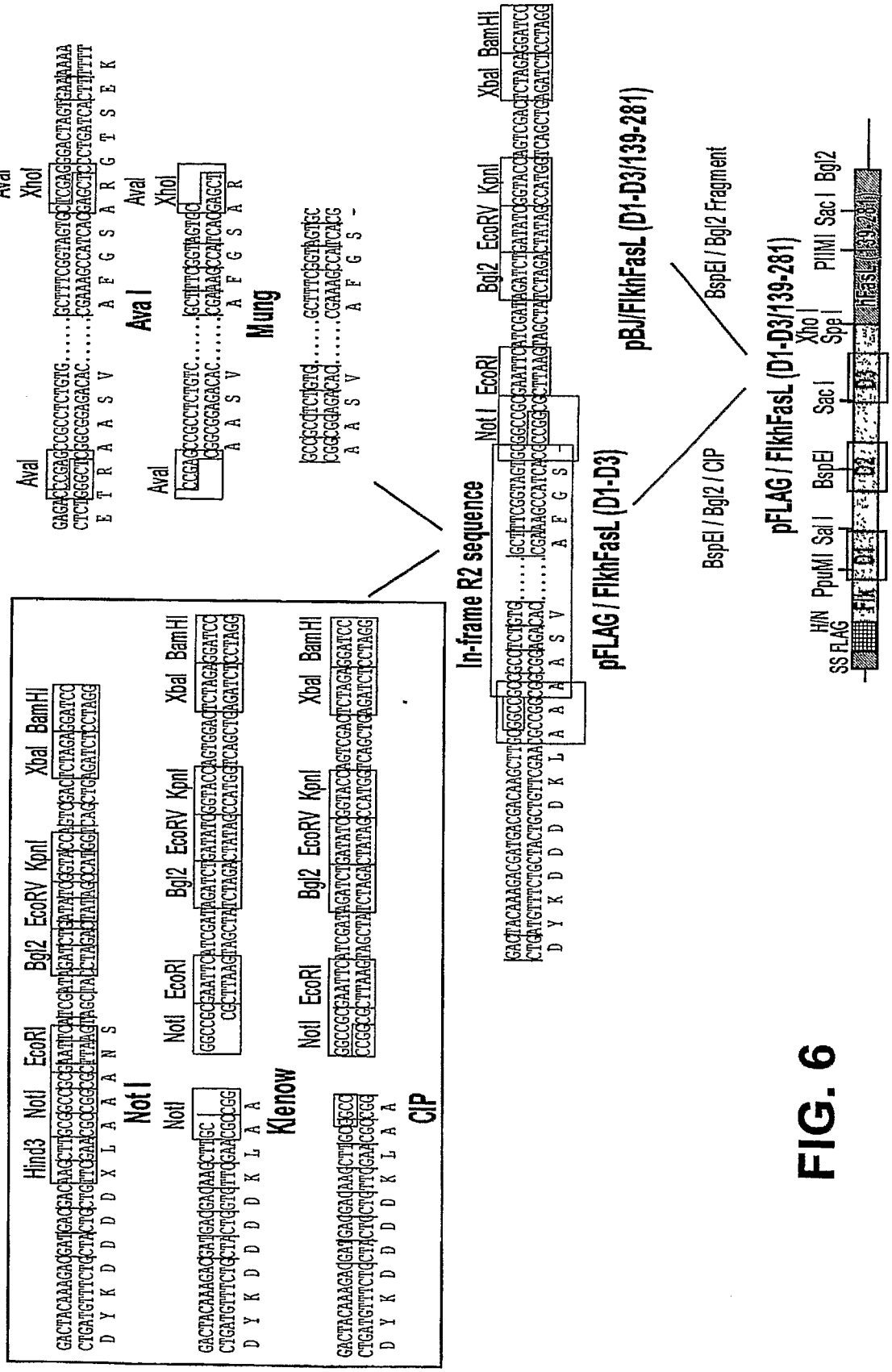

pFLAG-CMV-3
Hind3 NotI EcoRI Bgl2 EcoRV KpnI XbaI BamHI
D Y K D D D D K L A A A N S
Not I
NotI NotI EcoRI Bgl2 EcoRV KpnI XbaI BamHI
D Y K D D D D K L A A
Klenow
NotI EcoRI Bgl2 EcoRV KpnI XbaI BamHI
D Y K D D D D K L A A
CIP
pBJ/FlkhFasL (D1-D3/139-281)
AvaI XhoI
E T R A A S V A F G S A R G T S E S K
Ava I
AvaI AvaI XhoI
A A S V A F G S A R
Mung
A A S V A F G S -
In-frame R2 sequence
NotI EcoRI Bgl2 EcoRV KpnI XbaI BamHI
D Y K D D D D K L A A A S V A F G S
pFLAG / FlkhFasL (D1-D3)
pBJ/FlkhFasL (D1-D3/139-281)
BspEI / Bgl2 / CIP
BspEI / Bgl2 Fragment
pFLAG / FlkhFasL (D1-D3/139-281)
H/N SS FLAG
PpuMI SalI BspEI SacI XhoI SpeI PIMI SacI Bgl2

FIG. 6

1 ATGGAGAGCAAGGCGCTGCTAGCTGTCGCTCTGTGGTTCTGCGTGGAGACCCGAGCCGCC
61 TCTGTGGGTTTGCCTGGCGATTTTCTCCATCCCCCCAAGCTCAGCACACAGAAAGACATA
121 CTGACAATTTTGGCAAATACAACCCTTCAGATTACTTGCAGGGGACAGCGGGACCTGGAC
181 TGGCTTTGGCCCAATGCTCAGCGTGATTCTGAGGAAAGGGTATTGGTGACTGAATGCGGC
241 GGTGGTGACAGTATCTTCTGCAAAACACTCACCATTCCCAGGGTGGTTGGAAATGATACT
301 GGAGCCTACAAGTGCTCGTACCGGGACGTCGACATAGCCTCCACTGTTTATGTCTATGTT
361 CGAGATTACAGATCACCATTCATCGCCTCTGTCAGTGACCAGCATGGCATCGTGTACATC
421 ACCGAGAACAAGAACAAAACTGTGGTGATCCCCTGCCGAGGGTCGATTTCAAACCTCAAT
481 GTGTCTCTTTGCGCTAGGTATCCAGAAAAGAGATTTGTTCCGGATGGAAACAGAATTTCC
541 TGGGACAGCGAGATAGGCTTTACTCTCCCCAGTTACATGATCAGCTATGCCGGCATGGTC
601 TTCTGTGAGGCAAAGATCAATGATGAAACCTATCAGTCTATCATGTACATAGTTGTGGTT
661 GTAGGATATAGGATTTATGATGTGATTCTGAGCCCCCCGCATGAAATTGAGCTATCTGCC
721 GGAGAAAAACTTGTCTTAAATTGTACAGCGAGAACAGAGCTCAATGTGGGGCTTGATTTC
781 ACCTGGCACTCTCCACCTTCAAAGTCTCATCATAAGAAGATTGTAAACCGGGATGTGAAA
841 CCCTTTCCTGGGACTGTGGCGAAGATGTTTTTGAGCACCTTGACAATAGAAAGTGTGACC
901 AAGAGTGACCAAGGGGAATACACCTGTGTAGCGTCCAGTGGACGGATGATCAAGAGAAAT
961 AGAACATTTGTCCGAGTTCACACAAAGCCTTTTATTGCTTTCGGTAGTGCTCGAGGGACT
1021 AGT**GAAAAAAAGGAGCTGAGGAAAGTGGCCCATTTAACAGGCAAGTCCAACTCAAGGTCC**
1081 **ATGCCTCTGGAATGGGAAGACACCTATGGAATTGTCCTGCTTTCTGGAGTGAAGTATAAG**
1141 **AAGGGTGGCCTTGTGATCAATGAAACTGGGCTGTACTTTGTATATTCCAAAGTATACTTC**
1201 **CGGGGTCAATCTTGCAACAACCTGCCCCTGAGCCACAAGGTCTACATGAGGAACTCTAAG**
1261 **TATCCCCAGGATCTGGTGATGATGGAGGGGAAGATGATGAGCTACTGCACTACTGGGCAG**
1321 **ATGTGGGCCCGCAGCAGCTACCTGGGGGCAGTGTTCAATCTTACCAGTGCTGATCATTTA**
1381 **TATGTCAACGTATCTGAGCTCTCTCTGGTCAATTTTGAGGAATCTCAGACGTTTTTCGGC**
1441 **TTATATAAGCTCTAA**

FIG. 7

1 *MESKALLAVALWFCVETRA*ASVGLPGDFLHPPKLSTQKDILTILANTTLQITCRGQRDLD
61 WLWPNAQRDSEERVLVTECGGGDSIFCKTLTIPRVVGNDTGAYKCSYRDVDIASTVYVYV
121 RDYRSPFIASVSDQHGIVYITENKNKTVVIPCRGSISNLNVSLCARYPEKRFVPDGNRIS
181 WDSEIGFTLPSYMISYAGMVFCEAKINDETYQSIMYIVVVVGYRIYDVILSPPHEIELSA
241 GEKLVLNCTARTELNVGLDFTWHSPPSKSHHKKIVNRDVKPFPGTVAKMFLSTLTIESVT
301 KSDQGEYTCVASSGRMIKRNRTFVRVHTKPFIAFGSARGTS**EKKELRKVAHLTGKSNSRS**
361 **MPLEWEDTYGIVLLSGVKYKKGGLVINETGLYFVYSKVYFRGQSCNNLPLSHKVYMRNSK**
421 **YPQDLVMMEGKMMSYCTTGQMWARSSYLGAVFNLTSADHLYVNVSELSLVNFEESQTFFG**
481 **LYKL**

FIG. 8

```
1    ATGTCTGCACTTCTGATCCTAGCTCTTGTTGGAGCTGCAGTTGCTGACTACAAAGACGAT
61   GACGACAAGCTTGCGGCCGCCAGTGATACAGGTAGACCTTTCGTAGAGATGTACAGTGAA
121  ATCCCCGAAATTATACACATGACTGAAGGAAGGGAGCTCGTCATTCCCTGCCGGGTTACG
181  TCACCTAACATCACTGTTACTTTAAAAAAGTTTCCACTTGACACTTTGATCCCTGATGGA
241  AAACGCATAATCTGGGACAGTAGAAAGGGCTTCATCATATCAAATGCAACGTACAAAGAA
301  ATAGGGCTTCTGACCTGTGAAGCAACAGTCAATGGGCATTTGTATAAGACAAACTATCTC
361  ACACATCGACAAACCAATACAATCATAGCTCGAGGGACTAGTGAAAAAAAGGAGCTGAGG
421  AAAGTGGCCCATTTAACAGGCAAGTCCAACTCAAGGTCCATGCCTCTGGAATGGGAAGAC
481  ACCTATGGAATTGTCCTGCTTTCTGGAGTGAAGTATAAGAAGGGTGGCCTTGTGATCAAT
541  GAAACTGGGCTGTACTTTGTATATTCCAAAGTATACTTCCGGGGTCAATCTTGCAACAAC
601  CTGCCCCTGAGCCACAAGGTCTACATGAGGAACTCTAAGTATCCCCAGGATCTGGTGATG
661  ATGGAGGGGAAGATGATGAGCTACTGCACTACTGGGCAGATGTGGGCCCGCAGCAGCTAC
721  CTGGGGGCAGTGTTCAATCTTACCAGTGCTGATCATTTATATGTCAACGTATCTGAGCTC
781  TCTCTGGTCAATTTTGAGGAATCTCAGACGTTTTTCGGCTTATATAAGCTCTAA
```

FIG. 9

```
1    SDTGRPFVEMYSEIPEIIHMTEGRELVIPCRVTSPNITVTLKKFPLDTLIPDGKRIIWDS
61   RKGFIISNATYKEIGLLTCEATVNGHLYKTNYLTHRQTNTII
```

FIG. 10

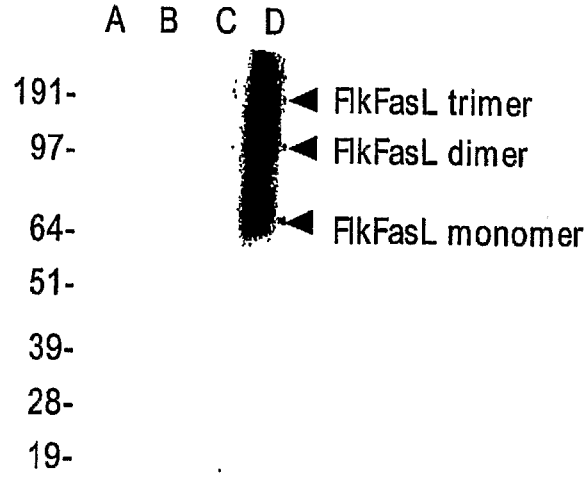


FIG. 11A

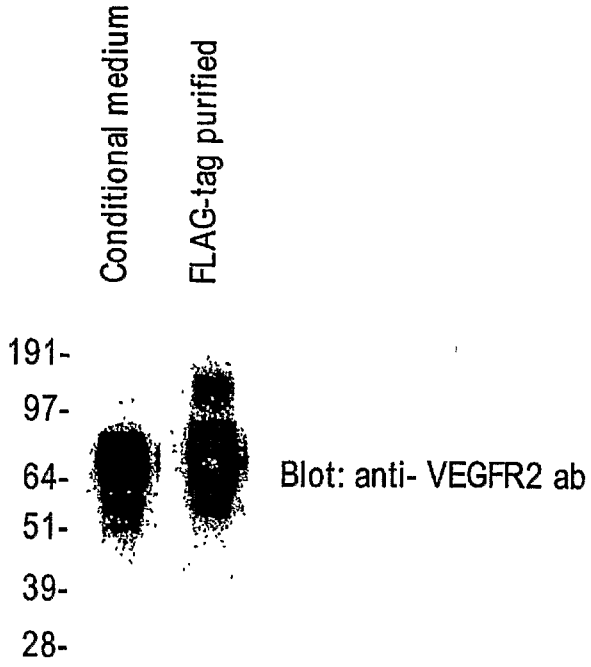


FIG. 11B

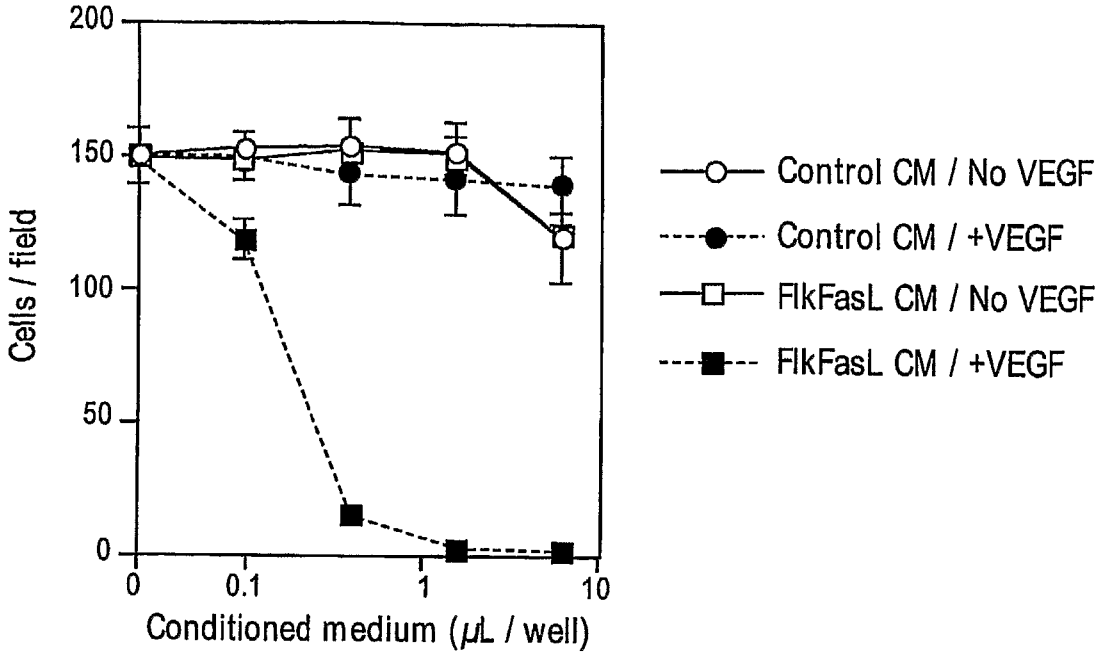

200
150
100
50
0
Cells / field
0
0.1
1
10
Conditioned medium (μL / well)
Control CM / No VEGF
Control CM / +VEGF
FlkFasL CM / No VEGF
FlkFasL CM / +VEGF

