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LIGHT, a new member of TNF superfamily, is highly expressed in activated lymphocytes but not in cancer cells. Soluble LIGHT was proved to inhibit the growth of cancer cells expressing both LTβR and THVEM/TR2 receptors. Local LIGHT gene transfer suppresses *in vivo* tumor formation. The anti-cancer effect of LIGHT correlates with its up-regulation of intercellular adhesion molecule-1 (ICAM-1) expression of cancer cells. The up-regulation of ICAM-1 expression is not only at ICAM-1 protein trafficking level on cell surface as showed by flow cytometry analysis, but also at ICAM-1 total protein level as confirmed by Western blot. We further confirmed that LIGHT enhancement up-regulation of ICAM-1 expression is STAT1 and JAK1 dependent using STAT1 deficient U3A and JAK1 deficient E2A4 cells. LIGHT-induced apoptosis of cancer cells resulted in down-regulation of anti-apoptosis Bcl-2 family members, such as Bcl-2, Bcl-X_L, Bag-1, and Mcl-1; up-regulation of pro-apoptosis Bcl-2 family member Bak. Extensive caspase activation is required in LIGHT-induced apoptosis of HT-29 colon cancer cells, but may not be required for LIGHT-induced apoptosis of MDA-MB-231 breast cancer cell. Activation of both DFF45 and PARP are involved in LIGHT-induced apoptosis. It was also proved that Bcl-2 down-regulation by LIGHT is STAT1 dependent.

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Introduction

Tumor necrosis factor (TNF) was originally discovered as a result of its anti-tumor activity, however, it is now generally recognized as a pleiotropic cytokine capable of numerous biological activities. The dose-limiting toxicity of TNFα and cytotoxicity of FasL for T-cells limits their clinical application as an anti-cancer agent. Clearly, there is a need for identification and characterization of new factors that regulate activation and differentiation of cells, both normally and in disease states. The recent remarkable progress in identifying new members of TNF and TNFR family together with the studies on their signal transduction pathways, has opened up a new opportunity and novel molecular targets for the treatment of cancer and inflammatory diseases. More importantly, LIGHT provides a novel system, distinct from the previously defined TNFα or Fas system, for the further understanding of the signaling pathways of TNF ligand-receptor interactions.

Specific Introduction for This Report.

We recently demonstrated that expression of LIGHT in MDA-MB-231 human breast carcinoma caused tumor suppression in *in vivo* xenograft tumor models. The LIGHT protein may play a role in the immune modulation and have a potential value in cancer therapy. The **specific aims** of this proposal are: 1) to investigate the expression patterns and clinical relevance of LIGHT and its recptors, LTβR or the HVEM/TR2; 2) to express and purify a large scale of soluble LIGHT protein and determine its therapeutic profile and efficacy in cancer therapy.

Findings from the proposed study would not only provide insights into the understanding of the function and role of this novel ligand, but lead to the development of an alternatively attractive approach for cancer therapy since LIGHT might selectively induce death of tumor cells and at the same time LIGHT triggers the co-stimulatory signal rather than the death signal to lymphocytes.

Body of Report

There were two technical objectives in the original proposal. The **technical objective 1** was to to investigate the expression patterns and clinical relevance of LIGHT and its recptors, LTβR or the HVEM/TR2 in human nomal, beign and malignant breast tissues. To achieve this goal, specific RT-PCR analyses were performed to study the expression patterns of LIGHT in various cells, the LIGHT mRNA was detected from fresh PBMC, which includes a mixture of T and B lymphocytes, natural killer cells, monocytes and granulocytes, which is consistent with Northern blot analysis as proviously reported (1). No LIGHT mRNA was found in resting PBMC, resting and activated Jurkat cells, or K562 cells. Increased LIGHT mRNA was found in activated PBMC, $CD3^+$ and $CD4^+$ T cells, two $CD8^+$ (TIL) cell lines, granulocytes, and monocytes. In addition, the presence of LIGHT mRNA was detected in LPS-activated neutrophils and PMA-stimulated U937 cells. There was little or nondetectable expression of LIGHT in various cancer cell lines derived from breast (MDA-MB-231, MCF-7, MDA-134, BT-474, and MDA-361), colon (HT-29 and SW-480), prostate (PC-3 and LNCaP), or 293T cells, except in one human breast epithelial-derived, nontumorigenic cell line MCF-10A cells. In addition, no LIGHT was found in three breast cancer tissue samples examined (2).

Collaborated with Dr. Heddy Zola in Women and Childrens Research Hospital of North Adelaide, Australia, We are now trying to get effective monoclonal antibodies against LIGHT to further address this topic.

We made both anti-LTβR and anti-HVEM/TR2 monoclonal antibodies to detect the wxpression of these two receptors on the cell surfece of this panel of cell lines using FACS analysis. It was shown that some cell lines express both LTβR and HVEM/TR2, some cell lines only express LTβR or HVEM/TR2, some cell lines are both LTβR and HVEM/TR2 double negative. Also, the expression level of LTβR and HVEM/TR2 varies from one cell line to the other cell line (Table I).

The technical objective 2 was to express soluble LIGHT protein and determine the therapeutic efficacy of soluble LIGHT protein in cancer therapy.

As previous report (2), we have prepared a large amount of purified soluble LIGHT protein, this protein has a molecular weight of 21kDa, it can induce apoptosis (programmed cell death) of HT-29 colon cancer cells at the ver low concentration of sLIGHT (~ng/ml), in the presence of IFNγ.

After testing the growth inhibition effect of sLIGHT to different cell lines, we found that sLIGHT can only inhibit the growth of cells with both LTβR and HVEM/TR2 (Table I).

