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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

The main goal of this proposal is to study the anti-tumor activity of p202 and its application in a breast cancer model system. Three specific aims are 1) To determine the effects of p202 on the sensitivity of breast cancer cells to anticancer agents; 2) To test the anti-tumor activity of p202 in breast cancer cells using preclinical gene therapy strategies in an orthotopic breast cancer animal model; and 3) To evaluate the therapeutic efficacy of p202 gene therapy in combination with other anti-cancer agents in an orthotopic breast cancer animal model. We have developed a p202-based therapeutic agent to study its efficacy in animal models in the first year of grant supported period. Our results have shown that adenovirus-mediated p202 gene transfer and its expression can cause growth-suppression and sensitization to TNF-α, taxol, CDDP and γ-irradiation induced apoptosis of breast cancer cells. In the last year, we have completed CMV-p202-liposome delivery system *in vivo* via intratumoral and i.v. injection, and immunohistostaining in p202-treated breast cancer cells.

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Introduction:

The main goal of this project is to study the anti-tumor activity of p202 and its application to gene therapy in breast cancer model systems. P202, an interferon (IFN)-inducible protein, interacts with several important regulatory proteins which participate signaling transduction pathways those are involved in cell cycle, cell differentiation and growth. These protein-protein interactions primarily lead to transcription repression of target genes, causing cellular growth arrest or differentiation (ref. 1-10). Our previous studies have shown that p202 could suppress both *in vitro* and *in vivo* cell growth and tumorigenicity of human breast cancer cells (ref. 11). We also found that p202 can directly interact with NF-κB and inhibit TNF-α induced NF-κB activation. The p202-mediated inactivation of NF-κB sensitizes breast cancer cells to TNF-α induced apoptosis (ref. 12). The major objectives of this grant application are to determine the effects of p202 on the sensitivity of breast cancer cells to anticancer agents; to test the anti-tumor activity of p202 in breast cancer cells using pre-clinical gene therapy strategies in an orthotopic breast cancer animal model; and to evaluate the therapeutic efficacy of p202 gene therapy in combination with other anti-cancer agents in an orthotropic breast cancer animal model. The success of the pre-clinical experiments in this grant application could lead to the translation of the laboratory research to clinical products and thus benefit breast cancer patients.

Body:

A. Specific Aims: (no changes)

Specific Aim 1: To determine the effect of p202 on the sensitivity of breast cancer cells to anti-cancer agents.

Specific Aim 2: To test the anti-tumor activity of p202 in breast cancer cells using pre-clinical gene therapy strategies in an orthotopic breast cancer animal model.

Specific Aim 3: To evaluate the therapeutic efficacy of p202 gene therapy in combination with other anti-cancer agents in an orthotopic breast cancer animal model.

B. Studies and Results:

In the last three years, we have developed a p202-mediated delivery system for therapeutic efficacy study in animal models. We further investigated the biological functions of p202. Our results have demonstrated that adenovirus-mediated p202 (Ad-p202) gene transfer and expression indeed could cause potent growth-suppression and sensitization to TNF-α induced apoptosis in breast cancer cells. In addition, p202 overexpression alone induces apoptosis and that may contribute to the p202-mediated multiple anti-tumor activity. Our data suggested that the activation of caspase (in particular, caspase-3) may be critical for Ad-p202 to exert full apoptotic effect in breast cancer cells. Importantly, Ad-p202 treatments resulted in significant tumor suppression in breast cancer xenograft model. A manuscript entitled "Pro-apoptotic and anti-tumor activities of adenovirus-mediated p202 gene transfer" by Ding, Y. et al. was published in *Clinical Cancer Research* 8:3290-3297, 2002 (Appendix 3). In the meantime, we also

initiated non-viral delivery system to avoid side effects, which were caused by the adenoviral vectors. In addition, in order to do the treatment in human body in the future clinical trials, we have cloned the human p202 genes, AIM2 (Absent In Melanoma) and MNDA (Myeloid Nuclear Differentiation Antigen). The AIM2 has demonstrated an anti-growth activity in human breast cancer cells. The progress of each specific aim is discussed below:

Specific Aim 1: To determine the effect of p202 on the sensitivity of breast cancer cells to anti-cancer agents.

As reported last year we have completed the biological effect of p202 *in vitro*, a manuscript has published in *Clinical Cancer Research* (Appendix 3). Our studies have shown that: 1) Ad-p202 delivery system is feasible and efficient (please see Appendix 3, Figure 1); 2) overexpression of p202 inhibits growth of breast cancer cell (Appendix 3, Figure 2); 3) Ad-p202 infected breast cancer cells are sensitized to TNF-α-induced apoptosis (Figure 3); and 4) Ad-p202 infected breast cancer cells are sensitized not only to γ-irradiation-induced apoptosis but also to anti-cancer drugs, such as Taxol and CDDP, -induced apoptosis (Appendix 3,Figure 6). To examine the molecular mechanism of p202's pro-apoptotic activity, we have treated Ad-p202 infected breast cancer cells with a pan caspase inhibitor, Z-VAD. We have found that activation of caspase is required for Ad-p202-mediated apoptosis in infected breast cancer cells. To further support our observation, we have infected MCF-7 cells, which are caspase-3 null breast cancer cells, with Ad-p202, the infected MCF-7 cells are unable to induce apoptosis. In addition, we treated Ad-p202 infected MDA-MB-468 cells with a caspase-3 specific inhibitor, Z-

DEVD-fmk, the treated cells failed to go into apoptosis (Appendix 3,Figure 4). Our results indicated that the caspase-3 is critical for Ad-p202-mediated apoptosis.

In addition, the p202 is a mouse gene and its human equivalent has not been identified. Considering potential application to human body in the future, we also initiated to clone human genes that are homologue to p202 family and examined their potential anti-tumor activities in breast cancer cells. We have used RT-PCR to clone these genes including AIM2 (Absent In Melanoma) and MNDA (Myeloid Nuclear Differentiation Antigen). When those two genes were tested their ability to inhibit cell growth using colony formation assay, we have found that AIM2 but not MNDA possesses activity to inhibit breast cancer cells, MDA-231 (Appendix 1). We will continue to compare anti-tumor activity of the human AIM2 and mouse p202 gene.

Specific Aim 2: To test the anti-tumor activity of p202 in breast cancer cells using pre-clinical gene therapy strategies in an orthotopic breast cancer animal model.

As reported in the last year we have completed the efficacy of Ad-p202 *in vivo*, the results were published in *Clinical Cancer Research* (Appendix 3). Our studies have demonstrated that p202 exhibits anti-tumor activity in intra-tumor and in tail vein injection (Appendix 3,Figures 5 & 7). To further understand the anti-tumor mechanism of p202 *in vivo*, the treated tumor section was studies by TUNEL assay and immunohistochemical analysis. Our results have suggested that the anti-tumor activity of p202 *in vivo* is correlated with tumor cell death and downregulation of an angiogenic factor, VEGF (Appendix 3,Figure 8).

We have successfully shown that the p202 gene delivered by adenoviral vector, which suppressed tumor development in an orthotopic breast cancer xenograft model through

i.v. systemic delivery (Appendix 3, Figure 5). The results provide a proof of concept for using p202 as a tumor suppression gene for mammary tumors. Considering potential side effects using adenoviral vectors through i.v. injection, we have also initiated a similar study using a non-viral delivery system, SN2. The preliminary results (Appendix 2) look promising.