FIG. 12

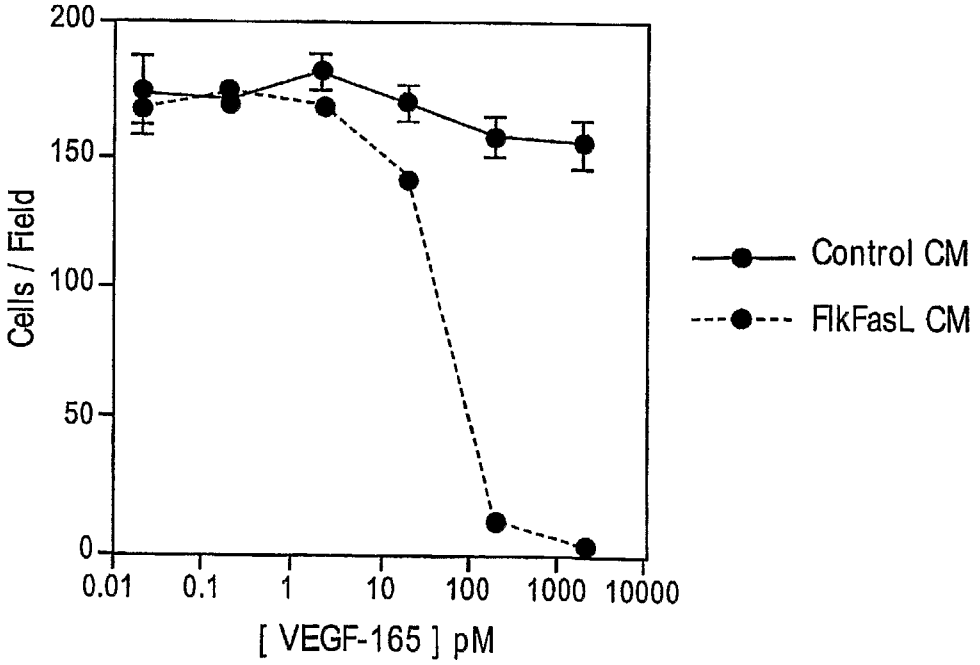

200
150
100
50
0
Cells / Field
0.01
0.1
1
10
100
1000
10000
[VEGF-165] pM
Control CM
FlkFasL CM

FIG. 13

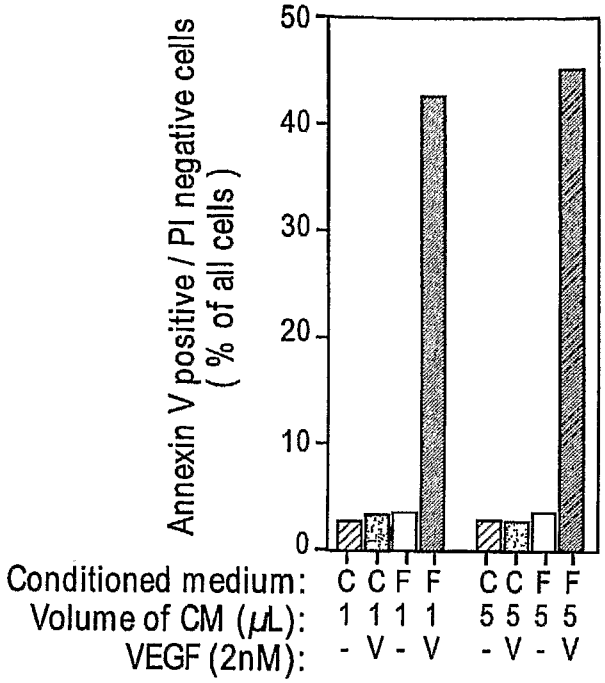

Annexin V positive / PI negative cells
(% of all cells)
50
40
30
20
10
0
Conditioned medium: C C F F C C F F
Volume of CM (μL): 1 1 1 1 5 5 5 5
VEGF (2nM): - V - V - V - V

FIG. 14

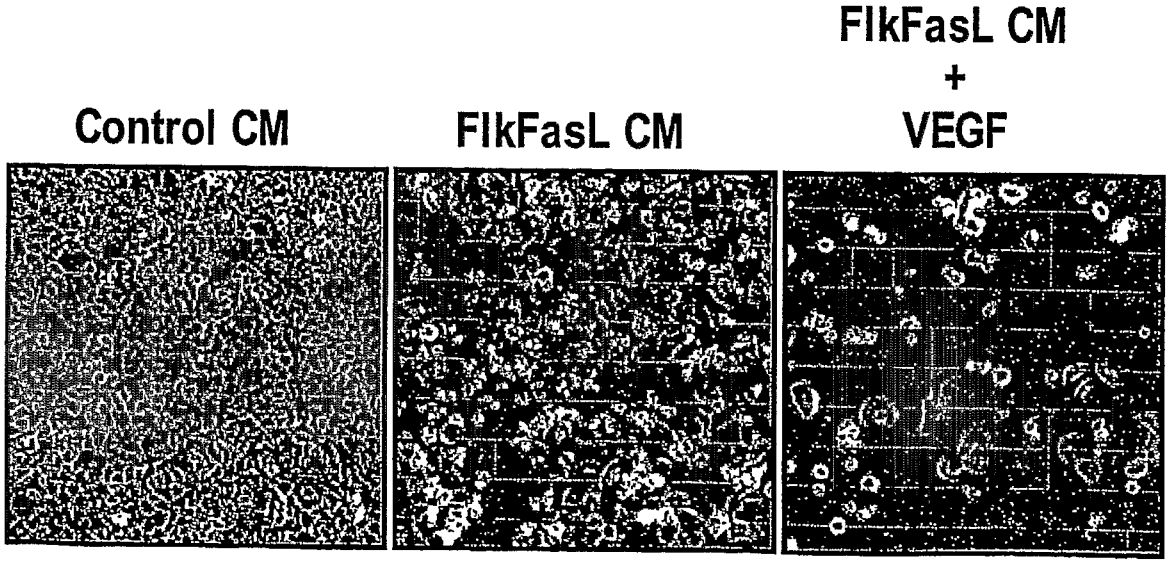

Control CM
FlkFasL CM
FlkFasL CM
+
VEGF

FIG. 15

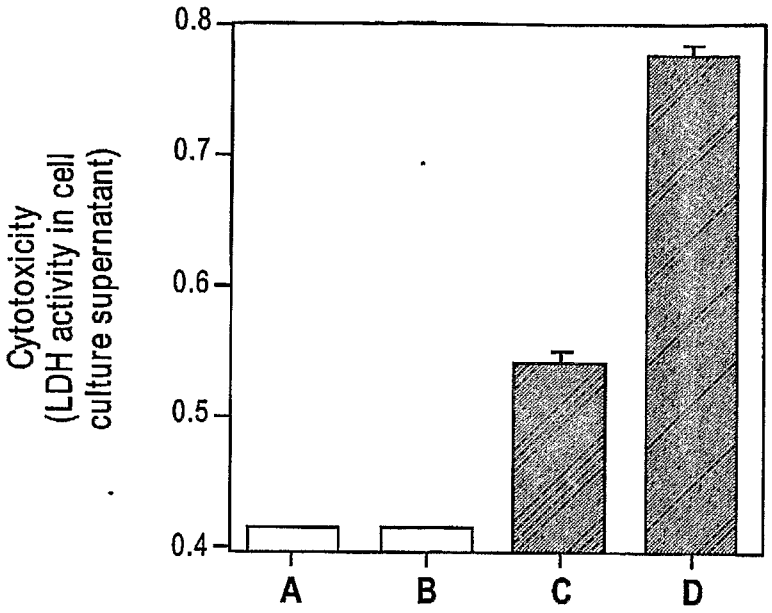

Cytotoxicity
(LDH activity in cell
culture supernatant)
0.8
0.7
0.6
0.5
0.4
A
B
C
D