Table I
Expression of LIGHT, LTβR, or TR2/HVEM and the Effects of sLIGHT on the In Vitro Proliferation of Various Human Cells

Cell lines	LIGHT expression*	LTβR expression*	TR2/HVEM expression*	Growth inhibition by sLIGHT‡
MDA-MB-231	–	++	++	+++
MCF-7	–	++	++	++
HT-29	–	+++	++	+++
HT3	n.d.	++	+	++
PC-3	–	++	–	–
PC-3/TR2/HVEM§	–	++	++	++
293T	–	–	–	–
MCF-10A	++	+	±	–
PBMC ¶	+	–	+	–
CD3+ T cells	+	–	++	–
TIL 1200	+	–	+	–
Jurkat	–	–	+	–

* Expression of LIGHT was determined by RT-PCR assay; expression of LTβR and TR2/HVEM was determined by FACS analysis; n.d., not done.

‡ Growth inhibition was carried out in the presence of LIGHT protein (100 ng/ml) plus INFγ (10 U/ml): +, 30% inhibition; ++, 50% inhibition; +++, 80% or more inhibition; –, < 10% inhibition.

§ PC-3/TR2/ HVEM cells were generated by stably transfected PC-3 cells with the full length of TR2/HVEM cDNA. Surface expression of TR2/HVEM on these cells was confirmed by FACS analysis.

¶ LIGHT was found only in the activated PBMC, not in the resting PBMC.

We then futher investigate the intercellular adhesion molecule-1 (ICAM-1) exxpression stimulation and apoptosis inducing effects of sLIGHT to achive our goal in technical objective 2.

1. sLIGHT Stimulates Intercellular Adhesion Molecule-1 (ICAM-1) Expression of Cancer Cells

Intercellular Adhesion Molecule-1 (ICAM-1) is essential for the activation of CD28 deficient cells (3, 4), it works as ligand for activating the killing activity of natural killer (NK) cells (5-8), in which the killer cell inhibitory receptor disrupts NK cells' adhesion to target cells (9), it also enhances MHC-peptide activation of CD8 (+) T cells without an organized immunological synapse (10) and enhances antibody-mediated lysis of tumor cells through a lymphocyte function-associated antigen-1 (LFA-1) dependent mechanism (11). Additionally, ICAM-1 is necessary for the adhesion and infiltration of neutrophils (12,13) and eosinophils (14) into the tumor cells. Most important, expression of ICAM-1 enhances *in vivo* lymphocyte adhesion to the cancer cells (15-17). Furthermore, ICAM-1 is involved in T cell-mediated B cell apoptosis by Fas/APO-1/CD95 (18). Cancer tissues or cell lines express significantly lower level of ICAM-1 than that of normal epithelium or benign cells (19-23). Therefore, to increase ICAM-1 expression level with various kinds of approaches in cancer cells is always the major effort for cancer therapy, such as radiation (24), use of cytokines like IL-12 (25) or other proinflammatory cytokines [31]. It has been reported that ICAM-1 gene transfection can inhibit metastasis of cancer cells and enhances their response to adoptive immunotherapy (26).

Previously, we reported that overexpression of LIGHT in MDA-MB-231 human breast cancer cells suppressed tumor growth *in vivo* and histological examination showed marked infiltration of neutrophil and necrosis in LIGHT expressing, but not in parental or the Neo-transfected MDA-MB-231 cells (2). To elucidate the mechanism how LIGHT induces cell growth suppression, we investigated the expression level of surface makers CD54, CD56, CD95, and CD119 of a group of cancer cell lines upon treatment of LIGHT, using flow cytometry analysis with specific antibodies against these cell surface makers. Our data showed that LIGHT treatment can dramatically increase the expression level of only CD54 (ICAM-1) in at least LTβR positive cancer cells, and this LIGHT-induced ICAM-1 up-regulation is STAT1 and JAK1 dependent. Hence, LIGHT-induced cell growth suppression is highly correlated with LIGHT-induced up-regulation of ICAM-1 expression.

Results

ICAM-1 is highly expressed in LIGHT transfected breast cancer cells and LIGHT expressing cancer cells are more susceptible to IFNγ stimulated ICAM-1 expression.

To correlate the anti-cancer effect of LIGHT with ICAM-1 expression, we detected the ICAM-1 expression in all three MDA-MB-231 breast cancer cells: wild type MDA-MB-231/wt, neo control MDA-MB-231/neo, and LIGHT transfectant MDA-MB-231/LIGHT. As can be seen in figure 1, significant expression of ICAM-1 was observed in LIGHT expressing cells, with average fluorescence intensity of 1240 ± 24.8, compared with that of 13.3 ± 22.9 of parental cells (MDA-MB-231/wt), and 14.3 ± 22.6 of Neo transfected cells, respectively. It is known that IFNγ can stimulate cancer cells to express ICAM-1, so it would be interesting to compare the susceptibility of these three cell lines to IFNγ stimulated ICAM-1 expression. To achieve this

goal, we treated all these three cell lines with different concentration of IFN γ, both our FACScan analysis and Western blot analysis showed that MDA-MB-231/LIGHT is the most susceptible cell line to IFNγ stimulated ICAM-1 expression among these three cell lines, and this up-regulation of ICAM-1 expression is not only at ICAM-1 protein level (higher intensity ICAM-1 specific band of MDA-MB-231/LIGHT in Western blot), but also at ICAM-1 protein trafficking level (higher cell surface expression level verified by FACScan analysis). For instance, at as low as 1.0 U/ml IFNγ, ICAM-1 expression level in MDA-MB-231/LIGHT cells was much higher than that of MDA-MB-231/wt and MDA-MB-231/neo cells. We did not observe any difference of expression level among these cell lines for cell surface makers CD56 (neural cell adhesion molecule N-CAM or NK cell marker), CD95 (Fas) and CD119 (IFNγ receptor α chain). Thus, there is a correlation between LIGHT anti-cancer effect and ICAM-1 expression.

sLIGHT stimulates ICAM-1 expression in different cancer cells.