Specific Aim 3: To evaluate the therapeutic efficacy of p202 gene therapy in combination with other anti-cancer agents in an orthotopic breast cancer animal model.

In the past three years, we have successfully established Ad-p202 and CMV-p202/SN2 delivery systems to test its pro-apoptotic and anti-tumor activities *in vitro* and *in vivo*. We will evaluate the therapeutic efficacy in combination with anti-cancer agents, such as Taxol, in an orthotopic breast cancer animal model. This Aim will be an important area of focus in the subsequent years of research within this grant supported period.

Key Research Accomplishments:

- One reportable outcome was published in *Clin. Cancer Res.* 8:3290-3297, 2002.
- Clone human homologues, AIM2 and MNDA of p202 mouse gene
- AIM2 has anti-growth ability in breast cancer cells, MDA-231.
- The feasibility *of in vivo* anti-tumor effect by a non-viral systemic delivery of p202.

Reportable Outcomes: 1

Ding, Y., Wen Y., Spohn. B., Wang, L., Xia, W., Kwong, K. Y., Shao, R., Li, Z., Hortobagyi, G. N., **Hung, M.-C.** and Yan, D.-H. Pro-apoptotic and Anti-tumor Activities of Adenovirus-Mediated p202 Gene Transfer. *Clin. Cancer Res.* 8:3290-3297, 2002.

Conclusions:

Based upon our studies, p202, an interferon-inducible protein, has pro-apoptotic and anti-tumor activities *in vitro* and *in vivo*, respectively. Its pro-apoptotic activity may require activation of caspase-3 pathway; and its anti-tumor activity may be related to induce apoptosis and inhibit angiogensis in tumor cells. P202 also sensitizes breast cancer cells to TNF-α, γ-irradiation, Taxol and CDDP-induced apoptosis *in vitro*. It is worth noting that in another our observation, the p202 suppresses metastasis and angiogensis in a human pancreatic cancer model system (ref. 13). Taken together, p202 could be a potentially therapeutic gene against breast cancer. We also clone the human homologues of mouse p202 gene, AIM2 and MNDA, and AIM2 has anti-growth activity in human

breast cancer cells using colony formation assay. Therefore, we will continue to investigate whether the combination of p202 (or AIM2) and anti-cancer drugs or TNF-α or γ-irradiation therapy might achieve synergistic (or additive) therapeutic efficacy against breast cancer *in vivo*. The success of the pre-clinical experiments in this proposal would fulfill the purpose of directing the basic science research in laboratory to clinical products to benefit breast caner patients.

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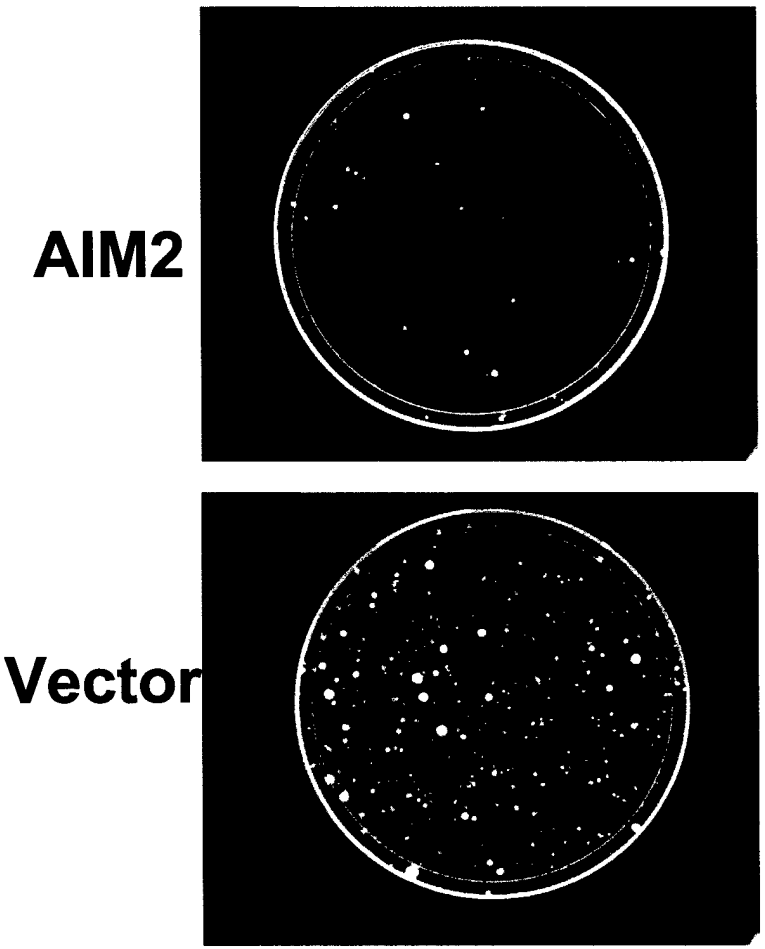
Appendices:

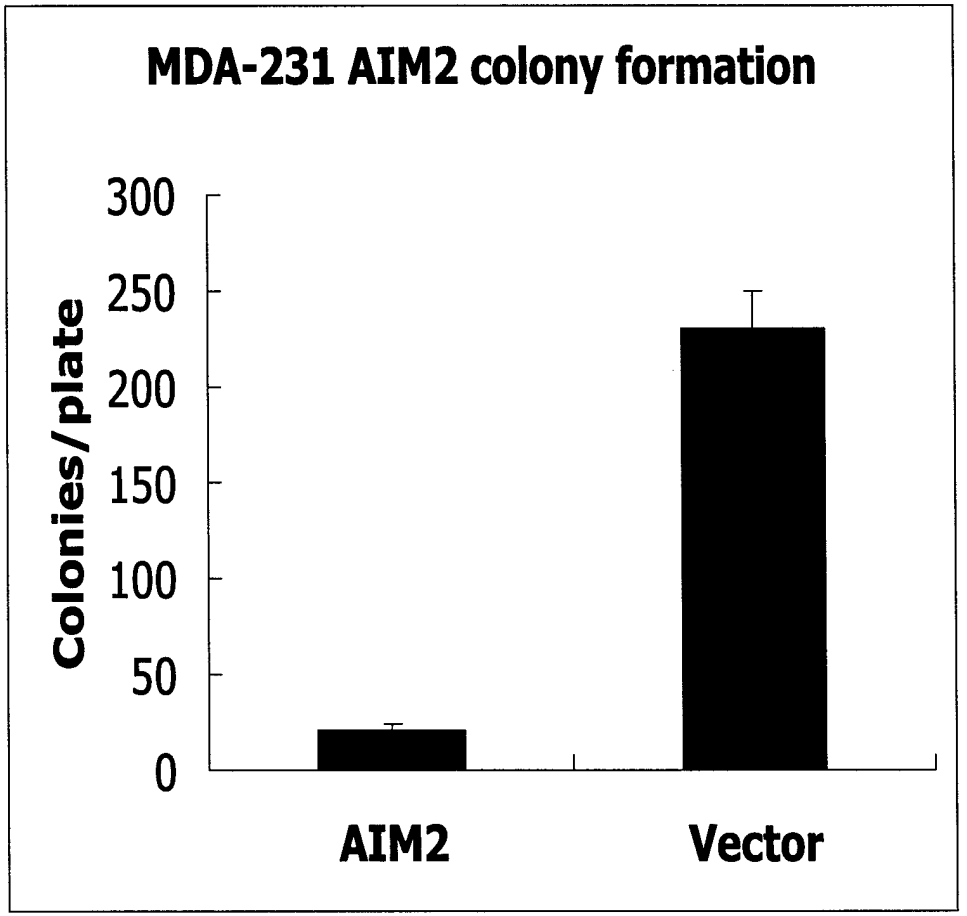
1. An anti-growth activity of AIM2 in breast cancer cells.