FIG. 16

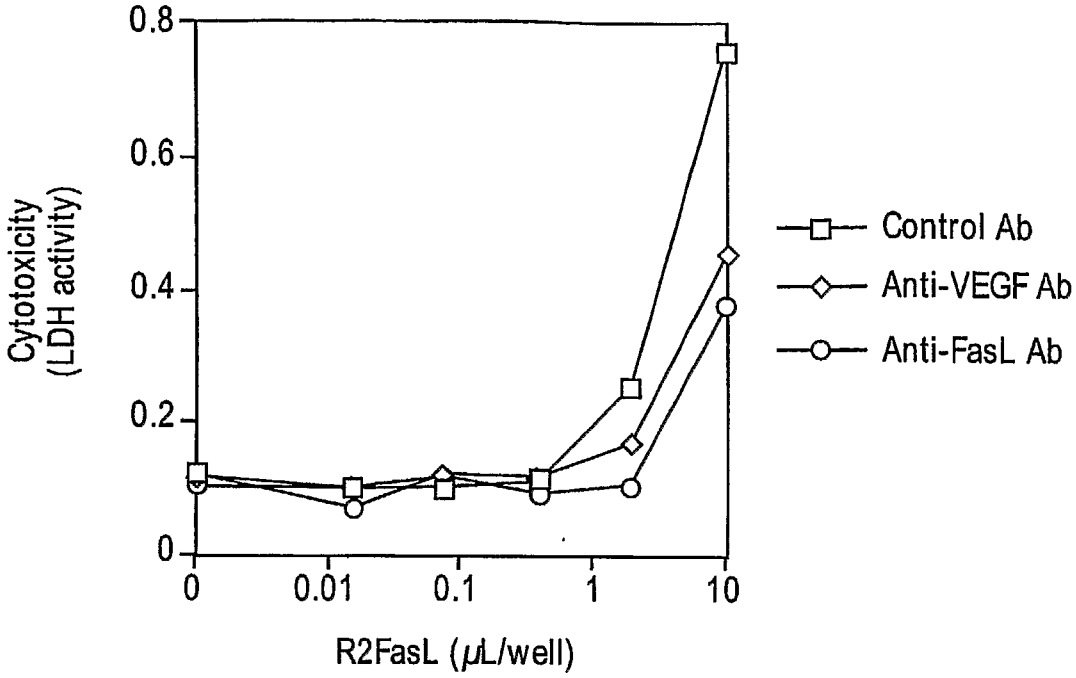


FIG. 17A

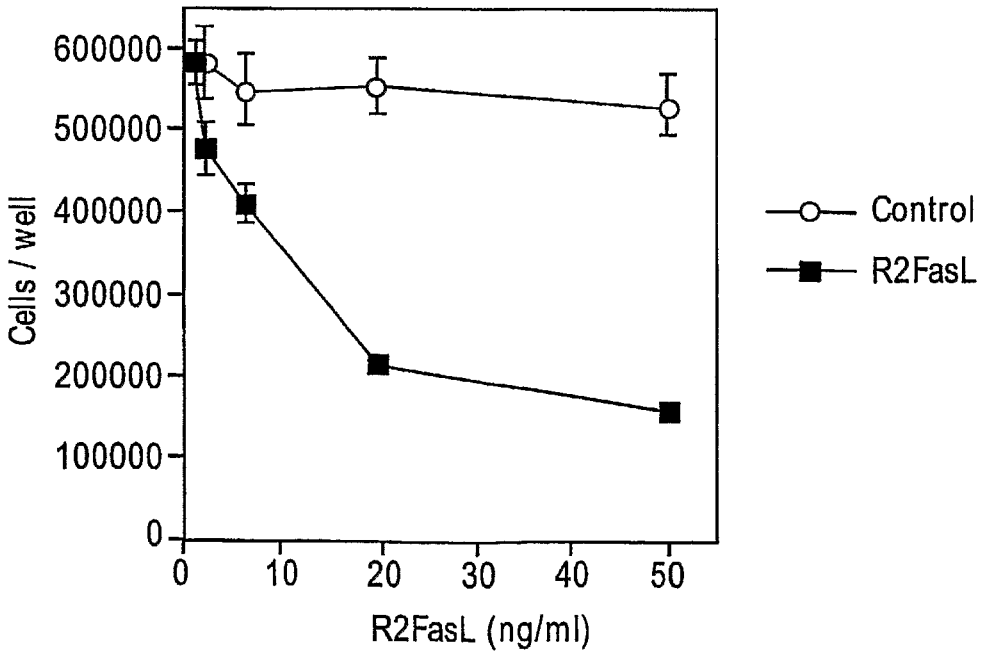


FIG. 17B

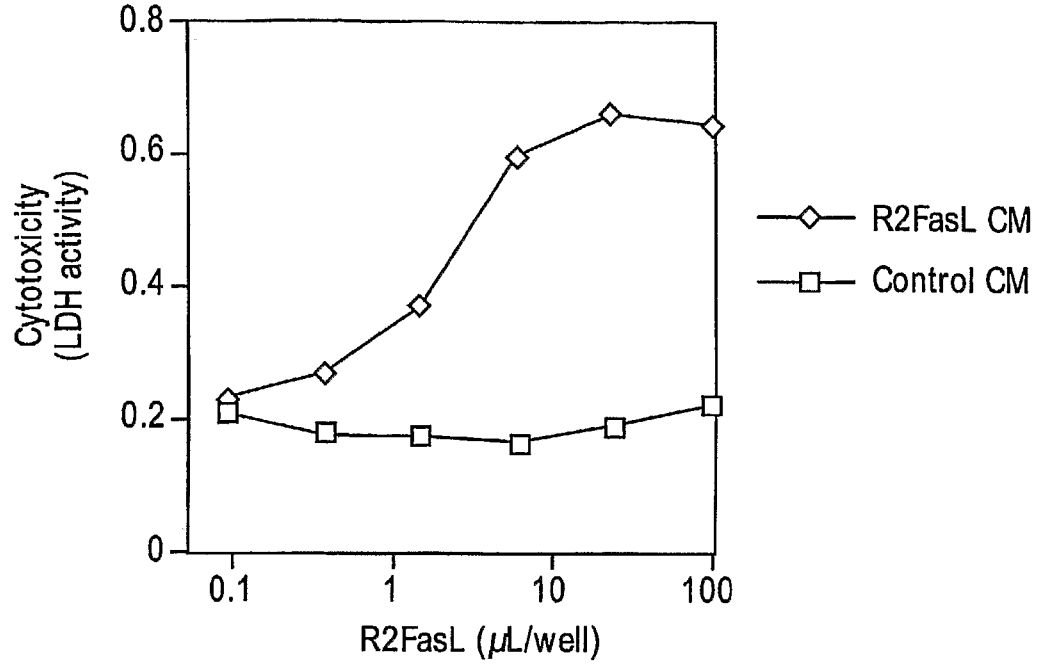


FIG. 17C

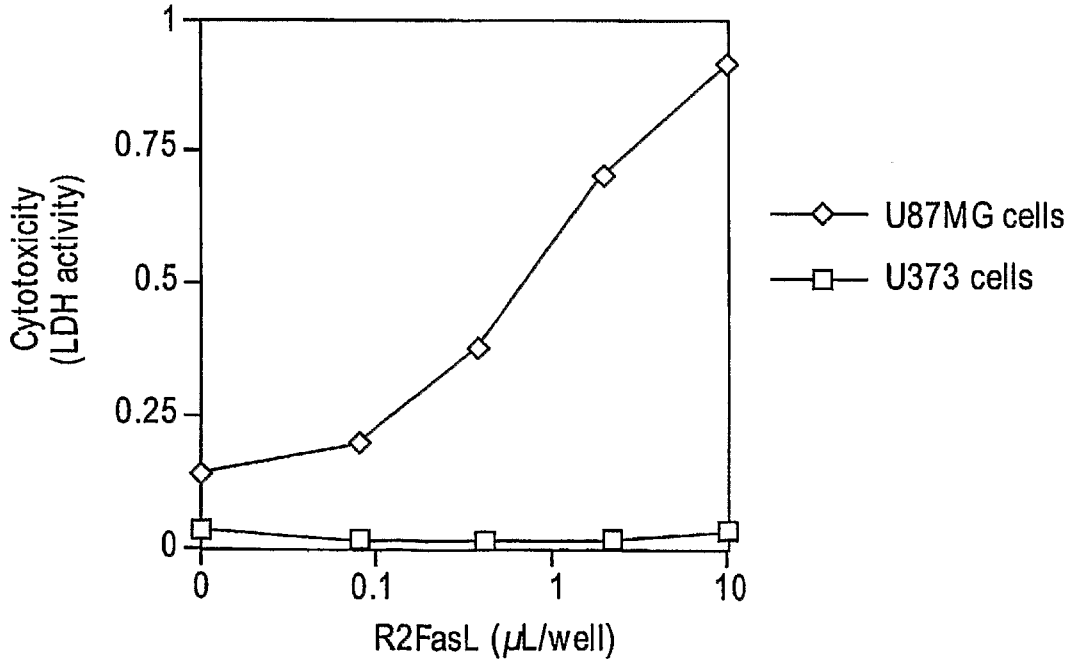


FIG. 17D

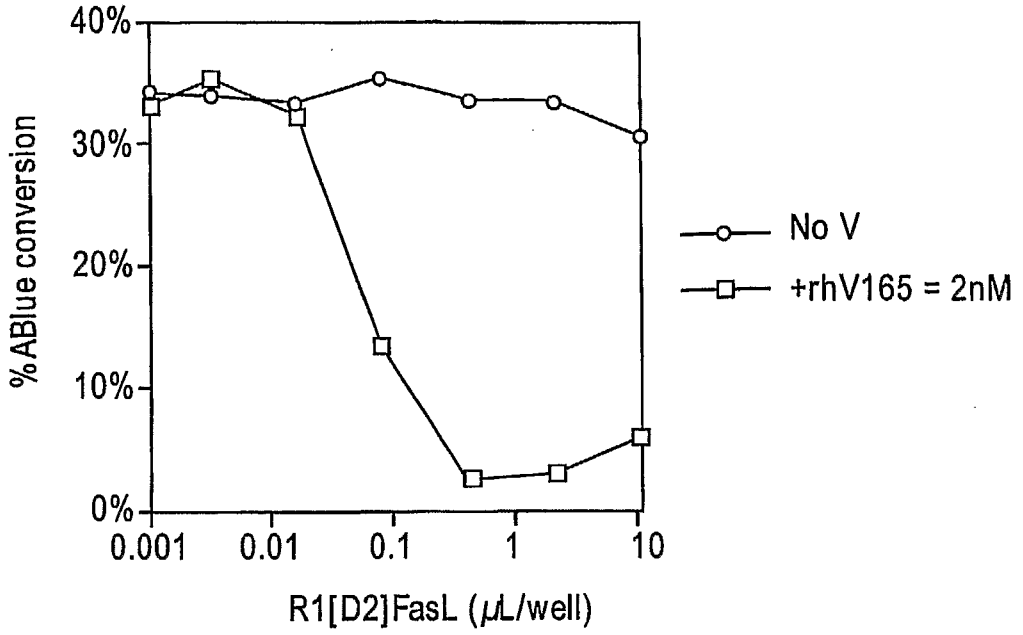


FIG. 18A

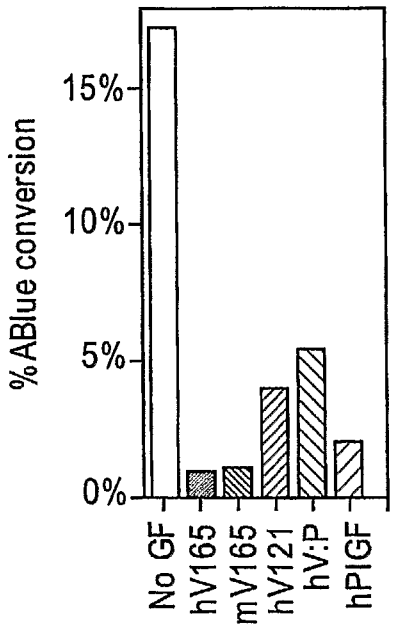


FIG. 18B

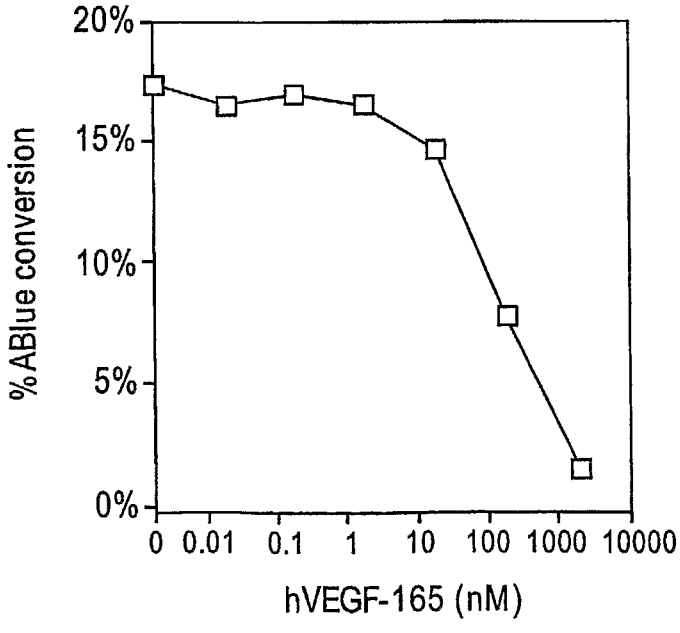


FIG. 18C

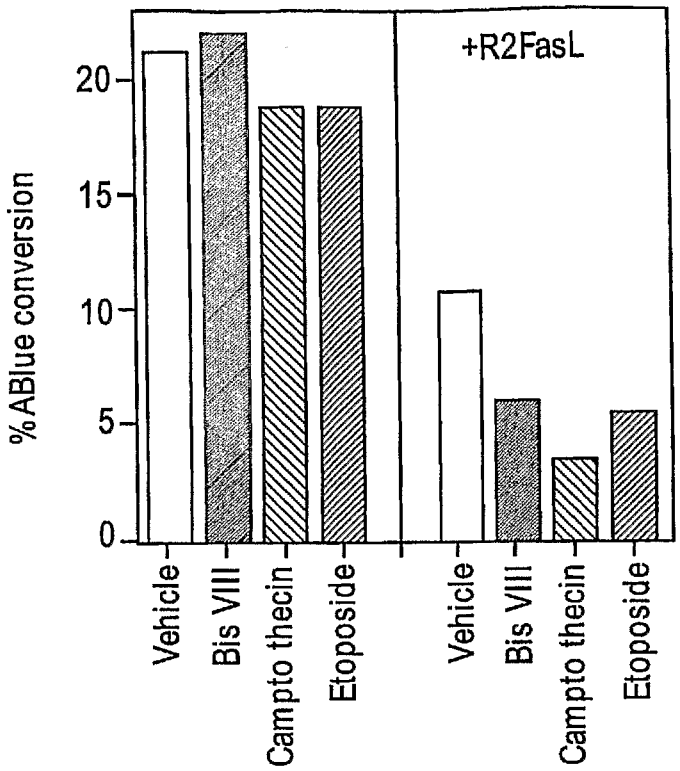


FIG. 19A

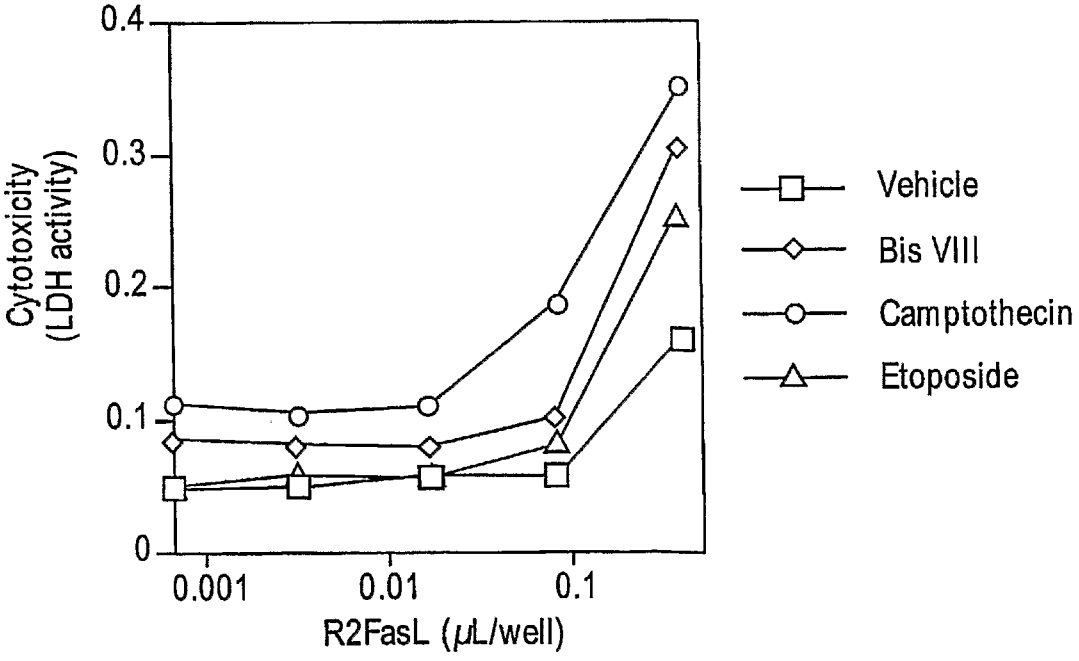


FIG. 19B

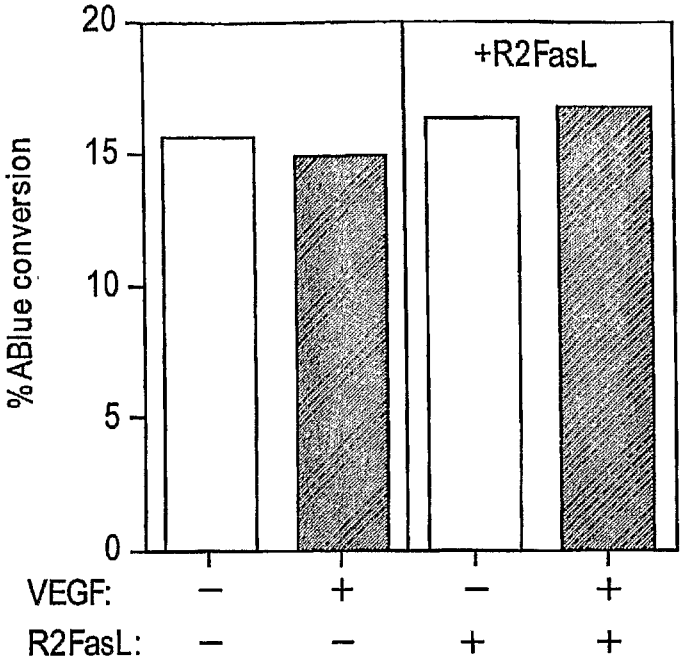


FIG. 20A

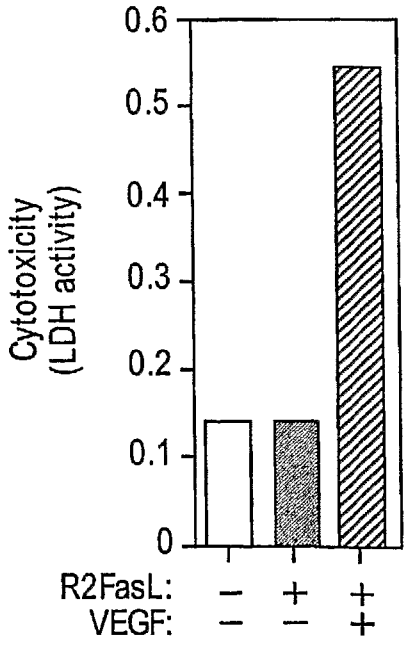


FIG. 20B

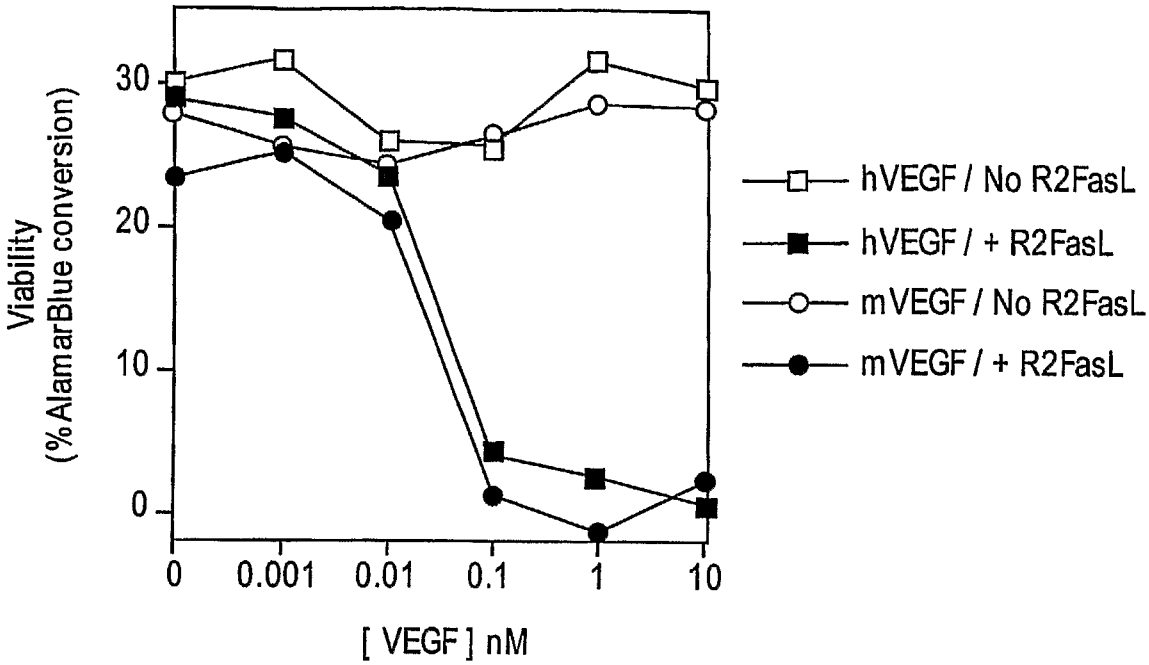


FIG. 21A

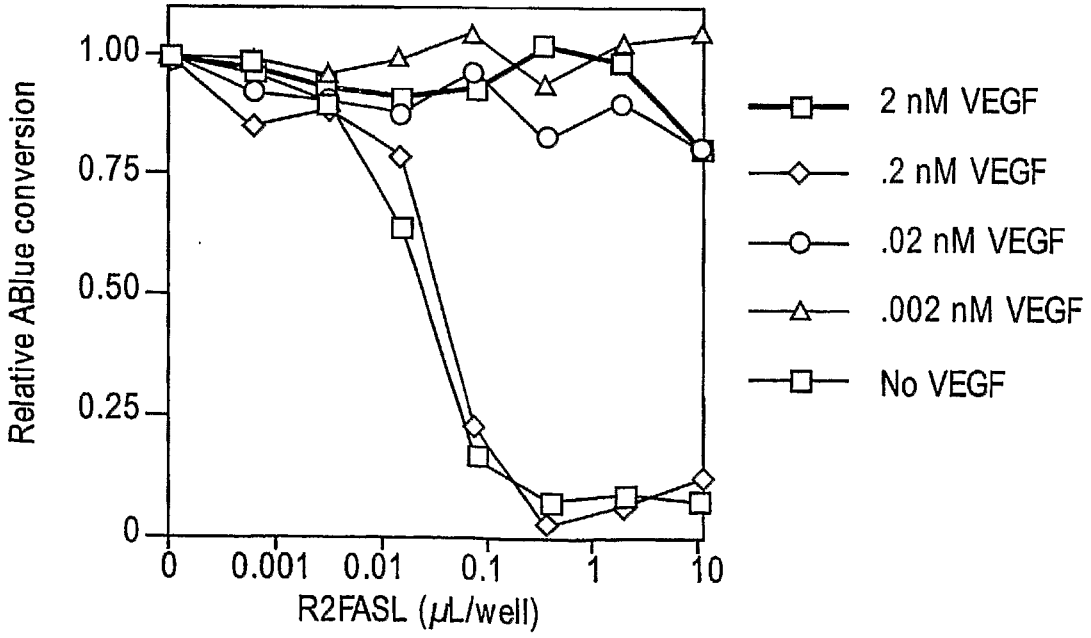


FIG. 21B

INFORMAL SEQUENCE LISTING

SEQ ID NO:1 Extracellular domain of human VEGF Receptor-2 protein sequence, including signal peptide (part of GenBank NP_002244)

```
1   MQSKVLLAVA LWLCVETRAA SVGLPSVSLD LPRLSIQKDI LTIKANTTLQ ITCRGQRDLD
61  WLWPNNQSGS EQRVEVTECS DGLFCKTLTI PKVIGNDTGA YKCFYRETDL ASVIYVYVQD
121 YRSPFIASVS DQHGVVYITE NKNKTVVIPC LGSISNLNVS LCARYPEKRF VPDGNRISWD
181 SKKGFTIPSY MISYAGMVFC EAKINDESYQ SIMYIVVVVG YRIYDVVLSP SHGIELSVGE
241 KLVLNCTART ELNVGIDFNW EYPSSKHQHK KLVNRDLKTQ SGSEMKKFLS TLTIDGVTRS
301 DQGLYTCAAS SGLMTKKNST FVRVHEKPFV AFGS
```

SEQ ID NO:2 Extracellular domain of mouse VEGF Receptor-2 protein sequence, including signal peptide (part of GenBank P35918)

```
1   MESKALLAVA LWFCVETRAA SVGLPGDFLH PPKLSTQKDI LTILANTTLQ ITCRGQRDLD
61  WLWPNAQRDS EERVLVTECG GGDSIFCKTL TIPRVVGNDT GAYKCSYRDV DIASTVYVYV
121 RDYRSPFIAS VSDQHGIVYI TENKNKTVVI PCRGSISNLN VSLCARYPEK RFVPDGNRIS
181 WDSEIGFTLP SYMISYAGMV FCEAKINDET YQSIMYIVVV VGYRIYDVIL SPPHEIELSA
241 GEKLVLNCTA RTELNVGLDF TWHSPPSKSH HKKIVNRDVK PFPGTVAKMF LSTLTIESVT
301 KSDQGEYTCV ASSGRMIKRN RTFVRVHTKP FIAFGS
```

SEQ ID NO:3 Extracellular domain of rat VEGF Receptor-2 protein sequence, including signal peptide (part of GenBank O08775)

```
1   MESRALLAVA LWFCVETRAA SVGLPGDSLH PPKLSTQKDI LTILANTTLQ ITCRGQRDLD
61  WLWPNTPRDS EERVLVTECG DSIFCKTLTV PRVVGNDTGA YKCFYRDTDV SSIVYVYVQD
121 HRSPFIASVS DEHGIVYITE NKNKTVVIPC RGSISNLNVS LCARYPEKRF VPDGNRISWD
181 SEKGFTIPSY MISYAGMVFC EAKINDETYQ SIMYIVLVVG YRIYDVVLSP PHEIELSAGE
241 KLVLNCTART ELNVGLDFSW QFPSSKHQHK KIVNRDVKSL PGTVAKMFLS TLTIDSVTKS
301 DQGEYTCTAY SGLMTKKNKT FVRVHTKPFI AFGS
```

SEQ ID NO:4 Extracellular domain of human VEGF Receptor-2 protein sequence (part of GenBank NP_002244)

```
1   ASVGLPSVSL DLPRLSIQKD ILTIKANTTL QITCRGQRDL DWLWPNNQSG SEQRVEVTEC
61  SDGLFCKTLT IPKVIGNDTG AYKCFYRETD LASVIYVYVQ DYRSPFIASV SDQHGVVYIT
121 ENKNKTVVIP CLGSISNLNV SLCARYPEKR FVPDGNRISW DSKKGFTIPS YMISYAGMVF
181 CEAKINDESY QSIMYIVVVV GYRIYDVVLS PSHGIELSVG EKLVLNCTAR TELNVGIDFN
241 WEYPSSKHQH KKLVNRDLKT QSGSEMKKFL STLTIDGVTR SDQGLYTCAA SSGLMTKKNS
301 TFVRVHEKPF VAFGS
```

SEQ ID NO:5 Extracellular domain of mouse VEGF Receptor-2 protein sequence (part of GenBank P35918)

```
1   ASVGLPGDFL HPPKLSTQKD ILTILANTTL QITCRGQRDL DWLWPNAQRD SEERVLVTEC
61  GGGDSIFCKT LTIPRVVGND TGAYKCSYRD VDIASTVYVY VRDYRSPFIA SVSDQHGIVY
121 ITENKNKTVV IPCRGSISNL NVSLCARYPE KRFVPDGNRI SWDSEIGFTL PSYMISYAGM
181 VFCEAKINDE TYQSIMYIVV VVGYRIYDVI LSPPHEIELS AGEKLVLNCT ARTELNVGLD
241 FTWHSPPSKS HHKKIVNRDV KPFPGTVAKM FLSTLTIESV TKSDQGEYTC VASSGRMIKR
```

```
301 NRTFVRVHTK PFIAFGS
```

SEQ ID NO:6 Extracellular domain of rat VEGF Receptor-2 protein sequence (part of GenBank O08775)

```
1   ASVGLPGDSL HPPKLSTQKD ILTILANTTL QITCRGQRDL DWLWPNTPRD SEERVLVTEC
61  GDSIFCKTLT VPRVVGNDTG AYKCFYRDTD VSSIVYVYVQ DHRSPFIASV SDEHGIVYIT
121 ENKNKTVVIP CRGSISNLNV SLCARYPEKR FVPDGNRISW DSEKGFTIPS YMISYAGMVF
181 CEAKINDETY QSIMYIVLVV GYRIYDVVLS PPHEIELSAG EKLVLNCTAR TELNVGLDFS
241 WQFPSSKHQH KKIVNRDVKS LPGTVAKMFL STLTIDSVTK SDQGEYTCTA YSGLMTKKNK
301 TFVRVHTKPF IAFGS
```

SEQ ID NO:7 Amino acid linker sequence

```
1   ARGTS
```