In order to elucidate the molecular mechanism of how LIGHT induces apoptosis, we examined the changes of cell surface marker expression level of a group of cancer cells upon treatment with sLIGHT. We found out that there was no difference of expression level among these cell lines for cell surface makers CD56, CD95 (Fas) and CD119. The only significant alteration we observed is also ICAM-1 (CD54). As can be seen in table2, sLIGHT alone slightly increased ICAM-1 expression in SKBR3, HT-29, HT3 and PC-3 cells, IFNγ alone increased the expression of ICAM-1 in MCF-10A, SKBR3, PC-3, HT-29, Jurkat, U937, and HT-3 cells; sLIGHT combined with IFNγ, ICAM-1 level went to a higher level in MCF-10A, SKBR3, PC-3, MCF-7, HT-29, and HT-3, no clear change was detected in Jurkat and U937. Neither IFNγ, sLIGHT alone nor their combination induced 293T cells to express ICAM-1. As addressed before, this up-regulation of ICAM-1 expression is not only at ICAM-1 protein

Table 2. sLIGHT Stimulates ICAM-1 Expression in Different Cancer Cells*

Cell lines	No treated	sLIGHT	IFNγ	IFNγ +LIGHT	LTβR	TR2
MCF-10A	5.62±16.5**	8.98 ±15.1	58.3±19.9	83.8±20.8	+ ***	+
MDA-MB-231	6.26±22.2	6.49±22.4	107±32.2	184±29.0	+	+
SKBR3	16.5±38.7	39.2±49.1	100±39.6	273±43.4	+	+
MCF-7	10.4±27.5	10.7±32.7	30.5±47.3	40.7±38.4	+	+
HT-29	7.23±23.7	14.3±26.7	264±25.9	421±27.9	+	+
HT3	7.50±18.3	21.3±21.5	26.4±29.1	60.4±29.6	+	+
PC-3	7.5±27.8	18.4±36.8	56.2±42.5	212±45.9	+	-***
Jurkat	7.23±20.6	6.49±21.1	14.3±18.7	13.8±34.5	-	+
U937	8.35±19.5	8.66±20.8	13.3±32.6	13.8±34.5	n.d.	n.d.
293T	5.42±20.0	5.42±19.9	5.42±19.8	5.62±20.1	-	-

* 1×10^6 cells were treated with IFNγ 10 U/ml, LIGHT 100ng/ml, or combined use of both for 48hrs.

** mean ± SD of fluorescence intensity

*** + positive or - negative

level (higher intensity ICAM-1 specific band of sLIGHT and IFNγ combined use in Western blot, data not shown.), but also at ICAM-1 protein trafficking level (higher cell surface expression level verified by FACScan analysis). It is of worthy to note that all those cells sensitive to LIGHT induced ICAM-1 up-regulation are both LTβR and HVEM/TR2 positive (table 2) (MCF-10A, MDA-MB-231, SKBR3, MCF-7, HT-29, HT-3), or at least LTβR positive; both LTβR and HVEM/TR2 negative cells (293T) are resistant to LIGHT-induced ICAM-1 up-regulation. Therefore, LTβR might be sufficient for LIGHT-induced ICAM-1 up-regulation in cancer cells

sLIGHT enhancement of ICAM-1 expression is dose-dependent

To make certain the up-regulation of ICAM-1 comes from whether sLIGHT or IFNγ alone or combined use of IFNγ and sLIGHT. We performed an experiment to test the dose-dependent pattern of the ICAM-1 upregulation, sLIGHT alone did not increase the expression level of ICAM-1 in MDA-MB-231 cells, even at as high as 1000ng/ml concentration. IFNγ alone did increase the expression level of ICAM-1 at only 5 U/ml. LIGHT was able to enhance this IFNγ mediated up-regulation of ICAM-1 expression, at IFNγ 5U/ml, as low as 1.0 ng/ml LIGHT could be able to augment the up-regulation of ICAM-1 expression, the more LIGHT concentration, the clearer this up-regulation. In a word, sLIGHT enhancement of ICAM-1 expression is dose-dependent

LIGHT enhancement of up-regulation of ICAM-1 is STAT1 and JAK1 dependent.

Since IFNγ increases LIGHT-induced ICAM-1 expression, in an effort to delineate the role of STAT1 and JAK1 in LIGHT-induced up-regulation of ICAM-1 expression, we evaluated the response to LIGHT-induced ICAM-1 expression in a serial of STAT1 and JAK1 deficient and reconstituted cells to investigate whether up-regulation of ICAM-1 expression is STAT1 and JAK1 dependent. As shown in figure 1, 2fTGH fibrosarcoma cells showed a nice pattern of sLIGHT-induced ICAM-1 expression, and this expression could be increased by use of IFNγ. U3A cells are STAT1 deficient; the ICAM-1 expression level induced by both sLIGHT alone and its combination with IFNγ was lowered dramatically. U3A1-1 cells are STAT1 reconstituted; we could be able to see a nice LIGHT-induced ICAM-1 expression pattern like that in 2fTGH. LIGHT slightly induced ICAM-1 expression, and this induction could also be increased by the use of IFNγ. E2A4 cells are JAK1 deficient; there was no such pattern. In addition, we did not observe any difference of expression level in 2fTGH, U3A, and U3A1-1 cells for cell surface maker CD95 (Fas), also CD56 and CD119, and similar observation in Hela and E2A4 cells. These results further suggest that ICAM-1 is the only cell surface maker, which expression is regulated by LIGHT, and LIGHT-induced up-regulation of ICAM-1 is STAT1 and JAK1 dependent.