2. The *In vivo* anti-tumor effect by a systemic delivery of p202 through a non-viral delivery system, SN2.

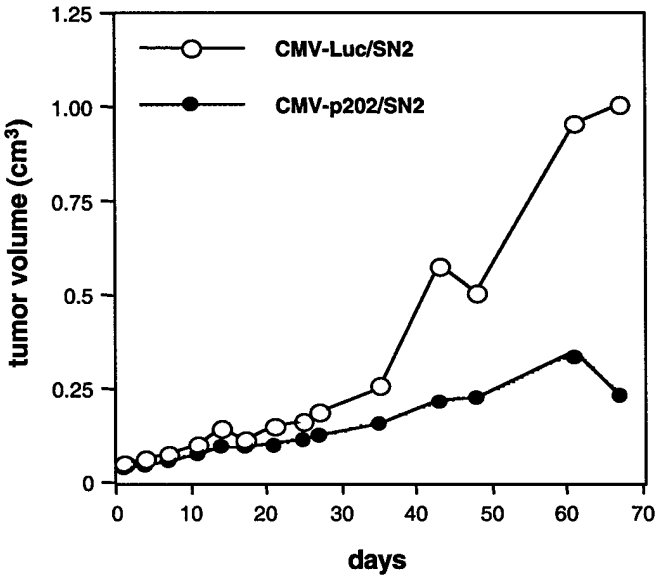
3. Reprint of Reportable Outcome- Ding, Y., Wen Y., Spohn. B., Wang, L., Xia, W., Kwong, K. Y., Shao, R., Li, Z., Hortobagyi, G. N., **Hung, M.-C.** and Yan, D.-H. Pro-apoptotic and Anti-tumor Activities of Adenovirus-Mediated p202 Gene Transfer. *Clin. Cancer Res.* 8:3290-3297, 2002.

MDA-231 colony formation





Appendix 1: Anti-growth activity of AIM2 in breast cancer cells



Appendix 2: *In vivo* anti-tumor effect by a systemic delivery of CMV-p202/SN2. MDA-MB-468 cells (1 x 10^6 cells) were implanted in each mammary fat pad of female nude mice. Tumor-bearing mice were divided into two treatment groups: CMV-Luc/SN2 (5 mice/10 tumors) or CMV-p202/SN2 (5 mice/10 tumors), at 20 μg DNA/52 μg SN2 in 200 μl PBS via tail vein injection. Treatment started when tumor reached 5-cm in diameter with a treatment schedule of twice a week for five weeks and once a wee thereafter.Tumor volume was recorded at the time indicated.

Appendix 3:

Reportable Outcome

Ding, Y., Wen Y., Spohn. B., Wang, L., Xia, W., Kwong, K. Y., Shao, R., Li, Z., Hortobagyi, G. N., **Hung, M.-C**. and Yan, D.-H. Pro-apoptotic and Anti-tumor Activities of Adenovirus-Mediated p202 Gene Transfer. *Clin. Cancer Res.* 8:3290-3297, 2002.

Proapoptotic and Antitumor Activities of Adenovirus-mediated p202 Gene Transfer[1]

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ABSTRACT

Purpose and Experimental Design: **p202, a mouse IFN-inducible protein, is a member of the 200-amino acid repeat family. Enforced p202 expression in stable cancer cell lines resulted in growth inhibition *in vitro* and tumor suppression *in vivo*. However, to study the immediate effect of p202 and test the potential efficacy of p202 treatment, an efficient gene delivery system for p202 is required. For these purposes, an adenoviral vector expressing the p202 gene (Ad-p202) was generated. We examined the effects of Ad-p202 infection on human breast cancer cells. Furthermore, we tested the efficacy of Ad-p202 treatment on breast and pancreatic cancer xenograft models.**

Results: **We found that Ad-p202 infection induces growth inhibition and sensitizes the otherwise resistant cells to tumor necrosis factor α-induced apoptosis. In addition, we demonstrated for the first time that Ad-p202 infection induces apoptosis and that activation of caspases is required for the full apoptotic effect. More importantly, we showed the efficacy of Ad-p202 treatment on breast cancer xenograft models, and this antitumor effect correlated well with enhanced apoptosis in Ad-p202-treated tumors.**

Conclusions: **We conclude that Ad-p202 is a potent growth-inhibitory, proapoptotic, and tumor-suppressing agent. Ad-p202 may be further developed into an efficient therapeutic agent for human cancer gene therapy.**

INTRODUCTION

IFN is known to exert antiproliferative and antiviral actions. It has both direct and indirect (immunological) antitumor activity in several human malignancies, including leukemia and lymphomas as well as solid tumors. Aside from the therapeutic effects of IFN in certain clinical settings, there are also undesirable side effects (*e.g.*, fever, chills, anorexia, and anemia) associated with the high-dose IFN treatment that is often required to obtain a significant response (1, 2). This has hampered IFN as an effective anticancer agent. In an attempt to circumvent this potential drawback and maintain the benefit of IFN-mediated antitumor activity, we have begun to explore the possibility of using an IFN-inducible protein, p202, as a potential therapeutic agent (3–5). p202 is a mouse IFN-inducible, chromatin-associated protein. It belongs to the 200-amino acid repeat family (6, 7). The unique feature of p202 is illustrated by its ability to interact with several important transcriptional regulators that include E2Fs, Rb, pocket proteins p130 and p107, Fos/Jun, c-Myc, NF-κB,[3] and p53BP-1 (reviewed in Ref. 8), resulting in transcriptional repression of genes that are up-regulated by these transcriptional regulators. The exact role of p202 in the IFN-mediated signal pathway is not well defined. However, consistent with the multiple antitumor activities of IFN (9), enforced expression of p202 in stable murine fibroblasts and human cancer cell lines leads to retardation of cell growth and suppression of transformation phenotype (3, 5, 10, 11). Furthermore, breast cancer cells stably transfected with p202 are sensitized to TNF-α-induced apoptosis (5), and that effect is associated with inactivation of the TNF-α-induced NF-κB via p202-NF-κB interaction. We postulated that p202 sensitizes cancer cells to TNF-α-induced apoptosis by inactivating NF-κB, which, in turn, turns off NF-κB-activated antiapoptotic gene expression, leading to enhanced TNF-α-induced cell killing (5).