SEQ ID NO:8 Human FasL protein sequence (GenBank P48023)

```
1   MQQPFNYPYP QIYWVDSSAS SPWAPPGTVL PCPTSVPRRP GQRRPPPPPP PPPLPPPPPP
61  PPLPPLPLPP LKKRGNHSTG LCLLVMFFMV LVALVGLGLG MFQLFHLQKE LAELRESTSQ
121 MHTASSLEKQ IGHPSPPPEK KELRKVAHLT GKSNSRSMPL EWEDTYGIVL LSGVKYKKGG
181 LVINETGLYF VYSKVYFRGQ SCNNLPLSHK VYMRNSKYPQ DLVMMEGKMM SYCTTGQMWA
241 RSSYLGAVFN LTSADHLYVN VSELSLVNFE ESQTFFGLYK L
```

SEQ ID NO:9 Mouse FasL protein sequence (GenBank A53062)

```
1   MQQPMNYPCP QIFWVDSSAT SSWAPPGSVF PCPSCGPRGP DQRRPPPPPP PVSPLPPPSQ
61  PLPLPPLTPL KKKDHNTNLW LPVVFFMVLV ALVGMGLGMY QLFHLQKELA ELREFTNQSL
121 KVSSFEKQIA NPSTPSEKKE PRSVAHLTGN PHSRSIPLEW EDTYGTALIS GVKYKKGGLV
181 INETGLYFVY SKVYFRGQSC NNQPLNHKVY MRNSKYPEDL VLMEEKRLNY CTTGQIWAHS
241 SYLGAVFNLT SADHLYVNIS QLSLINFEES KTFFGLYKL
```

SEQ ID NO:10 Rat FasL protein sequence (GenBank A49266)

```
1   MQQPVNYPCP QIYWVDSSAT SPWAPPGSVF SCPSSGPRGP GQRRPPPPPP PPSPLPPPSQ
61  PPPLPPLSPL KKKDNIELWL PVIFFMVLVA LVGMGLGMYQ LFHLQKELAE LREFTNHSLR
121 VSSFEKQIAN PSTPSETKKP RSVAHLTGNP RSRSIPLEWE DTYGTALISG VKYKKGGLVI
181 NEAGLYFVYS KVYFRGQSCN SQPLSHKVYM RNFKYPGDLV LMEEKKLNYC TTGQIWAHSS
241 YLGAVFNLTV ADHLYVNISQ LSLINFEESK TFFGLYKL
```

SEQ ID NO:11 Human FasL protein sequence in VEGFR-FasL fusion protein (part of GenBank P48023)

```
1   EKKELRKVAH LTGKSNSRSM PLEWEDTYGI VLLSGVKYKK GGLVINETGL YFVYSKVYFR
61  GQSCNNLPLS HKVYMRNSKY PQDLVMMEGK MMSYCTTGQM WARSSYLGAV FNLTSADHLY
121 VNVSELSLVN FEESQTFFGL YKL
```

SEQ ID NO:12 Mouse FasL protein sequence for VEGFR-FasL fusion protein (part of GenBank A53062)

```
1   EKKEPRSVAH LTGNPHSRSI PLEWEDTYGT ALISGVKYKK GGLVINETGL YFVYSKVYFR
61  GQSCNNQPLN HKVYMRNSKY PEDLVLMEEK RLNYCTTGQI WAHSSYLGAV FNLTSADHLY
121 VNISQLSLIN FEESKTFFGL YKL
```

SEQ ID NO:13 Rat FasL protein sequence for VEGFR-FasL fusion protein (part of GenBank A49266)

```
1   ETKKPRSVAH LTGNPRSRSI PLEWEDTYGT ALISGVKYKK GGLVINEAGL YFVYSKVYFR
61  GQSCNSQPLS HKVYMRNFKY PGDLVLMEEK KLNYCTTGQI WAHSSYLGAV FNLTVADHLY
121 VNISQLSLIN FEESKTFFGL YKL
```

SEQ ID NO:14 cDNA sequence encoding a VEGFR-FasL fusion protein Flk(D1-D3)FasL(139-281), including signal peptide and linker sequence

```
1    ATGGAGAGCA AGGCGCTGCT AGCTGTCGCT CTGTGGTTCT GCGTGGAGAC CCGAGCCGCC
61   TCTGTGGGTT TGCCTGGCGA TTTTCTCCAT CCCCCCAAGC TCAGCACACA GAAAGACATA
121  CTGACAATTT TGGCAAATAC AACCCTTCAG ATTACTTGCA GGGGACAGCG GGACCTGGAC
181  TGGCTTTGGC CCAATGCTCA GCGTGATTCT GAGGAAAGGG TATTGGTGAC TGAATGCGGC
241  GGTGGTGACA GTATCTTCTG CAAAACACTC ACCATTCCCA GGGTGGTTGG AAATGATACT
301  GGAGCCTACA AGTGCTCGTA CCGGGACGTC GACATAGCCT CCACTGTTTA TGTCTATGTT
361  CGAGATTACA GATCACCATT CATCGCCTCT GTCAGTGACC AGCATGGCAT CGTGTACATC
421  ACCGAGAACA AGAACAAAAC TGTGGTGATC CCCTGCCGAG GGTCGATTTC AAACCTCAAT
481  GTGTCTCTTT GCGCTAGGTA TCCAGAAAAG AGATTTGTTC CGGATGGAAA CAGAATTTCC
541  TGGGACAGCG AGATAGGCTT TACTCTCCCC AGTTACATGA TCAGCTATGC CGGCATGGTC
601  TTCTGTGAGG CAAAGATCAA TGATGAAACC TATCAGTCTA TCATGTACAT AGTTGTGGTT
661  GTAGGATATA GGATTTATGA TGTGATTCTG AGCCCCCCGC ATGAAATTGA GCTATCTGCC
721  GGAGAAAAAC TTGTCTTAAA TTGTACAGCG AGAACAGAGC TCAATGTGGG GCTTGATTTC
781  ACCTGGCACT CTCCACCTTC AAAGTCTCAT CATAAGAAGA TTGTAAACCG GGATGTGAAA
841  CCCTTTCCTG GGACTGTGGC GAAGATGTTT TTGAGCACCT TGACAATAGA AAGTGTGACC
901  AAGAGTGACC AAGGGGAATA CACCTGTGTA GCGTCCAGTG GACGGATGAT CAAGAGAAAT
961  AGAACATTTG TCCGAGTTCA CACAAAGCCT TTTATTGCTT TCGGTAGTGC TCGAGGGACT
1021 AGTGAAAAAA AGGAGCTGAG GAAAGTGGCC CATTTAACAG GCAAGTCCAA CTCAAGGTCC
1081 ATGCCTCTGG AATGGGAAGA CACCTATGGA ATTGTCCTGC TTTCTGGAGT GAAGTATAAG
1141 AAGGGTGGCC TTGTGATCAA TGAAACTGGG CTGTACTTTG TATATTCCAA AGTATACTTC
1201 CGGGGTCAAT CTTGCAACAA CCTGCCCCTG AGCCACAAGG TCTACATGAG GAACTCTAAG
1261 TATCCCCAGG ATCTGGTGAT GATGGAGGGG AAGATGATGA GCTACTGCAC TACTGGGCAG
1321 ATGTGGGCCC GCAGCAGCTA CCTGGGGGCA GTGTTCAATC TTACCAGTGC TGATCATTTA
1381 TATGTCAACG TATCTGAGCT CTCTCTGGTC AATTTTGAGG AATCTCAGAC GTTTTTCGGC
1441 TTATATAAGC TCTAA
```