DISCUSSION

IFNγ can stimulate the expression of ICAM-1, and this stimuli can be increased by TNFα, but this is only a synergism effect, since both IFNγ and TNFα alone can stimulate cancer cells to express ICAM-1 (27-29). In human airway epithelial cells, it was proposed that the signaling pathway of TNFα induced up-regulation of ICAM-1 expression is like this: TNFα → TNF-RI →

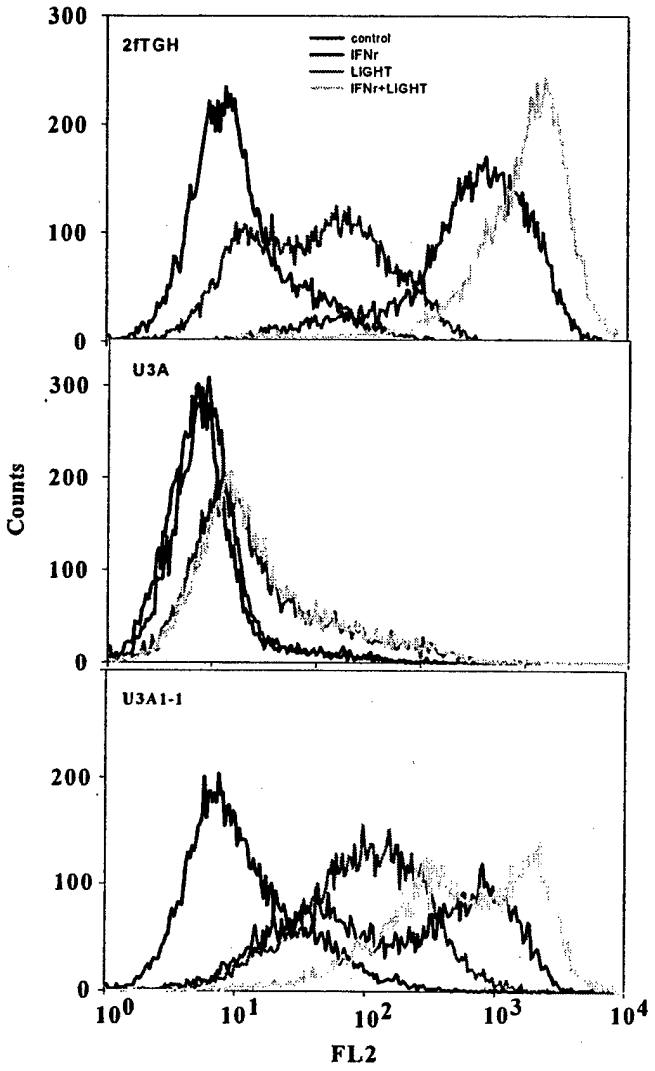

Figure 1. FACS analysis with ICAM-1 specific antibody. 2fTGH shows a nice pattern of LIGHT-induced up-regulation of ICAM-1 expression, U3A (STAT1 deficient) does not, U3A1-1 (STAT1 knock in) shows this pattern again.

PC-PLC → DAG → PKC → (NF-κB?) → ICAM-1 mRNA → ICAM-1 surface expression . This synergistic activation of ICAM-1 by TNFα and IFNγ is mediated by p65/p50 and p65/c-Rel and interferon-responsive factor STAT1α (p91) that can be activated by both IFNγ and TNFα (29). LIGHT is different from TNFα, because LIGHT only binds to LTβR, TR2 or newly discovered TR6. Furthermore, it seems that LIGHT-induced ICAM-1 expression in some group of cancer cells is different from other group of cancer cells, because sLIGHT alone slightly increased ICAM-1 expression in SKBR3, HT-29, HT3 and PC-3 cells, but not increase ICAM-1 expression in MCF-10A, MDA-MB-231, MCF-7 and other cancer cells (Table 1). It is not clear how LIGHT signaling from TRAF3 to ICAM-1 expression in these cells, and it would be interesting to see the mechanism of how these two group of cancer cells differently respond to LIGHT-induced ICAM-1 expression. Also IFNγ can dramatically enhance LIGHT induced up-

regulation of ICAM-1 expression, it is reasonable that the signaling is through STAT1-JAK1 pathway (30-32), but it remains unclear how IFNγ transmits its signal to LIGHT.

Previously, we reported that overexpression of LIGHT in MDA-MB-231 human breast cancer cells suppressed tumor growth *in vivo* and histological examination showed marked infiltration of neutrophil and necrosis in LIGHT expressing but not in parental or the Neo-transfected MDA-MB-231 cells [7]. We also observed clear apoptosis of MDA-MB-231/LIGHT treated with IFNγ and MDA-MB-231 treated with both IFNγ and LIGHT *in vitro* (publish separately). In this experiment, we observed that ICAM-1 expression was highly increased in MDA-MB-231/LIGHT cells, which were not treated with IFNγ or treated with IFNγ, and high level ICAM-1 expression in MDA-MB-231/wt cells which were treated with both IFNγ and LIGHT, we are assuming that it is the high level expression of ICAM-1 which leads to the *in vivo* and *in vitro* susceptibility of MDA-MB-231 cells (or other cancer cells) to the killing from "killing cytokines"(including LIGHT itself) and "killing effecter cells". To prove this, one quick approach is to neutralize ICAM-1 secretion using ICAM-1 blocking antibody, then to attack these cells with human NK cells to see whether they are still sensitive.

Our data indicate that LIGHT can up-regulate ICAM-1 expression, IFNγ remarkably enhance this up-regulation, higher level expression of ICAM-1 in turn results in the high susceptibility of cancer cells to the killings at various levels. We plan to further investigate the pathways of how ICAM-1 expression is up-regulated by LIGHT in cancer cells.

2. sLIGHT Enhances Apoptosis of Cancer Cells

Previously it was reported that overexpression of LIGHT in MDA-MB-231 breast cancer cells suppressed tumor growth *in vivo* Histological examination showed marked neutrophil infiltration and necrosis in LIGHT expressing but not in the parental or the Neo-transfected MDA-MB-231 tumors. Here it is reported that LIGHT induces apoptosis of MDA-MB-231 breast cancer cells and HT-29 colon cancer cells through down-regulation of anti-apoptotic Bcl-2 family members, and LIGHT-induced apoptosis involves the activation of both mitochondrial and death receptor apoptosis signal transduction pathways. Also, since IFNγ contributes to the apoptosis, this LIGHT-induced, IFNγ-mediated apoptosis is at least STAT1 dependent.