To generate a p202-based therapeutic agent for efficacy study in animal models and a tool to study the biological function of p202, we constructed Ad-p202. In this study, we

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[3] The abbreviations used are: NF-κB, nuclear factor κB; TNF-α, tumor necrosis factor α; i.t., intratumor; CMV, cytomegalovirus; GFP, green fluorescence protein; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MOI, multiplicity of infection; PARP, poly-(ADP-ribose) polymerase; pfu, plaque-forming unit(s); TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling; PI, postinfection; fmk, fluoromethyl ketone; Z-VAD, N-benzyloxycarbonyl-Val-Ala-Asp; Z-DEVD, N-benzyloxycarbonyl-Asp-Glu-Val-Asp.

show that Ad-p202 infection of breast cancer cells resulted in growth inhibition and sensitization to TNF-α-induced apoptosis. Interestingly, we found that Ad-p202 infection alone induces apoptosis in breast cancer cells, and the activation of caspases is critical for this process. More importantly, we demonstrated the efficacy of Ad-p202 treatment in human breast cancer xenograft models through either i.t. or i.v. injection. This antitumor activity correlated well with p202 expression and apoptosis in Ad-p202-treated tumors. Together, our results suggest that Ad-p202 is a potent growth-inhibitory, proapoptotic, antitumor agent that could be further developed to become an effective therapeutic agent for cancer gene therapy treatment.

MATERIALS AND METHODS

Generation of Ad-p202. Ad-p202 was constructed according to the protocol described previously (12). p202 cDNA (11) was subcloned into an adenovirus vector (pAdTrack-CMV) that carries a CMV promoter-driven GFP. A separate CMV promoter directs p202 cDNA. A control virus, an adenoviral vector expressing luciferase gene and GFP (Ad-Luc), was likewise generated. The expression of GFP gene enabled us to monitor the infection efficiency by direct observation using a fluorescence microscope.

***In Vitro* Growth Assays.** MDA-MB-468 human breast cancer cells were maintained in DMEM/Ham's F-12 (HyClone Laboratories, Inc.) supplemented with 10% (v/v) fetal bovine serum. MTT is a pale yellow substrate that can be cleaved by living cells (but not dead cells) to yield a dark blue formazan product. The extent of MTT cleavage determined colorimetrically (at 570 nm) can be used to measure cell proliferation. Briefly, 2×10^3 cells were plated in 96-well culture plates in 0.1 ml of culture medium. Ad-p202 or Ad-Luc was added at a MOI of 200 on the next day. At the different times indicated, 20 μl of MTT (5 mg/ml stock solution) were added to each well. Cells were cultured for an additional 2 h, and then 100 μl of lysis buffer [20% SDS in 50% *N,N*-dimethylformamide (pH 4.7)] were added to each well, followed by 5 h of incubation, and then absorbance was measured at 570 nm. [^{3}H]Thymidine incorporation assay was performed as described previously (13).

Apoptosis Assays. For flow cytometry analysis, cells were collected at the indicated times PI, washed once with PBS, and suspended in 0.5 ml of PBS containing 0.1% (v/v) Triton X-100 for nuclei preparation. The suspension was filtered through a nylon mesh and then adjusted to a final concentration of 0.1% (w/v) RNase and 50 μg/ml propidium iodide. Apoptotic cells were quantified by FACScan cytometer. The DNA fragmentation assay was carried out as described previously (13).

Western Blot Analysis. MDA-MB-468 cells treated with or without TNF-α (R&D Systems, Inc., Minneapolis, MN) were infected with Ad-p202 or Ad-Luc at a MOI of 200. Seventy-two h PI, cells were lysed with radioimmunoprecipitation assay lysis buffer. The protein extracts were subjected to SDS-PAGE followed by Western blotting according to the procedure described previously (5). Goat anti-p202 polyclonal antibody and anti-PARP antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and BD Transduction Laboratories (Lexington, KY), respectively. Caspase inhibitors Z-VAD and Z-DEVD-fmk were purchased from Enzyme Systems Products (Livermore, CA).

Gel-Shift Assay. The NF-κB gel-shift assay was performed as described previously (13).

Ad-p202 Gene Therapy in Human Cancer Xenograft Models. For the orthotopic breast cancer xenograft model, MDA-MB-468 cells (2×10^6 cells) were implanted in mammary fat pads (2 tumors/mouse) of female nude mice. Tumor-bearing mice were divided into two treatment groups: group 1, Ad-Luc; and group 2, Ad-p202. For i.t. injection, 1×10^9 pfu viruses/treatment was administered. Treatment started when tumor reached 0.3 cm in diameter with a treatment schedule of twice a week for 7 weeks and once a week thereafter. For tail vein injection, 5×10^8 pfu viruses/treatment were administered. Treatment started when tumor reached 0.5 cm in diameter with a treatment schedule of twice a week for 5 weeks and once a week thereafter.

Immunohistochemical Analysis of p202 Expression and Apoptosis. Mice were sacrificed 24 h after the last treatment. Tumors obtained from Ad-p202- or Ad-Luc-treated mice bearing either breast or pancreatic tumors were then excised and fixed with formalin and embedded in paraffin. Immunohistochemical analysis of p202 protein expression was performed according to the protocol described previously (14). Tumor sections were incubated with goat polyclonal antibody specific for p202 (Santa Cruz Biotechnology) followed by incubation with biotinylated rabbit antigoat IgG and subsequent incubation with avidin-biotin peroxidase before visualization. TUNEL assay was performed to detect the ends of degraded DNA fragments induced by apoptosis according to the protocol described previously (15).

RESULTS

Ad-p202 Mediates p202 Expression in Breast Cancer Cells. To test the efficiency and monitor the expression of p202 protein by Ad-p202 infection, we infected MDA-MB-468 breast cancer cells with either Ad-p202 or Ad-Luc followed by fluorescence microscopy and Western blot analysis, respectively. As shown in Fig. 1*A*, Ad-p202 and Ad-Luc infection at a MOI of 200, at 24-h PI, exhibited >90% infection efficiency as indicated by the GFP-positive cells shown in a representative field (Fig. 1*A*, *right panels*). The same cells are shown in phase-contrast images (Fig. 1*A*, *left panels*). The mock-infected cells (*Control*) showed no GFP expression. In addition to MDA-MB-468, we found that Ad-p202 could infect a panel of other human breast cancer cell lines (*e.g.*, MDA-MB-453, MDA-MB-435, MDA-MB-231, and MCF-7), albeit with various infection efficiency rates (data not shown). We chose the MDA-MB-468 cell line for subsequent studies because it is tumorigenic in the mouse xenograft model and has relatively high infection efficiency by Ad-p202. The expression of p202 protein in Ad-p202-infected cells was further analyzed by Western blot using p202-specific antibody. Fig. 1*B* shows that whereas the mock- and Ad-Luc-infected cells have no p202 expression, Ad-p202 infection efficiently directed p202 expression in MDA-MB-468 cells in a dose-dependent manner. These results clearly demonstrate that Ad-p202 infection adequately directs p202 expression in MDA-MB-468 cells.