SEQ ID NO:15 cDNA sequence encoding mouse extracellular domain of VEGF Receptor-2 (19-336)

```
1    GCCTCTGTGG GTTTGCCTGG CGATTTTCTC CATCCCCCCA AGCTCAGCAC ACAGAAAGAC
61   ATACTGACAA TTTTGGCAAA TACAACCCTT CAGATTACTT GCAGGGGACA GCGGGACCTG
121  GACTGGCTTT GGCCCAATGC TCAGCGTGAT TCTGAGGAAA GGGTATTGGT GACTGAATGC
181  GGCGGTGGTG ACAGTATCTT CTGCAAAACA CTCACCATTC CCAGGGTGGT TGGAAATGAT
241  ACTGGAGCCT ACAAGTGCTC GTACCGGGAC GTCGACATAG CCTCCACTGT TTATGTCTAT
301  GTTCGAGATT ACAGATCACC ATTCATCGCC TCTGTCAGTG ACCAGCATGG CATCGTGTAC
361  ATCACCGAGA ACAAGAACAA AACTGTGGTG ATCCCCTGCC GAGGGTCGAT TTCAAACCTC
421  AATGTGTCTC TTTGCGCTAG GTATCCAGAA AAGAGATTTG TTCCGGATGG AAACAGAATT
481  TCCTGGGACA GCGAGATAGG CTTTACTCTC CCCAGTTACA TGATCAGCTA TGCCGGCATG
541  GTCTTCTGTG AGGCAAAGAT CAATGATGAA ACCTATCAGT CTATCATGTA CATAGTTGTG
601  GTTGTAGGAT ATAGGATTTA TGATGTGATT CTGAGCCCCC CGCATGAAAT TGAGCTATCT
```

```
661  GCCGGAGAAA AACTTGTCTT AAATTGTACA GCGAGAACAG AGCTCAATGT GGGGCTTGAT
721  TTCACCTGGC ACTCTCCACC TTCAAAGTCT CATCATAAGA AGATTGTAAA CCGGGATGTG
781  AAACCCTTTC CTGGGACTGT GGCGAAGATG TTTTTGAGCA CCTTGACAAT AGAAAGTGTG
841  ACCAAGAGTG ACCAAGGGGA ATACACCTGT GTAGCGTCCA GTGGACGGAT GATCAAGAGA
901  AATAGAACAT TTGTCCGAGT TCACACAAAG CCTTTTATTG CTTTCGGTAG T
```

SEQ ID NO:16 cDNA sequence encoding human FasL (139-281)

```
1    GAAAAAAAGG AGCTGAGGAA AGTGGCCCAT TTAACAGGCA AGTCCAACTC AAGGTCCATG
61   CCTCTGGAAT GGGAAGACAC CTATGGAATT GTCCTGCTTT CTGGAGTGAA GTATAAGAAG
121  GGTGGCCTTG TGATCAATGA AACTGGGCTG TACTTTGTAT ATTCCAAAGT ATACTTCCGG
181  GGTCAATCTT GCAACAACCT GCCCCTGAGC CACAAGGTCT ACATGAGGAA CTCTAAGTAT
241  CCCCAGGATC TGGTGATGAT GGAGGGGAAG ATGATGAGCT ACTGCACTAC TGGGCAGATG
301  TGGGCCCGCA GCAGCTACCT GGGGGCAGTG TTCAATCTTA CCAGTGCTGA TCATTTATAT
361  GTCAACGTAT CTGAGCTCTC TCTGGTCAAT TTTGAGGAAT CTCAGACGTT TTTCGGCTTA
421  TATAAGCTC
```

SEQ ID NO:17 cDNA sequence encoding human extracellular domain of VEGF Receptor-2 (1-334), including signal peptide (part of GenBank NM_002253)

```
1    ATGCAGAGCA AGGTGCTGCT GGCCGTCGCC CTGTGGCTCT GCGTGGAGAC CCGGGCCGCC
61   TCTGTGGGTT TGCCTAGTGT TTCTCTTGAT CTGCCCAGGC TCAGCATACA AAAAGACATA
121  CTTACAATTA AGGCTAATAC AACTCTTCAA ATTACTTGCA GGGGACAGAG GGACTTGGAC
181  TGGCTTTGGC CCAATAATCA GAGTGGCAGT GAGCAAAGGG TGGAGGTGAC TGAGTGCAGC
241  GATGGCCTCT TCTGTAAGAC ACTCACAATT CCAAAAGTGA TCGGAAATGA CACTGGAGCC
301  TACAAGTGCT TCTACCGGGA AACTGACTTG GCCTCGGTCA TTTATGTCTA TGTTCAAGAT
361  TACAGATCTC CATTTATTGC TTCTGTTAGT GACCAACATG GAGTCGTGTA CATTACTGAG
421  AACAAAAACA AAACTGTGGT GATTCCATGT CTCGGGTCCA TTTCAAATCT CAACGTGTCA
481  CTTTGTGCAA GATACCCAGA AAAGAGATTT GTTCCTGATG GTAACAGAAT TTCCTGGGAC
541  AGCAAGAAGG GCTTTACTAT TCCCAGCTAC ATGATCAGCT ATGCTGGCAT GGTCTTCTGT
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721  AAGCTTGTCT TAAATTGTAC AGCAAGAACT GAACTAAATG TGGGGATTGA CTTCAACTGG
781  GAATACCCTT CTTCGAAGCA TCAGCATAAG AAACTTGTAA ACCGAGACCT AAAAACCCAG
841  TCTGGGAGTG AGATGAAGAA ATTTTTGAGC ACCTTAACTA TAGATGGTGT AACCCGGAGT
901  GACCAAGGAT TGTACACCTG TGCAGCATCC AGTGGGCTGA TGACCAAGAA GAACAGCACA
961  TTTGTCAGGG TCCATGAAAA ACCTTTTGTT GCTTTTGGAA GT
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SEQ ID NO:18 cDNA sequence encoding human extracellular domain of VEGF Receptor-2 (19-334) (part of GenBank NM_002253)

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241  GCCTACAAGT GCTTCTACCG GGAAACTGAC TTGGCCTCGG TCATTTATGT CTATGTTCAA
301  GATTACAGAT CTCCATTTAT TGCTTCTGTT AGTGACCAAC ATGGAGTCGT GTACATTACT
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541  TGTGAAGCAA AAATTAATGA TGAAAGTTAC CAGTCTATTA TGTACATAGT TGTCGTTGTA
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721  TGGGAATACC CTTCTTCGAA GCATCAGCAT AAGAAACTTG TAAACCGAGA CCTAAAAACC
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SEQ ID NO:19 Protein Sequence of human VEGF Receptor-1 (1-1338) including signal peptide (underlined) and Ig-like C2-type domains (32-123; 151-214; 230-327; 335-421; 428-553; 556-654; and 661-747 indicated in bold) (GenBank P17948)

```
1    MVSYWDTGVL LCALLSCLLL TGSSSGSKLK DPELSLKGTQ HIMQAGQTLH LQCRGEAAHK
61   WSLPEMVSKE SERLSITKSA CGRNGKQFCS TLTLNTAQAN HTGFYSCKYL AVPTSKKKET
121  ESAIYIFISD TGRPFVEMYS EIPEIIHMTE GRELVIPCRV TSPNITVTLK KFPLDTLIPD
181  GKRIIWDSRK GFIISNATYK EIGLLTCEAT VNGHLYKTNY LTHRQTNTII DVQISTPRPV
241  KLLRGHTLVL NCTATTPLNT RVQMTWSYPD EKNKRASVRR RIDQSNSHAN IFYSVLTIDK
301  MQNKDKGLYT CRVRSGPSFK SVNTSVHIYD KAFITVKHRK QQVLETVAGK RSYRLSMKVK
361  AFPSPEVVWL KDGLPATEKS ARYLTRGYSL IIKDVTEEDA GNYTILLSIK QSNVFKNLTA
421  TLIVNVKPQI YEKAVSSFPD PALYPLGSRQ ILTCTAYGIP QPTIKWFWHP CNHNHSEARC
481  DFCSNNEESF ILDADSNMGN RIESITQRMA IIEGKNKMAS TLVVADSRIS GIYICIASNK
541  VGTVGRNISF YITDVPNGFH VNLEKMPTEG EDLKLSCTVN KFLYRDVTWI LLRTVNNRTM
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661  PYLLRNLSDH TVAISSSTTL DCHANGVPEP QITWFKNNHK IQQEPGIILG PGSSTLFIER
721  VTEEDEGVYH CKATNQKGSV ESSAYLTVQG TSDKSNLELI TLTCTCVAAT LFWLLLTLLI
781  RKMKRSSSEI KTDYLSIIMD PDEVPLDEQC ERLPYDASKW EFARERLKLG KSLGRGAFGK
841  VVQASAFGIK KSPTCRTVAV KMLKEGATAS EYKALMTELK ILTHIGHHLN VVNLLGACTK
901  QGGPLMVIVE YCKYGNLSNY LKSKRDLFFL NKDAALHMEP KKEKMEPGLE QGKKPRLDSV
961  TSSESFASSG FQEDKSLSDV EEEEDSDGFY KEPITMEDLI SYSFQVARGM EFLSSRKCIH
1021 RDLAARNILL SENNVVKICD FGLARDIYKN PDYVRKGDTR LPLKWMAPES IFDKIYSTKS
1081 DVWSYGVLLW EIFSLGGSPY PGVQMDEDFC SRLREGMRMR APEYSTPEIY QIMLDCWHRD
1141 PKERPRFAEL VEKLGDLLQA NVQQDGKDYI PINAILTGNS GFTYSTPAFS EDFFKESISA
1201 PKFNSGSSDD VRYVNAFKFM SLERIKTFEE LLPNATSMFD DYQGDSSTLL ASPMLKRFTW
1261 TDSKPKASLK IDLRVTSKSK ESGLSDVSRP SFCHSSCGHV SEGKRRFTYD HAELERKIAC
1321 CSPPPDYNSV VLYSTPPI
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SEQ ID NO:20 Protein Sequence of extracellular domains of human VEGF Receptor-1 (27-758; without signal peptide) (part of GenBank P17948)