Results

LIGHT gene transferring enhances IFNr-mediated Bcl-2 down-regulation

Previously, It was reported that introduction of LIGHT cDNA into MDA-MB-231 cells (MDA-MB-231/LIGHT) caused complete tumor suppression *in vivo (2)*. It would be interesting to see whether Bcl-2 down-regulation is also involved in MDA-MB-231/LIGHT cell apoptosis, because Bcl-2 overexpression occurred in most of the breast cancer cells (33). We observed that as low as 10 U/ml IFNr could be able to induce Bcl-2 down-regulation in MDA-MB-231/LIGHT cells, however there was no change of Bcl-2 expression in MDA-MB-231/wt cells even being treated with 100 U/ml IFNγ. Also, cell cycle analysis indicated that there was no significant difference among MDA-MB-231/wt, MDA-MB-231/neo, and MDA-MB-231/LIGHT cells treated with IFNγ in terms of their distribution in G0-G1, G2-M, and S phase of the cell cycle (data not shown). These results suggest that IFNr treatment of MDA-MB-231/LIGHT cells does

not cause cell cycle arrest of MDA-MB-231/LIGHT cells, it is a process of apoptosis involved in Bcl-2 down-regulation.

LIGHT induces apoptosis of MDA-MB-231 cells.

In a previous work, it was reported that IFNγ (10 U/ml) alone slightly induced apoptosis of MDA-MB-231 (subclone 2LMP) cells (2.2%, but no significant difference with no treated cells), LIGHT (100 ng/ml) alone induced a high level of apoptosis (5.7%), combined use of both IFNγ (10 U/ml) and LIGHT (100 ng/ml) remarkably increased the apoptosis level (up to 13.2%), for HT-29, this level goes up to 79.1% (2) at 72 hrs of treatment. Also, cell cycle analysis indicated that there was no significant difference among no treated cells, cells treated with IFNγ alone, cells treated with LIGHT alone, and cells treated with both IFNγ and LIGHT, regarding to their distribution in G0-G1, G2-M, and S phase of the cell cycle. These findings demonstrate that LIGHT treatment does not cause cell cycle arrest, it is also a process of apoptosis.

Alterations of Bcl-2 family members is involved in LIGHT induced apoptosis

In order to elucidate the molecular mechanism of how LIGHT works to induce apoptosis, a serial of Western blot assay was performed to trace the changes of Bcl-2 family members upon treatment with LIGHT. We observed that LIGHT alone did not induce Bcl-2 down-regulation; and IFNγ did not induce Bcl-2 down-regulation, however, at IFNγ 10 U/ml, as low as 10 ng/ml LIGHT clearly induced down-regulation of Bcl-2. We further observed that Bcl-2 down-regulation started as early as 48hr, and as time going, Bcl-2 level became getting lower and lower. We used 96 hrs, 20 U/ml IFNγ and different concentration of LIGHT to study all the apoptosis signal transduction events in MDA-MB-231 and HT-29 cells, as shown in figure 2, Bcl-2 was down-regulated, also we observed other anti-apoptosis Bcl-2 family member members like Bcl-X_L, Bag-1, and Mcl-1 were all down-regulated. As for the proapoptosis Bcl-2 family members, Bak was up-regulated. Taken together, LIGHT treatment triggers extensive alterations of Bcl-2 family members in MDA-MB-231 cells.

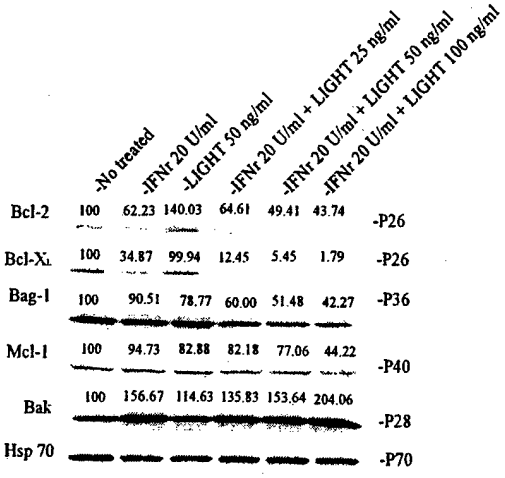

Figure 2. Western blot with specific antibodies showing LIGHT-Induced apoptosis of MDA-MB-231 (2LMP) cells results in Down-regulation of anti-apoptosis Bcl-2 members.

Extensive caspase activation is associated with, but their activity, especially caspase-3 activity may not be necessary for LIGHT-induced apoptosis of MDA-MB-231 cells, but necessary for HT-29 cells

Recent discoveries established that multiple distinct signaling pathways regulate apoptosis. Such pathways are activated in general by the formation of a death-inducing signaling complex (DISC). Activation of the DISC results in the early recruitment of inducer caspases (caspase-2, -8, -9, -12). These inducer caspasee then amplify the apoptosis signal by cleavage and activation of effector caspases (caspase-3, -6, -7) which execute apoptosis by degrading hundreds of regulatory proteins and activation of endonucleases and other proteins (34-36). Thus, caspases are very important in the execution of apoptosis, at least in some of the apoptosis signal tansduction pathways. To investigate the roles of caspases in LIGHT-induced apoptosis of MDA-MB-231 cells and HT-29 cells, the expression of caspase-3, -6, -7, -8, and -9 in MDA-MB-231and HT-29 cells treated with different concentration of LIGHT in the presence of IFNγ was probed with Western blot. If being activated, both P21 and P17 cleavage part of caspase-3, P12 cleavage part of caspase-6, P20 cleavage part of caspase-7, both P43/P41 and P18 cleavage parts of caspase-8, both P37 and P35 cleavage parts of caspase-9 should be visible. As shown in figure 3, caspase-3 and caspase-7 were activated, since either caspase-3 cleavage parts P21 and P17, or caspase-7 cleavage part P20 were observed; Caspase-8 and caspase-9 were activated, as caspase-8 cleavage parts P43/P41 and P18, and caspase-9 cleavage parts P37 and P35 were observed; Caspase-6 was also activated, since P12 cleavage part of caspase-6 was observed. The difference among the activation of these caspases is that the activation of casapase-6 and caspase-8 already existed in no treated cells (96 hrs), LIGHT alone, IFNγ alone, or combined use of both of them may cause their up-regulation to some extent, but with no significant difference compared with no treated cells. On the other hand, the activation of caspase-3, caspase-7, and caspase-9 came from the treatment of LIGHT in the presence of IFNγ. These findings demonstrate that extensive caspase activation takes part in LIGHT-induced apoptosis of MDA-MB-231 cells.