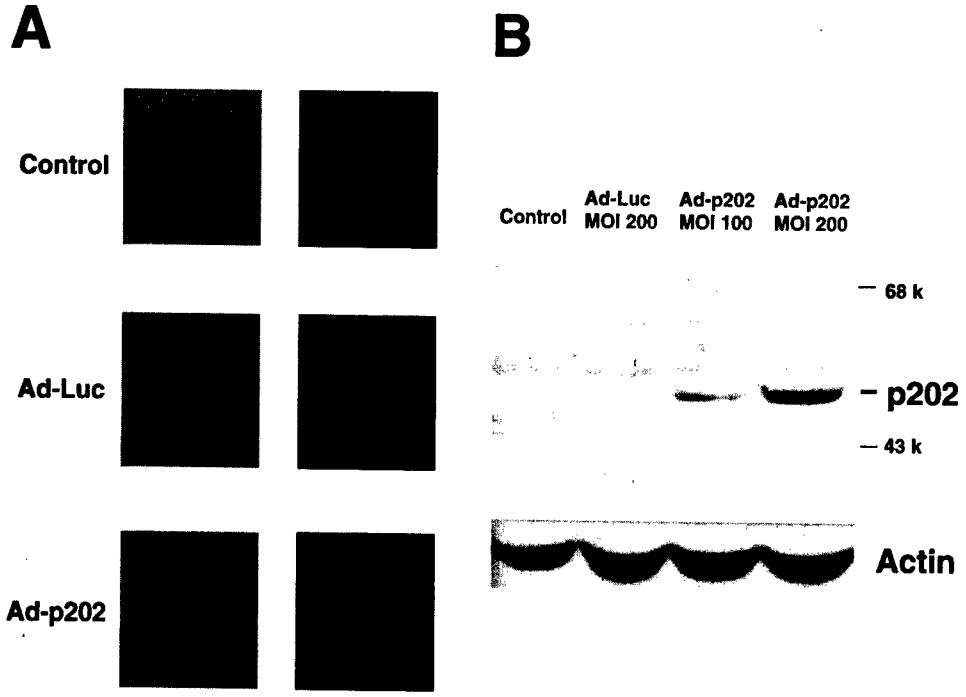

Fig. 1 Ad-p202 construction, p202 expression, and infection efficiency. *A*, Ad-p202 was generated according to the protocol described previously (12). The pAdTrack-CMV vector contains two independent CMV promoter-driven transcription units, one for GFP and one for p202 cDNA. MDA-MB-468 human breast cancer cells were infected by Ad-Luc or Ad-p202 at a MOI of 200. Twenty-four h PI, >90% of cells were found to be GFP positive as visualized by fluorescence microscopy (*right panels*), indicating that the infection efficiency is >90%. *Left panels*, phase-contrast microscopy. *Control*, mock-infected cells. *B*, p202 protein is expressed in Ad-p202 infected cells. MDA-MB-468 cells infected with Ad-Luc or Ad-p202 for 72 h were analyzed for p202 protein expression by Western blot. *Control*, mock-infected cells. Actin protein was used as an equal loading control.

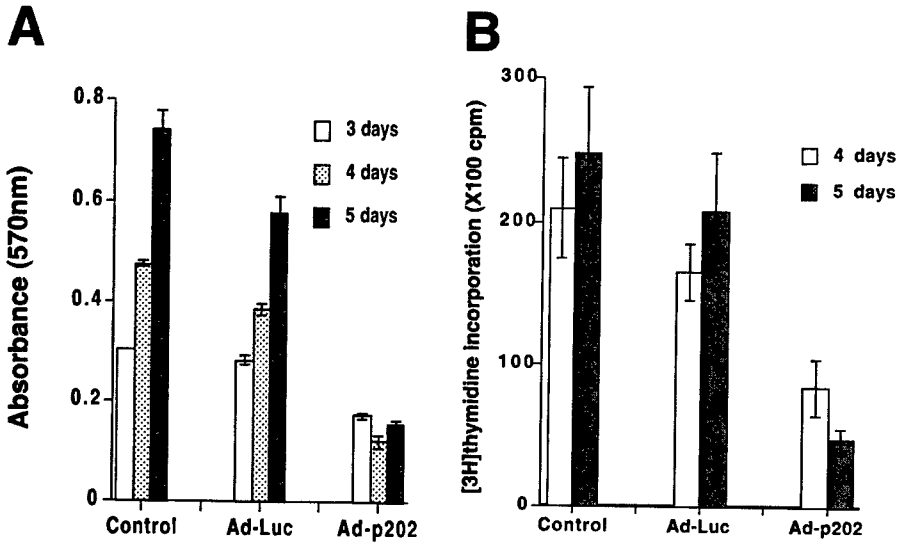

Fig. 2 Ad-p202 infection inhibits cell proliferation. MDA-MB-468 cells were infected with Ad-Luc or Ad-p202 at a MOI of 200. Cell growth was monitored at the indicated PI time (3–5 days) by (*A*) MTT assay [means ± SD (n = 3)] or (*B*) [^{3}H]thymidine incorporation assay; data are presented as the means of quadruplicates.

Ad-p202 Infection Reduces Breast Cancer Cell Growth. To assess the effect of Ad-p202 infection on cell growth, we infected MDA-MB-468 cells with either Ad-p202 or Ad-Luc followed by *in vitro* growth assays such as the MTT assay and [^{3}H]thymidine incorporation assay at different time points, *i.e.*, 3–5 days PI. As shown in Fig. 2, whereas the mock infection (*Control*) and Ad-Luc infection have no growth-inhibitory effect on MDA-MB-468 cells, Ad-202 infection significantly hampered cell growth (Fig. 2*A*) and DNA synthesis rates (Fig. 2*B*). This observation strongly indicates that Ad-p202 infection inhibits cell growth in breast cancer cells and is congruent with our previous findings using stable cancer cell lines (3, 5).