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61   QFCSTLTLNT AQANHTGFYS CKYLAVPTSK KKETESAIYI FISDTGRPFV EMYSEIPEII
121  HMTEGRELVI PCRVTSPNIT VTLKKFPLDT LIPDGKRIIW DSRKGFIISN ATYKEIGLLT
181  CEATVNGHLY KTNYLTHRQT NTIIDVQIST PRPVKLLRGH TLVLNCTATT PLNTRVQMTW
241  SYPDEKNKRA SVRRRIDQSN SHANIFYSVL TIDKMQNKDK GLYTCRVRSG PSFKSVNTSV
301  HIYDKAFITV KHRKQQVLET VAGKRSYRLS MKVKAFPSPE VVWLKDGLPA TEKSARYLTR
361  GYSLIIKDVT EEDAGNYTIL LSIKQSNVFK NLTATLIVNV KPQIYEKAVS SFPDPALYPL
421  GSRQILTCTA YGIPQPTIKW FWHPCNHNHS EARCDFCSNN EESFILDADS NMGNRIESIT
481  QRMAIIEGKN KMASTLVVAD SRISGIYICI ASNKVGTVGR NISFYITDVP NGFHVNLEKM
541  PTEGEDLKLS CTVNKFLYRD VTWILLRTVN NRTMHYSISK QKMAITKEHS ITLNLTIMNV
601  SLQDSGTYAC RARNVYTGEE ILQKKEITIR DQEAPYLLRN LSDHTVAISS STTLDCHANG
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721  TVQGTSDKSN LE
```

SEQ ID NO:21 cDNA Sequence encoding extracellular domains of human VEGF Receptor-1 (27-758) (part of GenBank NM_002019)

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1    TCAAAATTAA AAGATCCTGA ACTGAGTTTA AAAGGCACCC AGCACATCAT GCAAGCAGGC
61   CAGACACTGC ATCTCCAATG CAGGGGGGAA GCAGCCCATA AATGGTCTTT GCCTGAAATG
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421  GTTACTTTAA AAAAGTTTCC ACTTGACACT TTGATCCCTG ATGGAAAACG CATAATCTGG
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541  TGTGAAGCAA CAGTCAATGG GCATTTGTAT AAGACAAACT ATCTCACACA TCGACAAACC
601  AATACAATCA TAGATGTCCA AATAAGCACA CCACGCCCAG TCAAATTACT TAGAGGCCAT
661  ACTCTTGTCC TCAATTGTAC TGCTACCACT CCCTTGAACA CGAGAGTTCA AATGACCTGG
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1021 GTTGTATGGT TAAAAGATGG GTTACCTGCG ACTGAGAAAT CTGCTCGCTA TTTGACTCGT
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1741 CAAAAAATGG CCATCACTAA GGAGCACTCC ATCACTCTTA ATCTTACCAT CATGAATGTT
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2041 ATTATTTTAG GACCAGGAAG CAGCACGCTG TTTATTGAAA GAGTCACAGA AGAGGATGAA
2101 GGTGTCTATC ACTGCAAAGC CACCAACCAG AAGGGCTCTG TGGAAAGTTC AGCATACCTC
2161 ACTGTTCAAG GAACCTCGGA CAAGTCTAAT CTGGAG
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SEQ ID NO:22 Amino acid sequence of Flk(D1-D3)+FasL(139-281), including signal peptide

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121 RDYRSPFIAS VSDQHGIVYI TENKNKTVVI PCRGSISNLN VSLCARYPEK RFVPDGNRIS
181 WDSEIGFTLP SYMISYAGMV FCEAKINDET YQSIMYIVVV VGYRIYDVIL SPPHEIELSA
241 GEKLVLNCTA RTELNVGLDF TWHSPPSKSH HKKIVNRDVK PFPGTVAKMF LSTLTIESVT
301 KSDQGEYTCV ASSGRMIKRN RTFVRVHTKP FIAFGSARGT SEKKELRKVA HLTGKSNSRS
361 MPLEWEDTYG IVLLSGVKYK KGGLVINETG LYFVYSKVYF RGQSCNNLPL SHKVYMRNSK
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481 LYKL
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SEQ ID NO:23 Amino acid sequence of Flk(D1-D3)+FasL(139-281); without signal peptide

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181 VFCEAKINDE TYQSIMYIVV VVGYRIYDVI LSPPHEIELS AGEKLVLNCT ARTELNVGLD
241 FTWHSPPSKS HHKKIVNRDV KPFPGTVAKM FLSTLTIESV TKSDQGEYTC VASSGRMIKR
301 NRTFVRVHTK PFIAFGSARG TSEKKELRKV AHLTGKSNSR SMPLEWEDTY GIVLLSGVKY
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361 KKGGLVINET GLYFVYSKVY FRGQSCNNLP LSHKVYMRNS KYPQDLVMME GKMMSYCTTG
421 QMWARSSYLG AVFNLTSADH LYVNVSELSL VNFEESQTFF GLYKL

SEQ ID NO:24 Amino acid sequence of the extracellular domain of human neuropilin-1, including signal peptide sequence (part of GenBank AAC12921)

1 MERGLPLLCA VLALVLAPAG AFRNDKCGDT IKIESPGYLT SPGYPHSYHP SEKCEWLIQA
61 PDPYQRIMIN FNPHFDLEDR DCKYDYVEVF DGENENGHFR GKFCGKIAPP PVVSSGPFLF
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181 KMSEIILEFE SFDLEPDSNP PGGMFCRYDR LEIWDGFPDV GPHIGRYCGQ KTPGRIRSSS
241 GILSMVFYTD SAIAKEGFSA NYSVLQSSVS EDFKCMEALG MESGEIHSDQ ITASSQYSTN
301 WSAERSRLNY PENGWTPGED SYREWIQVDL GLLRFVTAVG TQGAISKETK KKYYVKTYKI
361 DVSSNGEDWI TIKEGNKPVL FQGNTNPTDV VVAVFPKPLI TRFVRIKPAT WETGISMRFE
421 VYGCKITDYP CSGMLGMVSG LISDSQITSS NQGDRNWMPE NIRLVTSRSG WALPPAPHSY
481 INEWLQIDLG EEKIVRGIII QGGKHRENKV FMRKFKIGYS NNGSDWKMIM DDSKRKAKSF
541 EGNNNYDTPE LRTFPALSTR FIRIYPERAT HGGLGLRMEL LGCEVEAPTA GPTTPNGNLV
601 DECDDDQANC HSGTGDDFQL TGGTTVLATE KPTVIDSTIQ SEFPTYGFNC EFGWGSHKTF
661 CHWEHDNHVQ LKWSVLTSKT GPIQDHTGDG NFIYSQADEN QKGKVARLVS PVVYSQNSAH
721 CMTFWYHMSG SHVGTLRVKL RYQKPEEYHQ LVWMAIGHQG DHWKEGRVLL HKSLKLYQVI
781 FEGEIGKGNL GGIAVDDISI NNHISQEDCA KPADLDKKNP EIKIDETGST PGYEGEGEGD

SEQ ID NO:25 Amino acid sequence human VEGFR-3 (GenBank NP_891555)

1 MQRGAALCLR LWLCLGLLDG LVSDYSMTPP TLNITEESHV IDTGDSLSIS CRGQHPLEWA
61 WPGAQEAPAT GDKDSEDTGV VRDCEGTDAR PYCKVLLLHE VHANDTGSYV CYYKYIKARI
121 EGTTAASSYV FVRDFEQPFI NKPDTLLVNR KDAMWVPCLV SIPGLNVTLR SQSSVLWPDG
181 QEVVWDDRRG MLVSTPLLHD ALYLQCETTW GDQDFLSNPF LVHITGNELY DIQLLPRKSL
241 ELLVGEKLVL NCTVWAEFNS GVTFDWDYPG KQAERGKWVP ERRSQQTHTE LSSILTIHNV
301 SQHDLGSYVC KANNGIQRFR ESTEVIVHEN PFISVEWLKG PILEATAGDE LVKLPVKLAA
361 YPPPEFQWYK DGKALSGRHS PHALVLKEVT EASTGTYTLA LWNSAAGLRR NISLELVVNV
421 PPQIHEKEAS SPSIYSRHSR QALTCTAYGV PLPLSIQWHW RPWTPCKMFA QRSLRRRQQQ
481 DLMPQCRDWR AVTTQDAVNP IESLDTWTEF VEGKNKTVSK LVIQNANVSA MYKCVVSNKV
541 GQDERLIYFY VTTIPDGFTI ESKPSEELLE GQPVLLSCQA DSYKYEHLRW YRLNLSTLHD