To further verify whether caspase activation is necessary for LIGHT-induced apoptosis, MDA-MB-231 cells were treated with LIGHT in the presence of IFNγ and caspase-3 inhibitor Z-DEVD-FMK, or a broad range caspase inhibitor Z-VAD-FMK, then cell survival rate was measured to see whether the antiproliferative effect of LIGHT was prevented. We observed that LIGHT-induced cell death was not inhibited by Z-DEVD-FMK or Z-VAD-FMK, instead cell death was slightly enhanced (but no significant difference if compared with the control), since cell survival rate was increased, with cells more susceptible to the treatment of Z-VAD-FMK than that of Z-DEVD-FMK. Caspase activity of one of the important caspases, caspase-3 was then detected to see whether caspase-3 activity was related to LIGHT-induced apoptosis, we observed that caspase-3 activity was decreased in the above-mentioned treatment of MDA-MB-231 cells, with cells treated with Z-DEVD-FMK clearer than treated with Z-VAD-FMK. These results suggest that casapase activation and caspase activity, especially caspase-3 activity is not necessary for LIGHT-induced apoptosis of MDA-MB-231 cells. However, either Z-DEVD-FMK or Z-VAD-FMK inhibited LIGHT-induced cell death, thus, caspase activity is necessary for LIGHT-induced apoptosis of HT-29 cells.

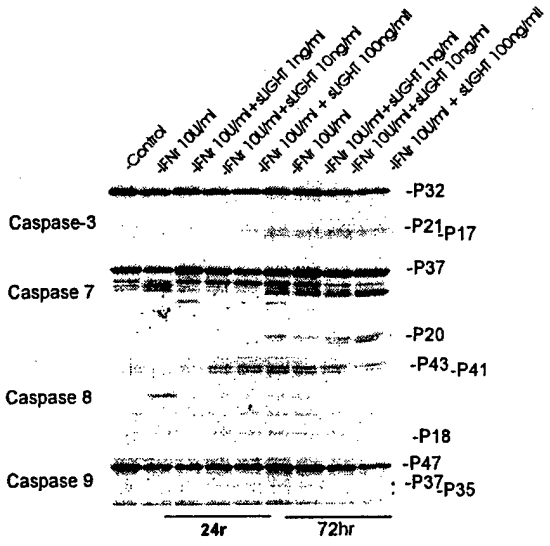

Fig. 3. Western blot with specific antibodies showing LIGHT-induced apoptosis of HT-29 cells invoves extensive Caspase activation

Cleavage of both DFF45 and PARP are involved in LIGHT induced apoptosis

Caspase-3 and caspase-7 are two executive caspases receiving apoptosis signal from mitochondria or death receptors, they will then transmit the apoptosis signal to caspase-6, DFF45 or PARP (37, 38) to act on DNA level. Upon being activated, DFF45 produces cleaved part P12, PARP produces cleaved part P89 (and P24). Our results showed that P12 cleavage part of DFF45 was observed at IFNγ treated alone, and this activation of DFF45 was enhanced by the treatment of LIGHT, since the P12 cleavage part was up-regulated. PARP cleavage part P89, on the other hand only existed with the treatment of LIGHT in the presence of IFNγ. These results demonstrate that cleavage of both DFf45 and PARP are involved in LIGH-induced apoptosis of MDA-MB-231 cells.

LIGHT induced apoptosis is STAT1 dependent

As described above, IFNγ contributes to LIGHT-induced, IFNγ-mediated apoptosis of MDA-MB-231 cells. To investigate the role of IFNγ in the apoptosis, Western blot analysis of STAT1 deficient U3A and STAT1 knock in U3A1-1fibrosarcoma cells treated with LIGHT in the presence of IFNγ were performed to see whether this IFNγ signaling adaptor takes effect in the process of apoptosis. As demonstrated in our experiment, combined use of LIGHT and IFNγ did not induces apoptosis of U3A cells as Bcl-2 was not down-regulated; However, combined use of LIGHT and IFNγ induced apoptosis of U3A1-1 cells as shown by Bcl-2 down-regulation. This result confirms that LIGHT-induced Bcl-2 down-regulation is STAT1 dependent, and IFNγ does take part in LIGHT-induced, IFNγ-mediated apoptosis of MDA-MB-231 cells.

LIGHT treatment does not change the expression level of LTβR or HVEM/TR2 on cancer cell surface

LTβR and HVEM/TR2 are two receptors that LIGHT binds to (1, 2). To elucidate whether LIGHT-induced apoptosis involves the expression alteration of these two receptors or not, a

serial of flow cytometry analysis was performed to examine the expression level of LTβR or HVEM/TR2 before and after treatment with INFγ alone, LIGHT alone, or combined use of LIGHT and INFγ. As shown in our experiment, both U3A and U3A1-1 cells are LTβR positive and HVEM/TR2 negative, the expression level of LTβR or HVEM/RTR2 was not changed with the above-mentioned treatment. MDA-MB-231 cells are LTβR and HVEM/TR2 double positive, the expression level of LTβR or HVEM/RTR2 also was not changed with the above-mentioned treatment. These experiments demonstrate that LIGHT treatment does not increase the expression level of LTβR or HVEM/TR2 on cancer cell surface, that is, LIGHT signaling is a event at the down-stream of LIGHT binds to LTβR or HVEM/TR2.