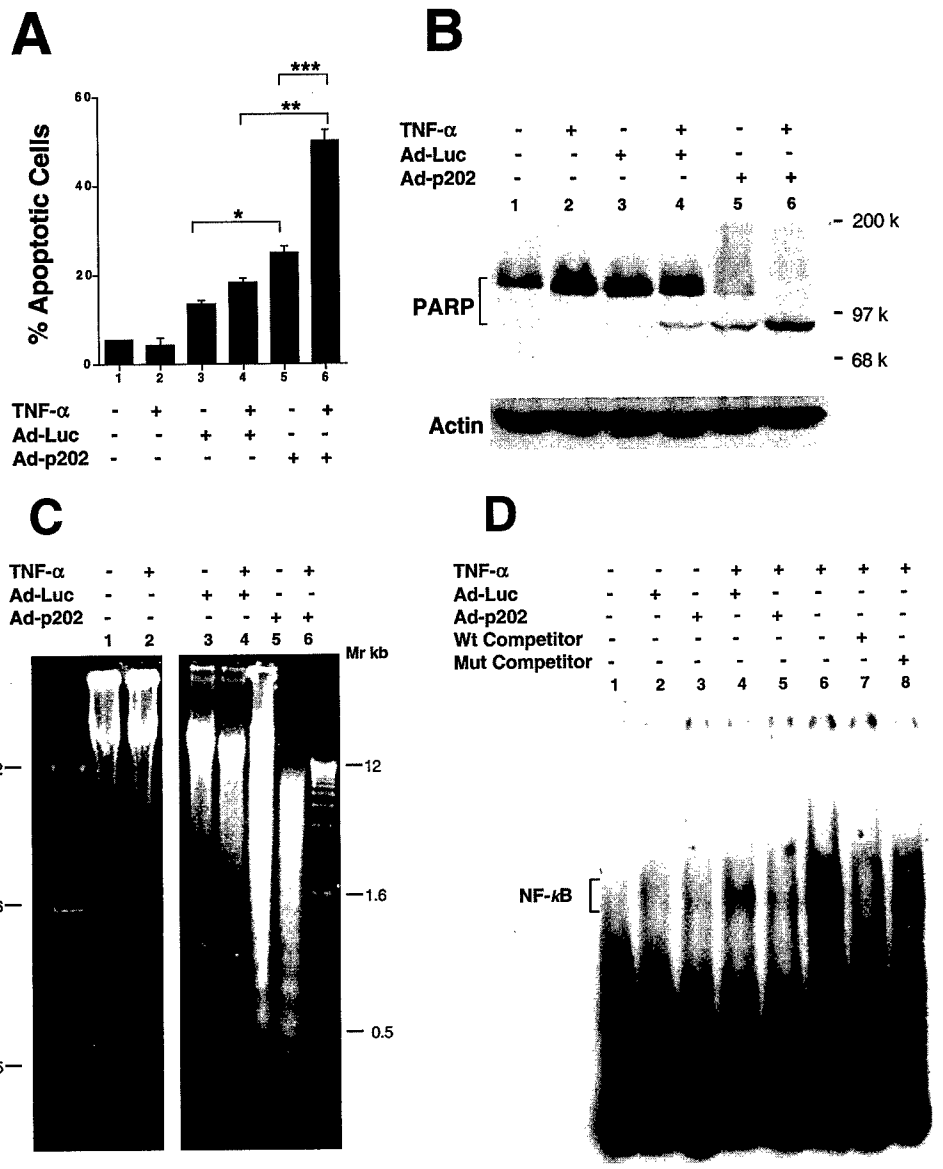

Fig. 3 Ad-p202 infection induces apoptosis and sensitizes cells to apoptosis induced by TNF-α. MDA-MB-468 cells were infected with Ad-Luc or Ad-p202 at a MOI of 200. *A*, 24 h PI, TNF-α (50 ng/ml) was added to the medium and incubated for 48 h (72 h PI). Apoptosis was then monitored by flow cytometry analysis (done in triplets; *bars*, SD). *t* test: *, $P < 0.001$; **, $P < 0.0002$; and ***, $P < 0.0005$. *B*, PARP cleavage assay. Twenty-four h PI, TNF-α (50 ng/ml) was added to the medium and incubated for 24 h. Treated cells were harvested 48 h PI. The PARP protein (M_r 116,000) was cleaved into M_r 85,000 product in the event of apoptosis. *C*, DNA fragmentation assay. Twenty-four h PI, TNF-α (50 ng/ml) was added to the medium and incubated for 24 h. Treated cells were harvested 48 h PI. *D*, Ad-p202 infection inhibits TNF-α-induced NF-κB DNA binding activity. MDA-MB-468 cells were infected with Ad-p202 or Ad-Luc in the presence or absence of TNF-α (50 ng/ml) 24 h PI for 30 min. The nuclear extracts were then isolated and incubated with a radioactive-labeled oligonucleotide containing NF-κB binding site (13). The excess cold wild-type or mutant NF-κB binding site was added to the incubation to demonstrate the specific NF-κB DNA binding activity. The NF-κB-DNA complex is indicated.

Ad-p202 Infection Induces Apoptosis in Breast Cancer Cells. Without stress signals, the p202 stable cancer cell lines do not exhibit apoptotic phenotype (3, 5). It is possible that p202 stable cell lines isolated after a vigorous selection process may possess a physiologically tolerant level of p202. The fact that only a small number of p202 stable cell lines were obtained by colony-forming assay (3, 5) raises the possibility that p202 expression alone may induce apoptosis. To test that possibility, we infected MDA-MB-468 cells with Ad-p202 or Ad-Luc followed by flow cytometry analysis at 72-h PI to detect apoptosis by measuring the cell population in the sub-G_1 phase of cell cycle. As shown in Fig. 3*A*, although Ad-Luc infection induced modest apoptosis (compare *Lane 1* with *Lane 3*), Ad-p202 infection (*Lane 5*) caused significantly more apoptosis (>20%) than Ad-Luc infection (*Lane 3*; $P < 0.001$, *t* test). That observation was further confirmed by two other apoptosis assays: (*a*) the PARP cleavage assay, in which full-length PARP (M_r 116,000) is cleaved by caspases into a fragment of approximately M_r 85,000 (Fig. 3*B*); and (*b*) a DNA fragmentation assay that is based on the activated endonucleases during apoptosis (Fig. 3*C*). Ad-p202 infection resulted in a marked increase of the PARP cleavage product (M_r 85,000; Fig. 3*B*, *Lane 5* and Fig. 4*A*, *Lane 3*) and an enhancement of DNA fragmentation (Fig. 3*C*, *Lane 5*). In contrast, Ad-Luc infection yielded a minimum amount of M_r 85,000 PARP cleavage product (Fig. 3*B*, *Lane 3* and Fig. 4*A*, *Lane 2*) as well as a near basal level of DNA fragmentation (Fig. 3*C*, compare *Lanes 1* and *3*). Together, our results strongly indicate that Ad-p202 alone induces apoptosis in MDA-MB-468 cells. Given that the MDA-MB-468 cell line harbors mutant p53 (16), Ad-p202-mediated apoptosis thus appears to be independent of p53 status.

p202-mediated Apoptosis Is Caspase-dependent. Because caspases are activated during apoptosis and have a variety

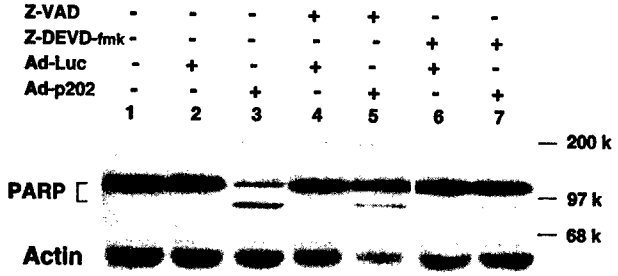

Fig. 4 The activation of caspases is critical for Ad-p202-mediated apoptosis. Z-VAD (100 μM) and Z-DEVD-fmk (80 μM) inhibit Ad-p202-mediated apoptosis in MDA-MB-468 cells. Western blot analysis of PARP cleavage and actin expression was performed 48 h PI. The intensity of full-length PARP and PARP cleavage product bands was measured using NIH Image 1.62 software.