601 AHGNPLLLDC KNVHLFATPL AASLEEVAPG ARHATLSLSI PRVAPEHEGH YVCEVQDRRS
661 HDKHCHKKYL SVQALEAPRL TQNLTDLLVN VSDSLEMQCL VAGAHAPSIV WYKDERLLEE
721 KSGVDLADSN QKLSIQRVRE EDAGPYLCSV CRPKGCVNSS ASVAVEGSED KGSMEIVILV
781 GTGVIAVFFW VLLLLIFCNM RRPAHADIKT GYLSIIMDPG EVPLEEQCEY LSYDASQWEF
841 PRERLHLGRV LGYGAFGKVV EASAFGIHKG SSCDTVAVKM LKEGATASEQ RALMSELKIL
901 IHIGNHLNVV NLLGACTKPQ GPLMVIVEFC KYGNLSNFLR AKRDAFSPCA EKSPEQRGRF
961 RAMVELARLD RRRPGSSDRV LFARFSKTEG GARRASPDQE AEDLWLSPLT MEDLVCYSFQ
1021 VARGMEFLAS RKCIHRDLAA RNILLSESDV VKICDFGLAR DIYKDPDYVR KGSARLPLKW
1081 MAPESIFDKV YTTQSDVWSF GVLLWEIFSL GASPYPGVQI NEEFCQRVRD GTRMRAPELA
1141 TPAIRHIMLN CWSGDPKARP AFSELVEILG DLLQGRGLQE EEEVCMAPRS SQSSEEGSFS
1201 QVSTMALHIA QADAEDSPPS LQRHSLAARY YNWVSFPGCL ARGAETRGSS RMKTFEEFPM
1261 TPTTYKGSVD NQTDSGMVLA SEEFEQIESR HRQESGFSCK GPGQNVAVTR AHPDSQGRRR
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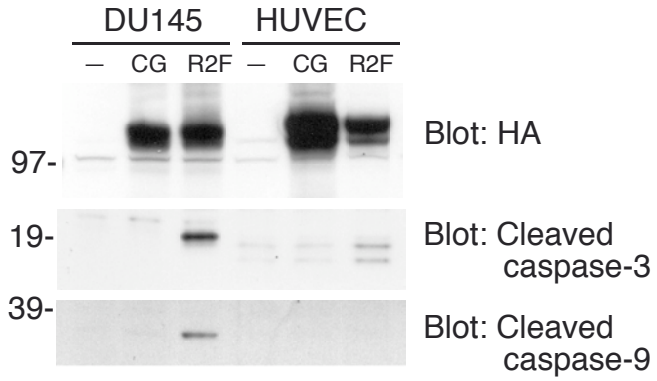


Figure 1. Adenoviral-directed expression of R2Fas receptor in DU145 prostate cancer cells and endothelial cells. DU145 prostate cancer cells and human umbilical vein endothelial cells (HUVEC) grown in 6-well plates were infected with AdV/CG-R2Fas or AdV/R2Fas at MOI = 40. Cells were lysed 48 hours after infection and lysates were electrophoresed by SDS/PAGE. Immunoblotting was performed with: HA antibody to visualize receptor expression (top panel); antibody against cleaved, active caspase-3 (middle panel); or antibody against cleaved, active caspase-9 (lower panel). Compared to DU145 prostate cancer cells, expression of R2Fas in endothelial cells stimulated much less activation of caspase-3 and caspase-9.

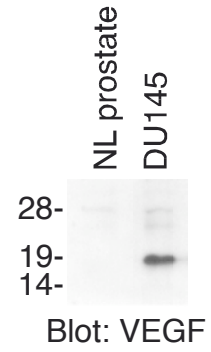


Figure 2. Overexpression of VEGF in DU145 prostate cancer cells. Lysates of normal prostate epithelial cells or DU145 prostate cancer cells were electrophoresed by SDS-PAGE and immunoblotted with antibody against VEGF.

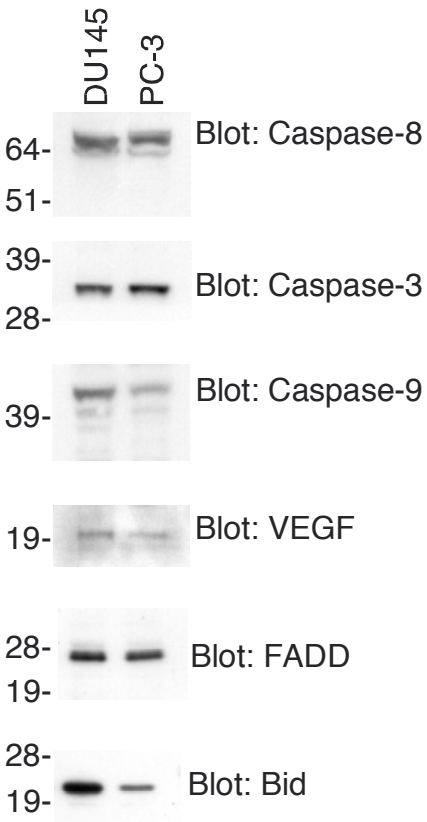


Figure 3. Expression of apoptosis proteins in DU145 and PC-3 prostate cancer cells. DU145 cells and PC-3 cells were grown in 6-well plates, lysed, and equal amounts of lysate protein were electrophoresed by SDS/PAGE. Immunoblotting was performed with antibodies to: Caspase-8, Caspase-3, Caspase-9, or VEGF. The two prostate cancer cell lines express similar level of these proteins.

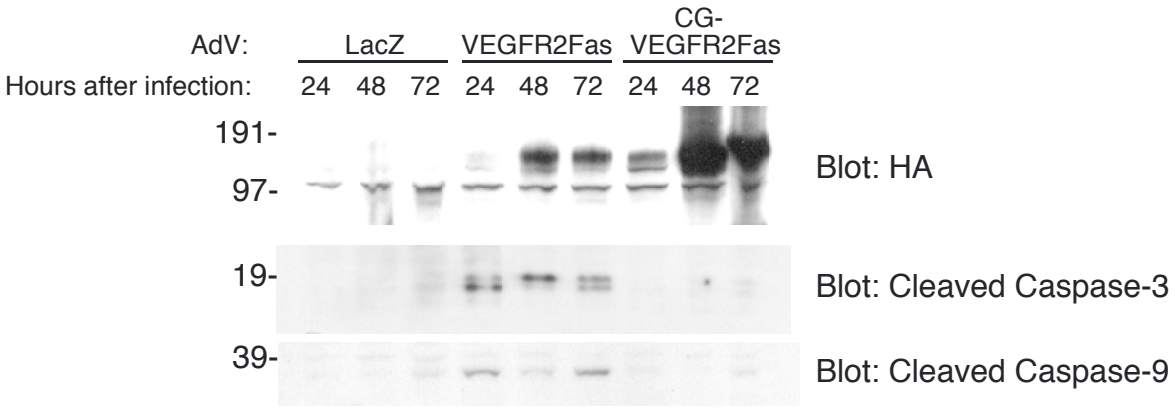


Figure 4. Adenoviral expression of VEGFR2Fas in DU145 prostate cancer cells stimulates activation of apoptotic signaling.
AdV / VEGFR2Fas, AdV / CG-VEGFR2Fas, or the control virus AdV / LacZ were put onto DU145 prostate cancer cells. Cells were lysed at 24, 48, or 72 hours, and electrophoresed by SDS/PAGE. Lysates were immunoblotted with HA antibody to demonstrate receptor expression, and with antibodies to the activated, cleaved forms of caspase-3 and caspase-9. Both caspase-3 and caspase-9 were activated in cells expressing VEGFR2Fas, but were not activated in the LacZ control cells or the cells expressing the CG-VEGFR2Fas receptor, which has an inactivating point mutation in the Fas death domain.

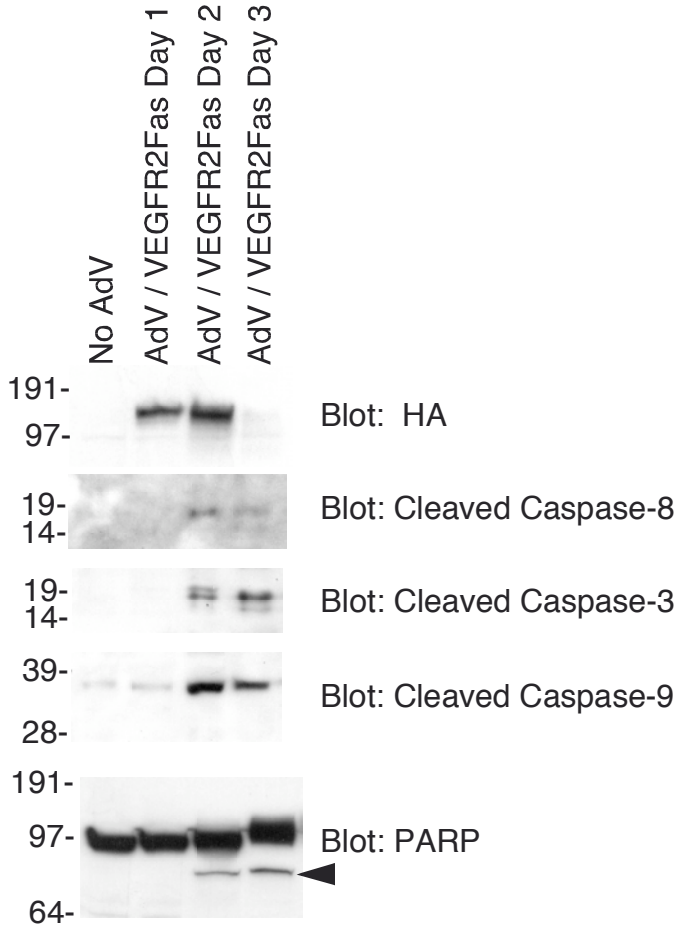


Figure 5. Adenoviral expression of VEGFR2Fas in DU145 cells stimulates activation of caspases-8, -3, and -9, and cleavage of PARP.
DU145 prostate cancer cells were infected with AdV / VEGFR2Fas or left uninfected as control (left lane). On days 1, 2, and 3 after infection cells were lysed and electrophoresed by SDS/PAGE. Immunoblot with HA antibody demonstrates expression of VEGFR2Fas beginning on day 1 after infection. Immunoblot with antibodies against the active, cleaved forms of caspase-8, caspase-3, and caspase-9 demonstrate activation beginning on day 2 after infection. Immunoblot with antibody against the caspase substrate PARP demonstrates cleavage of PARP (black arrowhead) beginning on day 2 after infection.

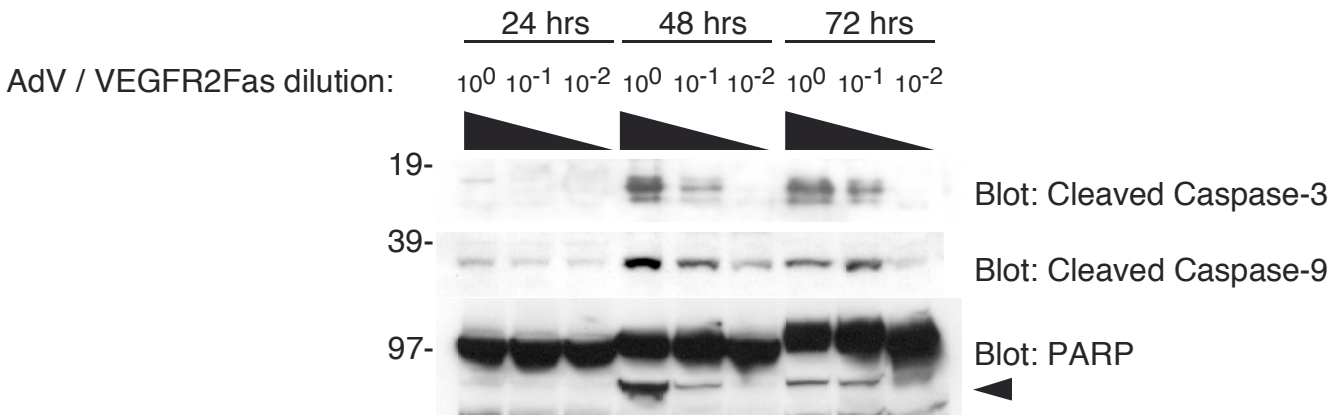


Figure 6. Adenoviral expression of VEGFR2Fas in DU145 cells stimulates caspase and PARP cleavage in a time-dependent and titer-dependent manner.
DU145 prostate cancer cells were infected with AdV / VEGFR2Fas at three titers (6.6 X 10^7, 6.6 X 10^6, and 6.6 X 10^5 virions/well) and lysed at 24, 48, or 72 hours after infection. Lysates were electrophoresed by SDS/PAGE. Immunoblot with antibodies against active, cleaved caspase-3 and caspase-9, and against the PARP substrate demonstrate that higher titers of AdV / VEGFR2Fas stimulate more activation of caspase-3 and caspase-9, and more cleavage of PARP. Peak activation occurs 48 hours after AdV / VEGFR2Fas infection.

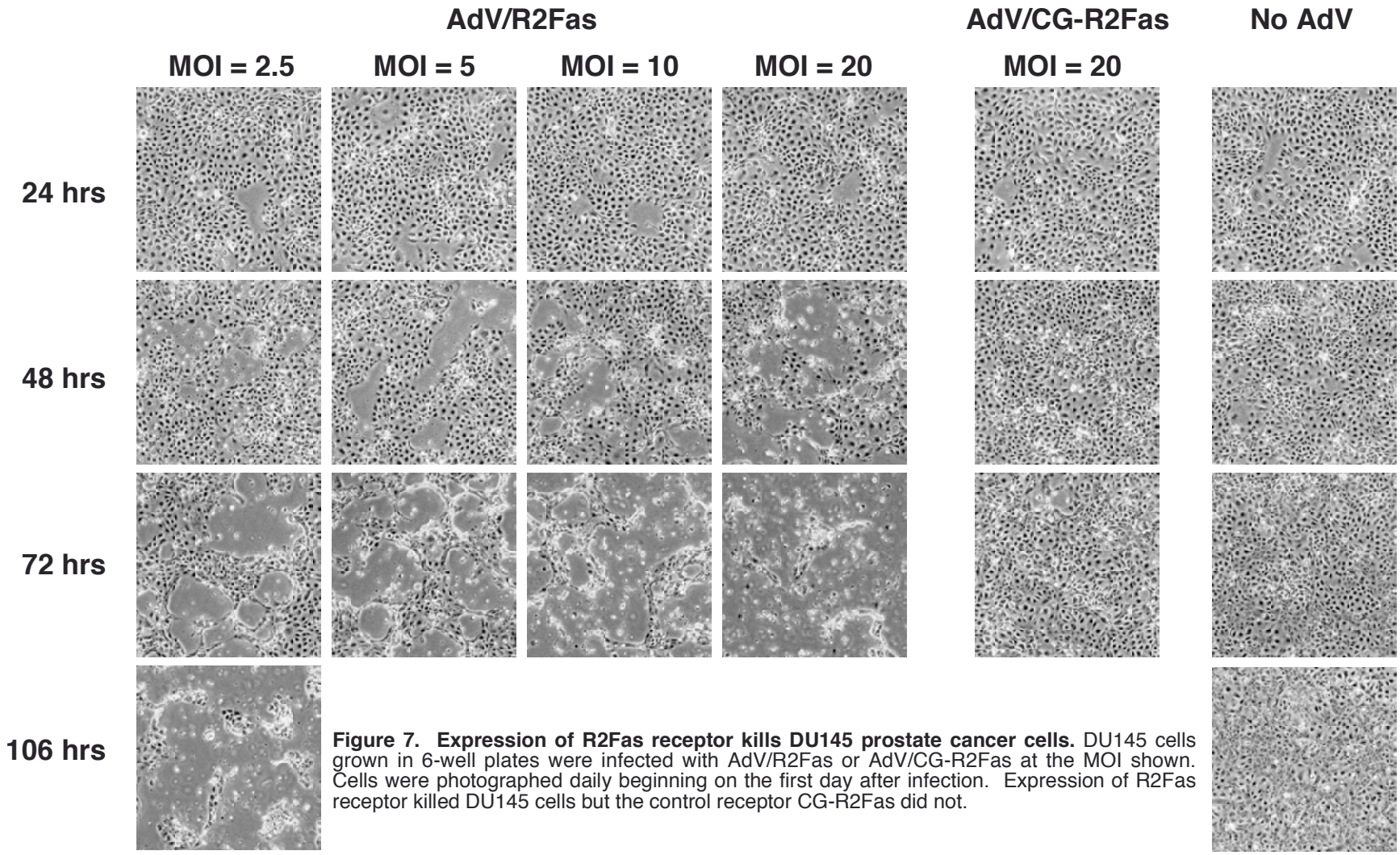


Figure 7. Expression of R2Fas receptor kills DU145 prostate cancer cells. DU145 cells grown in 6-well plates were infected with AdV/R2Fas or AdV/CG-R2Fas at the MOI shown. Cells were photographed daily beginning on the first day after infection. Expression of R2Fas receptor killed DU145 cells but the control receptor CG-R2Fas did not.

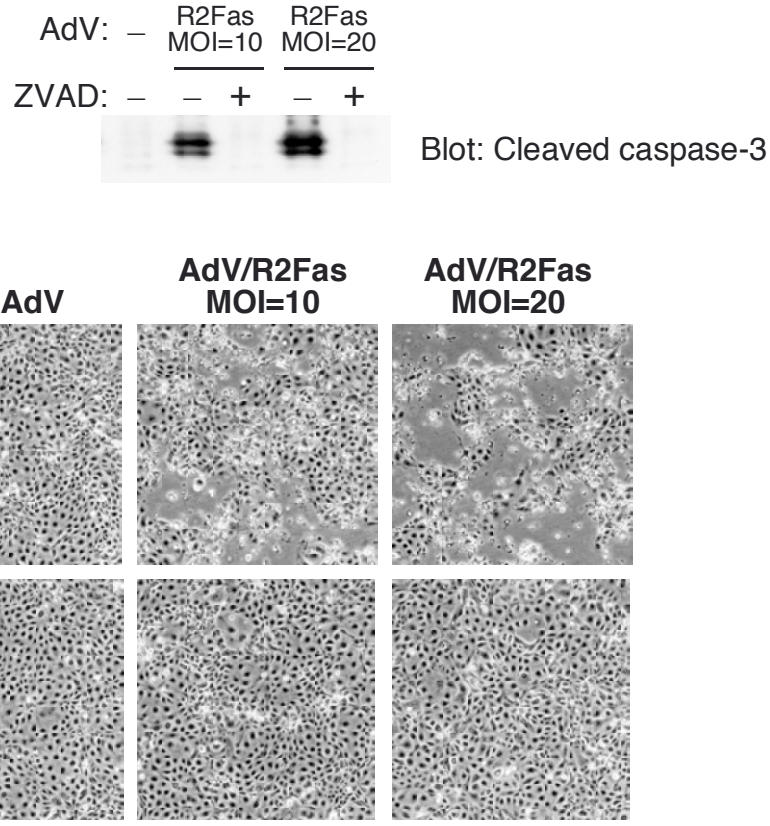