DISCUSSION

LIGHT induced MDA-MB-231 cell apoptosis is not only involved in Bcl-2 down-regulation, but also involved in Bcl-X_L, Bag-1, and Mcl-1 down-regulation, this is consistent with the reports that breast cancer cells overexpress anti-apoptosis Bcl-2 family member Bcl-2, Bcl-X_L, Bag-1, and Mcl-1 (33, 39, 40). Furthermore, Bag-1 seems to be more important to the survival of MDA-MB-231 cells than the other anti-apoptosis Bcl-2 family members, because its expression level is much higher than that of the other three Bcl-2 family members Bcl-2, Bcl-X_L, and Mcl-1, therefore, targeting Bag-1 is probably the major contriburion of LIGHT induced apoptosis in MDA-MB-231 cells.

Bcl-2 cleavage was not observed as reported in Hep3BT2 cells (41) in MDA-MM-231 cells. Unaltered Bid and Ser (112)-phosphor-bad up-regulation indicated that they might not antagonize the anti-apoptotic effect of anti-apoptosis Bcl-2 family members in MDA-MB-231 cells. Surprisingly, Bax was down-regulated, this is different from other reports that Bax is up-regulated or remains unaltered (42-44) when cells are induced to perform apoptosis. It was assumed that this Bax down-regulation might come from the growth-stimulating effect of IFNγ. It seems that Bak is the only pro-apoptosis molecule which was up-regulated in MDA-MB-231 cells by LIGHT, and higher level (the highest expression level among four pro-apoptosis Bcl-2 family members in MDA-MB-231 cells) of Bak should be enough to antagonize the anti-apoptotic effect of anti-apoptosis Bcl-2 family members.

Three major apoptotic pathways originating from three separate subcellular compartments have been identified: death receptor-mediated pathway, mitochondria pathway, and endoplasmic reticulum pathway. LIGHT-induced apoptosis of MDA-MB-231 cells is apparently involved in at least death receptor pathway and mitochondria pathway, because clear activation of caspase-8 and extensive alterations of Bcl-2 family members were observed. Furthermore, since caspase-9 was activated, but Apaf-1 remained unaltered, it is predicted that other factors like Smac/DIABLO might take effect, other than cytochrome c and AIF.

Since LIGHT treatment does not increase the expression level of LTβR or HVEM/TR2 on cancer cell surface, LIGHT signaling is a event at the down-stream after LIGHT binds to LTβR or HVEM/TR2. But it remains unclear how LIGHT signaling is connected to the down-stream of apoptosis pathways, especially from TRAF3 to caspase-8, and from TRAF3 to mitochondria, leading to the dramatic alterations Bcl-2 family members. The other important

issue is that the expression of TR6 must have been depressed by LIGHT induced apoptosis, whether TR6 was down-regulated and how this signal transmitted to apoptosis cascade is another important question.

Broad range caspase inhibitor, caspase-1 inhibitor, and caspase-3 inhibitor did not completely block LIGHT/IFNγ induced apoptosis in Hep3BT2 cells, so the authors proposed that there might exist a caspase-independent apoptosis pathway through reactive oxygen species (ROS) and other inducers, the signaling could even bypass the alteration of mitochondria (41), like reported by others. Extensive Bcl-2 family member alteration, caspase activation that include caspase-3, -6, -7, -8, and -9, in LIGHT-induced apoptosis of MDA-MB-231 cells were observed, but like in Hep3BT2 cells, caspase activation, especially caspase-3 activity is not necessarily required for the apoptosis, it is possible that there might exist a caspase-independent apoptosis, this is in concert with a previous observation that LIGHT induced apoptosis of MCF-7 breast cancer cells, which is caspase-3 deficient (2). The other possibility is that the caspase inhibitors used here could not rule out all the functions of the entire caspase family in the cell, this is different with the observation in HT-29 colon carcinoma cells, in which caspase activation and caspase activity are necessary for LIGHT-induced apoptosis (Zhang, et al, unpublished data].

LIGHT does not induce clear apoptosis in MDA-MB-231 cells, it must get the help from IFNγ, it was confirmed that LIGHT-induced, IFNγ-mediated apoptosis is STAT1 dependent, which is an important adaptor in the cascade of IFNγ signaling IFNγ is a pleiotropic cytokine, it can both inhibit and stimulate cell growth, LIGHT-induced IFNγ-mediated apoptosis is obviously not a synergistic cytotoxic effect of IFNγ and LIGHT, because we observed that IFNr down-regulated pro-apoptotic molecule Bax. It was reported that IFNγ-induced apoptosis signaling is through Fas/CD95 [59-60], how IFNγ and LIGHT signaling conduct their cross-talk to activate the down-stream apoptosis pathway is another interesting research topic.

Cancer cells do not express LIGHT (2), if LIGHT is overexpressed in cancer cells, they will become more susceptible to the attack from "killing factors (include cytokines and killing effector cells)", that is why tumor cells are suppressed *in vivo*. Here it was demonstrated LIGHT cDNA introduction clearly induced IFNγ-mediated Bcl-2 down-regulation in MDA-MB-231/LIGHT cells, it is easy to understand that IFNγ or other regulatory or "killing factors" are accessible to tumor cells *in vivo*, actually, LIGHT can stimulate T cells to produce IFNγ (2), and LIGHT cDNA introduction also highly up-regulates MDA-MB-231 cells to express ICAM-1, which is necessary for lymphocyte adhesion and infiltration. Therefore, LIGHT overexpression in cancer cells might represent a potential cancer therapy strategy.

Because Bcl-2 has been strongly implicated in the resistance of breast and many other forms of cancer to treatment with radiation and chemotherapy, a number of Bcl-2 small molecule inhibitors, this inhibitors has been developed in this Lab. which dramatically induce apoptosis in Bcl-2 expression cancer cells (publish separately). To elucidate whether Bcl-2 inhibitors have synergistic effect on LIGHT-induced apoptosis of MDA-MB-231 cells, a Annnexin V-propidium iodide assay of MDA-MB-231 cells treated with both LIGHT-IFNγ and a typical Bcl-2 inhibitor BL-106 5 uM for 72 hrs was performed, it was observed that BL-106 highly augmented the apoptosis inducing effect of LIGHT alone and combined use of LIGHT and IFNγ.