of substrates including PARP (17), the cleavage of PARP in Ad-p202-infected cells suggests that the activation of caspase may be involved in Ad-p202-induced apoptosis. To test that hypothesis, we infected MDA-MB-468 cells with Ad-p202 or Ad-Luc in the presence or absence of a pan-caspase inhibitor, Z-VAD. At 48 h PI, the intensity of full-length PARP and PARP cleavage product bands on Western blot was measured using NIH Image 1.62 software. The percentage of M_r 85,000 product was calculated by setting the total intensity of both M_r 116,000 and M_r 85,000 bands in each lane at 100%. As shown in Fig. 4, the addition of Z-VAD attenuates Ad-p202-induced apoptosis, as indicated by the reduced (but not completely eliminated) level of PARP M_r 85,000 cleavage product from 57.4% (*Lane 3*) to 13.3% (*Lane 5*), whereas Z-VAD has no effect on PARP cleavage in Ad-Luc-infected cells (*Lane 4*). This result supports the idea that the activation of caspases, at least in part, is required for Ad-p202 to induce full apoptotic effect. Because PARP is also a substrate for caspase-3, which is considered to be a crucial enzyme commonly activated during apoptosis (17), we examined whether the activation of caspase-3 plays a role in Ad-p202-induced apoptosis. To that end, we infected MDA-MB-468 cells with either Ad-p202 or Ad-Luc in the presence of a caspase-3-specific inhibitor, Z-DEVD-fmk (18). As shown in Fig. 4, the level of PARP cleavage product in Ad-p202-infected MDA-MB-468 cells is significantly reduced to 8.9% (*Lane 7*) with Z-DEVD-fmk treatment as compared with that without Z-DEVD-fmk (57.4%; *Lane 3*). As a control, no detectable PARP cleavage was observed in Ad-Luc-infected cells treated with Z-DEVD-fmk (*Lane 6*). Thus, this result suggests that the activation of caspase-3 is critical for Ad-p202-mediated apoptosis. To further confirm this observation, we infected a caspase-3-null breast cancer cell line, MCF-7 (19), with Ad-p202 or Ad-Luc, followed by flow cytometry analysis. We observed that although p202 was readily expressed as determined by Western blot, the Ad-p202-infected MCF-7 cells yielded the similar level of apoptosis as that of the controls, *i.e.*, mock and Ad-Luc infection (data not shown). Therefore, our data suggest that the activation of caspases is critical for Ad-p202 to exert full apoptotic effect.

Ad-p202 Infection Sensitizes Breast Cancer Cells to TNF-α-induced Apoptosis. We tested whether Ad-p202 infection could also sensitize breast cancer cells to TNF-α-induced apoptosis (5). Although MDA-MB-468 cells appear to be resistant to TNF-α (50 ng/ml; added at 24 h PI for 48 h)-induced apoptosis (Fig. 3*A*, *Lanes 1* and *2*; Fig. 3*B*, *Lanes 1* and *2*; and Fig. 3*C*, *Lanes 1* and *2*), the combination of TNF-α and Ad-p202 induced massive cell killing [compare Fig. 3*A*, *Lanes 5* and *6* ($P < 0.0005$); Fig. 3*B*, *Lanes 5* and *6*; and Fig. 3*C*, *Lanes 5* and *6*]. These results suggest that Ad-p202 infection sensitizes MDA-MB-468 cells to TNF-α-induced apoptosis. In contrast, the apoptosis resulting from the combined treatment of TNF-α and Ad-Luc (Fig. 3*A*, *Lane 4*) was significantly less than that from combined treatment with TNF-α and Ad-p202 (Fig. 3*A*, *Lane 6*; $P < 0.0002$). This observation was confirmed by PARP cleavage and DNA fragmentation assays (Fig. 3*B*, *Lanes 4* and *6*; Fig. 3*C*, *Lanes 4* and *6*). These data indicate that the sensitization to TNF-α-induced apoptosis is specific to p202 expression. Because p202-mediated sensitization to TNF-α-induced apoptosis correlated with the inactivation of NF-κB, specifically, via the loss of NF-κB DNA binding activity (5), we next tested whether Ad-p202 infection affects TNF-α-induced NF-κB DNA binding activity. At 24 h PI, TNF-α was added for 30 min, and the nuclear extract was then isolated and subsequently incubated with a radioactive-labeled oligonucleotide containing the NF-κB binding sites. A gel-shift assay was then performed to detect the NF-κB DNA binding activity. As shown in Fig. 3*D*, we observed a complete abolishment of TNF-α-induced NF-κB DNA binding activity in Ad-p202-infected MDA-MB-468 cells (Fig. 3*D*, compare *Lanes 1, 3, 5,* and *6*). As controls, TNF-α-induced NF-κB DNA binding activity (*Lane 6*) can be readily competed by cold wild-type NF-κB DNA binding site (*Lane 7*) and, to a lesser extent, by cold mutant probe (*Lane 8*). Ad-Luc infection also reduces the TNF-α-induced NF-κB DNA binding activity somewhat, but to a lesser extent than Ad-p202 (*Lanes 4* and *5*). Together, our data suggest that Ad-p202 infection could sensitize otherwise resistant MDA-MB-468 cells to apoptosis induced by TNF-α, which correlates with a loss of TNF-α-induced NF-κB DNA binding activity.

Antitumor Activity of Ad-p202 in Cancer Xenograft Models. To test the efficacy of Ad-p202 treatment in an orthotopic breast cancer xenograft model, we implanted MDA-MB-468 cells (2×10^6 cells) into mammary fat pads of female nude mice. Treatment began when tumor size reached ~0.5 cm in diameter (about 2 weeks after implantation). We then treated tumor-bearing mice (7 tumors/treatment group) with either Ad-p202 or control virus Ad-Luc (1×10^9 pfu/treatment) via i.t. injection. Treatments were administered twice per week for 7 weeks and once a week thereafter. Tumor size was measured by using the following formula: tumor size = $1/2 \times L \times S^2$, where L and S are the longest and shortest diameters measured, respectively. The tumor size distribution with Ad-p202 or Ad-Luc treatment at two time points (day 25 and day 67) is presented. Whereas there was little difference at the early stage of treatment (Fig. 5*A*, day 25; $P = 0.13$), the Ad-p202-treated tumors grew significantly slower than those treated with Ad-Luc on day 67 ($P = 0.04$). This result supports the idea of a p202-based gene therapy in breast cancer treatment. Because breast cancer is a metastatic disease, it is critical to develop a systemic delivery system for p202 gene transfer. Although the antitumor effect by i.t. treatment is encouraging, no report has shown a therapeutic effect by systemic administration of p202 in a cancer xenograft model. We then performed systemic gene therapy