Figure 8. ZVAD blocks R2Fas-mediated caspase activation and cell death. DU145 cells grown in 6-well plates were infected with AdV/R2Fas at the MOI shown in the presence of ZVAD (40 μM) or vehicle (DMSO). Cells were photographed and lysed 48 hours after infection. Lysates were electrophresed by SDS/PAGE and immunoblotted with antibody against cleaved caspase-3 (top panel). The pancaspase inhibitor ZVAD blocked caspase-3 activation (top panel) and blocked cell death (lower panel), confirming that R2Fas expression stimulates apoptosis via caspase activation.

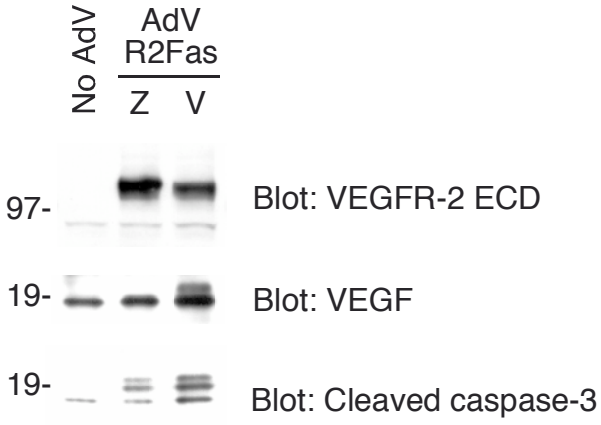


Figure 9. Co-expression of VEGF-165 potentiates R2Fas-mediated caspase-3 activation. DU145 cells grown in 6-well plates were infected with AdV/R2Fas at MOI=10 (lanes 2 and 3), and co-infected with either AdV/LacZ at MOI=10 (lane 2) or AdV/VEGF-165 at MOI=10 (lane 3). Cells were lysed 32 hours after infection. Increased expression of VEGF-165 by AdV/VEGF is seen in lane 3 in the middle panel. The lower panel shows increased caspase-3 cleavage in cells over-expressing VEGF.

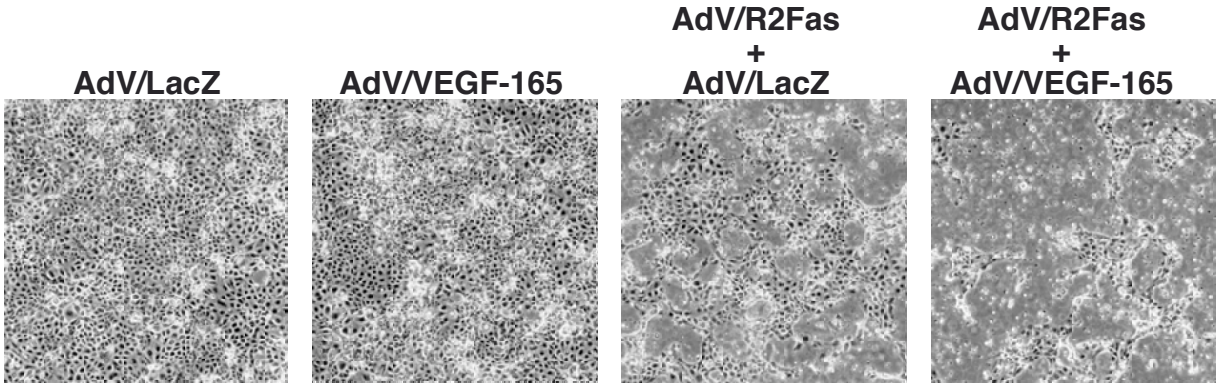


Figure 10. Co-expression of VEGF-165 potentiates R2Fas-mediated cell death. DU145 cells grown in 6-well plates were infected with either: AdV/LacZ alone (MOI=40); AdV/VEGF alone (MOI=40); AdV/R2Fas plus AdV/LacZ (MOI=20 for each); or AdV/R2Fas plus AdV/VEGF (MOI=20 for each). Cells were photographed 96 hours after infection. Co-infection of AdV/VEGF increased cell death mediated by expression of R2Fas, confirming that R2Fas activity responds to the level of VEGF expression.

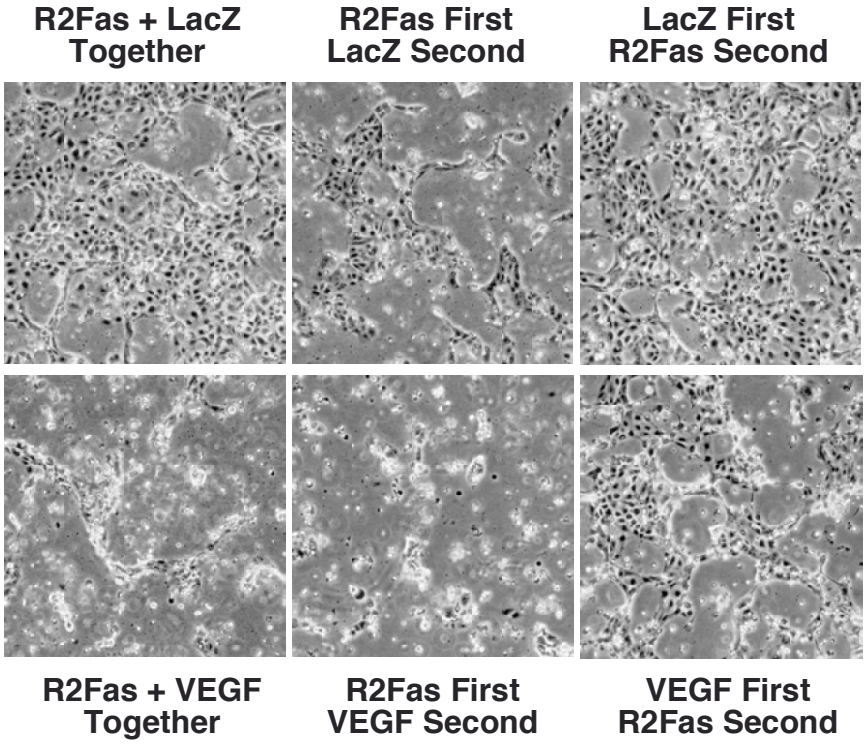


Figure 11. Co-expression of VEGF-165 potentiates R2Fas-mediated cell death. DU145 cells grown in 6-well plates were infected with AdV/ R2Fas (MOI=10) plus either AdV/LacZ (MOI=10) or AdV/VEGF (MOI=10). Cells on left were infected at the same time with both viruses. Cells in center panel were infected with AdV/R2Fas 24 hours before AdV/LacZ or AdV/VEGF. Cells on right were infected with AdV/LacZ or AdV/VEGF 24 hours before AdV/R2Fas. Increased expression of VEGF potentiated R2Fas-mediated apoptosis, especially when VEGF expression was simultaneous with or after R2Fas expression (left and center panels).

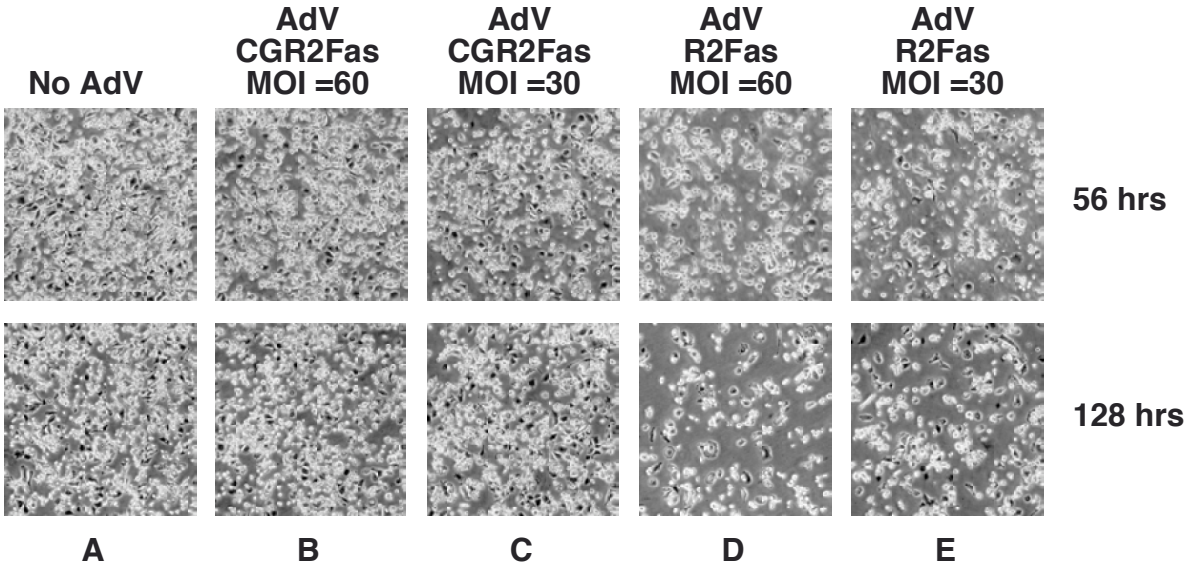


Figure 12. Expression of R2Fas receptor decreases PC-3 cell numbers. PC-3 cells grown in 6-well plates were infected with AdV/CG-R2Fas or AdV/R2Fas at the MOI indicated. Cells were photographed 56 and 128 hours after infection. Expression of R2Fas led to fewer total cells compared to expression of the control CG-R2Fas receptor (D vs. B, or E vs. C). We are investigating whether this is due to apoptosis or decreased cell proliferation.

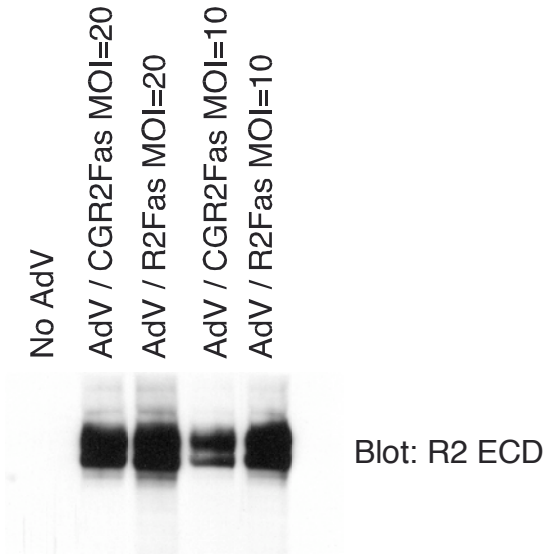


Figure 13. Expression of R2Fas receptor or control CG-R2Fas receptor in normal prostate epithelial cells. Normal primary human prostate epithelial cells grown in 6-well plates were infected with AdV/R2Fas or AdV/CG-R2Fas at MOI=10 or MOI=20 as indicated. Cells were lysed 48 hours after infection and lysates were electrophoresed by SDS/PAGE. Immunoblotting was performed with an antibody against the VEGFR-2 extracellular domain to visualize receptor expression.

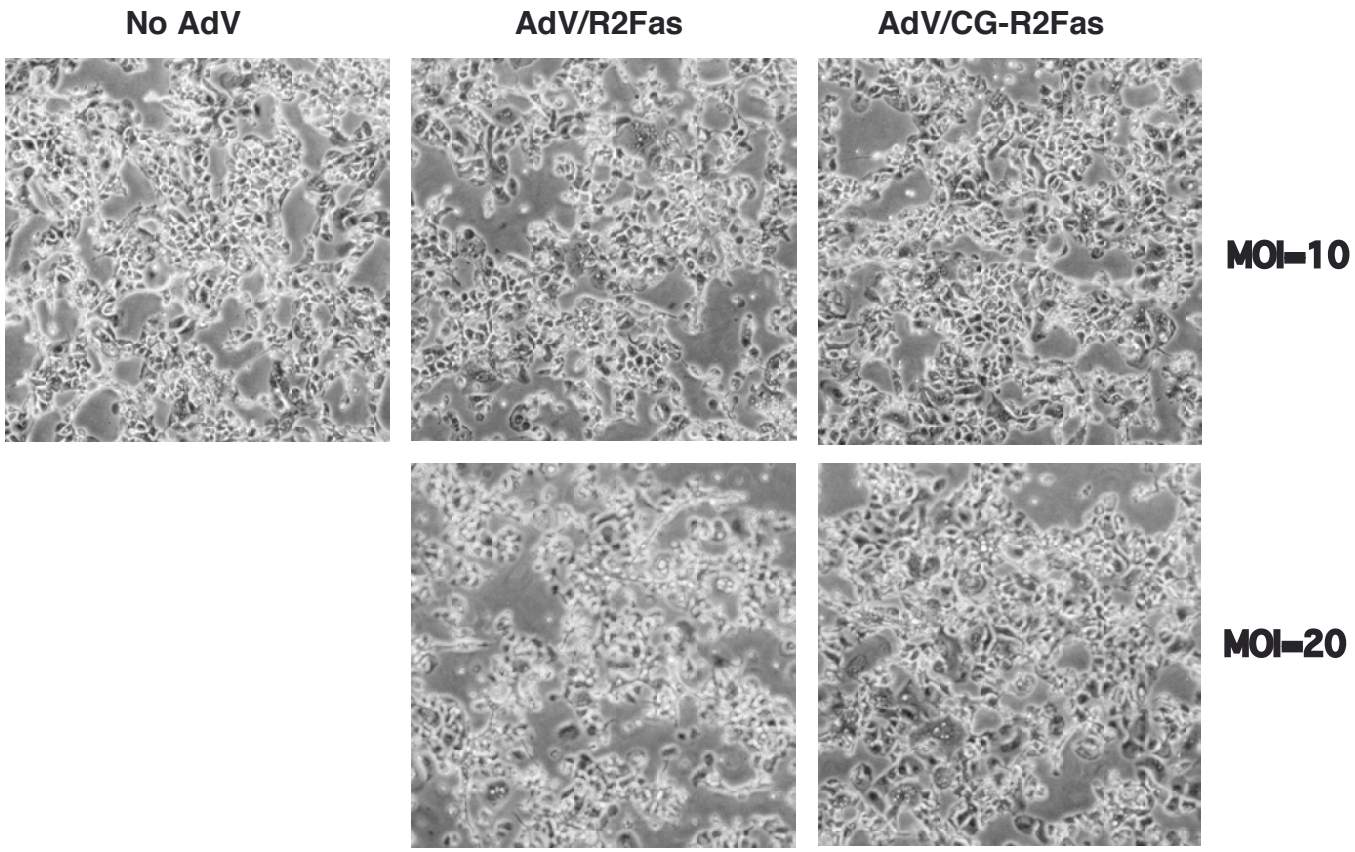


Figure 14. Expression of R2Fas does not stimulate apoptosis in normal human primary prostate epithelial cells. Normal human primary prostate epithelial cells grown in 6-well plates were infected with AdV/R2Fas or AdV/CG-R2Fas at MOI=10 or MOI=20 as indicated. Cells were photographed 72 hours after infection. Unlike DU145 prostate cancer cells, expression of R2Fas in normal prostate cells did not stimulate apoptosis.

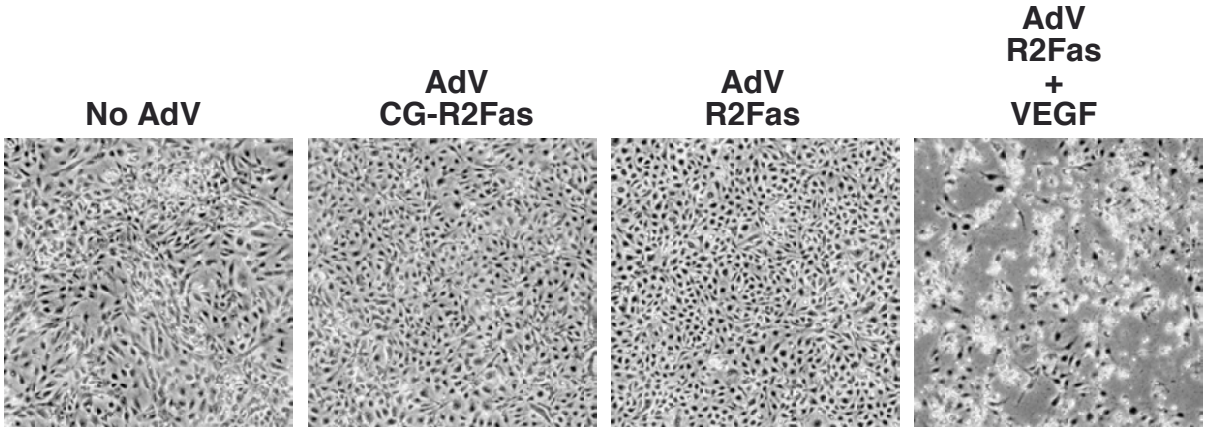


Figure 15. Expression of R2Fas receptor does not kill HUVEC cells but renders them killable by VEGF. Human umbilical vein endothelial cells (HUVEC) grown in 6-well plates were infected with AdV/CG-R2Fas or AdV/R2Fas at MOI = 40. Unlike DU145 cells, expression of R2Fas did not kill HUVEC cells (third panel; 100 hours after infection). However, R2Fas expression rendered HUVEC cells sensitive to killing by VEGF treatment (right panel, 1 nM VEGF-165 X 24 hours).

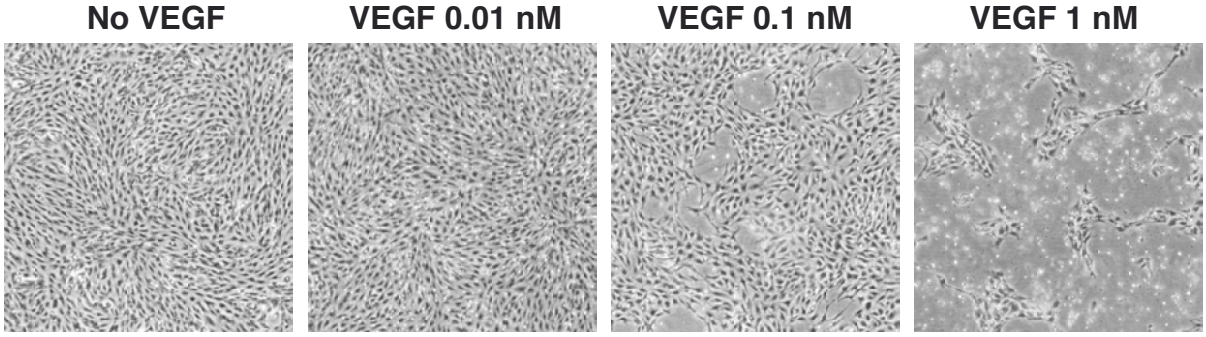


Figure 16. Expression of R2Fas receptor in the presence of VEGF kill endothelial cells. Porcine aortic endothelial cells were infected with AdV/R2Fas (MOI=20) in media containing no VEGF-165 (left panel) or the indicated concentrations of VEGF-165. Cells were photographed 96 hours after infection. Expression of R2Fas in cells treated with VEGF at 1 nM stimulated apoptosis (right panel).

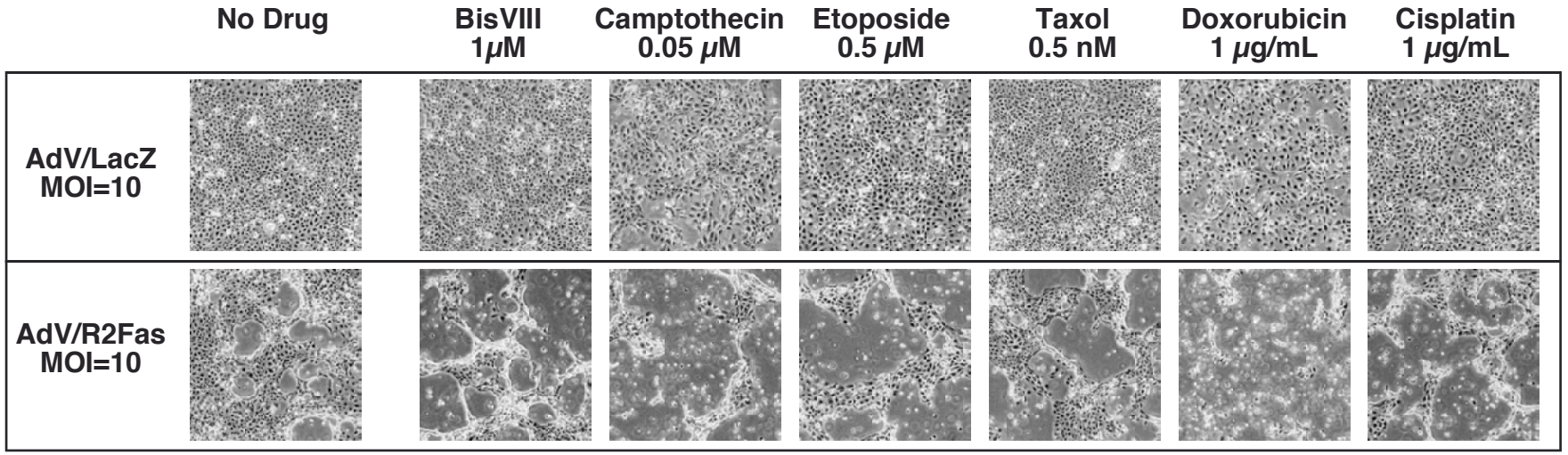


Figure 17. Potentiation of AdV/R2Fas-mediated cell death by pharmacologic agents. DU145 cells grown in 6-well plates were pretreated with the indicated agents for 12 hours before infection with AdV/LacZ or AdV/R2Fas at an MOI=10. Cells were photographed 36 hours after infection. All agents potentiated R2Fas-mediated apoptosis, especially BisVIII, camptothecin, etoposide, doxorubicin, and cisplatin.