It is of great significance to see that BL-106 augments LIGHT-induced (LIGHT alone or combined with IFNγ) apoptosis in MDA-MB-231 cells, as we know the apoptosis-inducing effect of LIGHT is dependent on IFNγ, LIGHT alone does not induce apoptosis *in vitro*, but it seems that BL-106 some how can replace IFNγ to enhance MDA-MB-231 cell apoptosis induced by LIGHT. The IC_{50} of BL-106 to MDA-MB-231 cells is about 20 uM (publish separately); here the concentration of BL-106 was only 5 uM. The other important point is: Except targeting Bcl-2, this small molecule drug might probably target other Bcl-2 family members and facilitate the apoptosis signaling from LIGHT to induce a higher level of apoptosis. It would be interesting to investigate the mechanism of how LIGHT works when it is combined with this group of small molecule anti-cancer drugs.

KEY RESEARCH ACCOMPLISHMENTS:

- Revealed that LIGHT is highly expressed in activated lymphocytes but not in cancer cells.
- Made soluble LIGHT and monoclonal antibodies against LTβ and HVEM/TR2, tested the growth inhibition of soluble LIGHT to different cell lines, proposed that both LTβ and HVEM/TR2 expression is necessary for LIGHT function.
- Correlated the anti-cancer effect of LIGHT with its upregulation of ICAM-1 expression of cancer cells.
- Demonstrated that LIGHT-induced apoptosis involves down-regulation of anti-apoptosis Bcl-2 family members and extensive caspase activation.

REPORTABLE OUTCOMES:

1. Zhai, Yifan, Ribo Guo, Tsui-Ling Yu, Jian Ni, Byoung S. Kwon, Gong-wei Jiang, Jiamo Lu, Jie Tan, Meena Ugustus Kent Carter, Lorena Rojas, Feng Zhu, Clint Lincoln, Greg Endress, Lily Xing, Sa Wang, Kwi-O. Oh, Reiner Gentz, Steve Ruben, Marc E. Lippman, Shie-Liang Hsieh, and **Dajun Yang**. LIGHT, anovel ligand for lymphotoxin β recptor and TR2/HVEM induces apoptosis and suppresses in vivi tumor formation via gene tansfer. J. Clin. Invest. 102: 1142-1151, 1998.
2. **Yang, Dajun**., Yifan. Zhai, and Manchao.Zhang. New TNF member LIGHT. J. Biol. Regulators and Homeostatic Agents. 2002 (in press).
3. Zhang, Manchao, Ribo Guo, and **Dajun Yang**. New TNF member LIGHT enhances IFNr-mediated up-regulation of intercellular adhesion molecule-1 (ICAM-1) expression of cancer cells. Clinical Cytometry Society,16th Annual Meeting of Clinical applications in Cytometry, Orlando, FL Nov 11-13, 2001.
4. Zhang, Manchao., and **Dajun Yang**. New TNF member LIGHT enhances IFN□-mediated apoptosis of HT-29 colon carcinoma cells through downregulation of Bcl-XL. 2001 Programmed Cell Death. Cold Spring Harbor, NY
5. Zhang, Manchao, Yifan. Zhai, and **Dajun.Yang**. New TNF member LIGHT-induced apoptosis of HT-29 colon carcinoma cells involves extensive caspase activation (submitted to Int. J. Cancer).
6. Zhang, Manchao., Ribo Guo, Yifan Zhai, Xin-Yuan Fu, and **Dajun Yang**. New TNF member LIGHT induces apoptosis of MDA-MB-231 breast cancer cells through downregulation of antiapoptosis Bcl-2 members (submitted to J Biol.Chem).

CONCLUSIONS:

LIGHT is highly expressed in activated lymphocytes but not in cancer cells. LIGHT can induce apoptosis or inhibit the growth of cancer cells *in vitro*, local LIGHT gene transfer suppresses the tumor formation *in vivo*.both LTβ and HVEM/TR2 expression is necessary for LIGHT function. The anti-cancer effect of LIGHT correlates with its upregulation of ICAM-1 expression of cancer cells. LIGHT-induced apoptosis involves down-regulation of anti-apoptosis Bcl-2 family members and extensive caspase activation.

Understanding the biological functions of LIGHT is complicated by the fact that each of its receptors also binds to other TNF family ligands. For example, in addition to LIGHT, the LTβR binds the LTα1β2 heterotrimer, while HVEM/TR2 can also bind the secreted homotrimeric form of LT, LTα3. Therefore, the presence of LIGHT might either synergize with or antagonize the signals delivered by these alternate ligands for the receptors that bind LIGHT (45).

Except its anti-cancer effect, LIGHT has been accepted as a very potent immunological modulator, which takes part in T cell and dendritic cell development (45), but there is no report of the role of LIGHT in nature killer (NK) cell development and its interaction with cancer cells. Since LIGHT can stimulate the expression of ICAM-1 on cancer cells, which is a ligand of LFA-1 of NK cells, this remind us it is of great significance to study the function of LIGHT in NK cell development and function.

We will continue to do some work on the *in vivo* anti-cancer effect of LIGHT. Based on the complexcity of LIGHT with its receptors, we only observed little or no effect of LIGHT in MDA-MB-231 (2LMP) xenograft tumor-bearing mice. Probably it is a better idea to get clear response if we combine the use of LIGHT with some chemotherapy drugs, like the protocol for some cytokines. The other strategy, which might be very effective, is to combine the use of LIGHT with our small molecule Bcl-2/Bcl-X_L inhibitors, which are currently developing in our Lab. Our preliminary data already showed that the apoptosis rate of 2LMP cells was highly increased, much higher than LIGHT and small molecule drug alone.

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