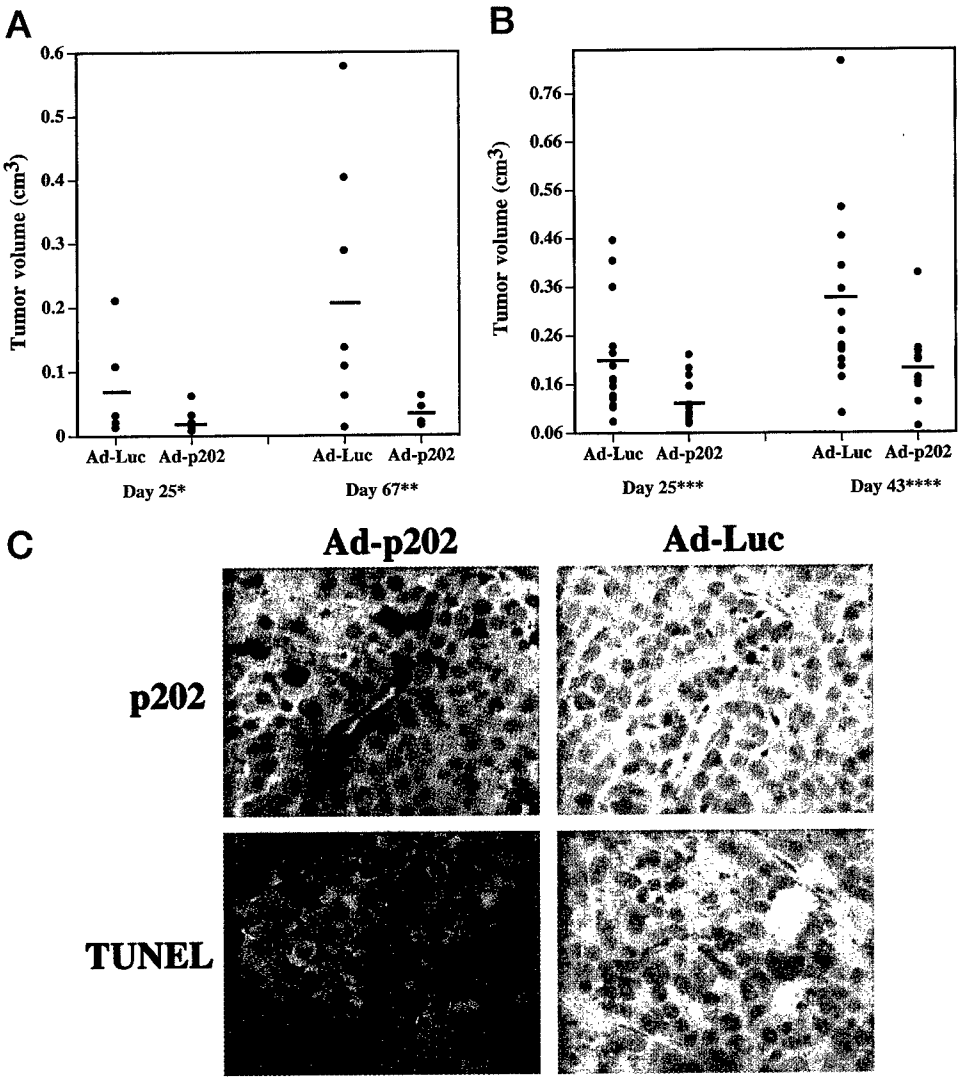

Fig. 5 Antitumor effect by systemic delivery of Ad-p202 in an orthotopic breast cancer xenograft model. *A,* Ad-p202-mediated antitumor effect on breast cancer xenografts by i.t. treatment. MDA-MB-468 cells (2×10^6 cells) were implanted in mammary fat pads of each female nude mouse. Tumor-bearing mice were divided into two treatment groups, Ad-Luc (total, 7 tumors) and Ad-p202 (total, 7 tumors), at 1×10^9 pfu/treatment via i.t. injection. Treatment started when tumor reached 0.3 cm in diameter with a treatment schedule of twice a week for 7 weeks and once a week thereafter. Tumor size in each treatment group was presented at the indicated time, *i.e.*, day 25 and day 67. *t* test: *, $P = 0.13$; and **, $P = 0.04$. *B,* Ad-p202-mediated antitumor effect on breast cancer xenografts by systemic treatment. MDA-MB-468 cells (2×10^6 cells) were implanted in mammary fat pads (2 tumors/mouse) of female nude mice. Tumor-bearing mice were divided into two treatment groups, Ad-Luc (total, 14 tumors) and Ad-p202 (total, 14 tumors), at 5×10^8 pfu via tail vein injection. Treatment started when tumor reached 0.5 cm in diameter with a treatment schedule of twice a week for 5 weeks and once a week thereafter. Tumor size in each treatment group was presented at the indicated time, *i.e.*, day 25 and day 43. *t* test: ***, $P = 0.0097$; and ****, $P = 0.014$. *C,* apoptosis correlates with p202 expression in Ad-p202-treated breast tumors. Mice were sacrificed 24 h after the last systemic treatment as described above. Tumors were then excised and fixed for the subsequent immunohistochemical analysis. p202 expression was analyzed by using an antibody specific for p202 on tumor samples obtained from Ad-p202- or Ad-Luc-treated mice (14). The TUNEL assay was also performed to detect apoptotic cells in these tumors (15). The *arrows* indicate the representatives of apoptotic cells.

experiments by treating tumor-bearing mice with Ad-p202 or Ad-Luc (5×10^8 pfu/treatment, 14 tumors/group) through tail vein injection. Treatments were administered twice per week for 5 weeks and once a week thereafter. As shown in Fig. 5*B*, Ad-p202-treated mice had a significantly reduced tumor growth rate as compared with the Ad-Luc-treated mice on day 25 ($P = 0.0097$) and day 43 ($P = 0.014$). The above-mentioned observation strongly suggests the feasibility of using a systemic p202-based gene therapy treatment for breast cancer. Because Ad-p202 induces apoptosis *in vitro* (Fig. 3), it is likely that the observed antitumor activity may correlate with enhanced apoptosis in Ad-p202-treated tumors. To test this possibility, we examined the presence of apoptosis in breast tumors treated systemically with Ad-p202. Immunostaining for p202 protein and apoptotic cells were performed 24 h after the last Ad-p202 and Ad-Luc treatment. As shown in Fig. 5*C*, p202 expression was readily detected by immu-

nohistochemical staining in Ad-p202-treated tumors but not in tumors treated with Ad-Luc. Interestingly, strong p202 expression was found in endothelial cells of a tumor blood vessel. It may be due to systemic delivery of Ad-p202. As predicted, apoptosis, as determined by TUNEL assay, is prevalent in Ad-p202-treated tumors but not in Ad-Luc-treated tumors (Fig. 5*C*). The *arrows* indicate the representatives of apoptotic cells. This observation is consistent with our *in vitro* data showing that p202 expression induces apoptosis (Fig. 3). We have also performed a similar Ad-p202 preclinical gene therapy treatment in a human pancreatic cancer xenograft model (4). Consistent with the data presented here, Ad-p202 treatment (by i.t. injection) inhibited tumor growth and induced apoptosis in tumors (data not shown). Taken together, the above-mentioned observations strongly indicate that p202 is a potent tumor-suppressing agent, and its apoptosis-inducing activity contributes to the multiple p202-mediated antitumor activities.

DISCUSSION

In this report we showed that, consistent with our previous findings using p202 stable breast cancer cell lines (5), Ad-p202 infection in MDA-MB-468 breast cancer cells resulted in growth inhibition and sensitization to TNF-α-induced apoptosis. Importantly, we demonstrated for the first time that Ad-p202 infection alone induces apoptosis *in vitro*. The correlation between p202 expression and enhanced apoptosis observed in Ad-p202-treated tumors also supports the *in vitro* observation. However, it is possible that the apoptosis could be the result of an artifact caused by coexpression of p202 and adenoviral proteins. We ruled out that possibility because Ad-Luc infection of a p202 stable cell line, 453-p202 (5), did not result in enhanced apoptosis as compared with that of the vector control cells infected by Ad-Luc (data not shown). This result thus strongly suggests that the Ad-p202-induced apoptosis is not likely due to cooperation between p202 and certain adenoviral proteins during infection. Rather, it indicates that a certain cellular apoptotic pathway was activated by p202 expression. Indeed, as shown in Fig. 4, Ad-p202-induced apoptosis requires caspase-3 activation to achieve a full apoptotic effect.

Here, we demonstrated the feasibility of using Ad-p202 in preclinical gene therapy settings. In particular, Ad-p202 treatment by i.t. or i.v. injection resulted in significant tumor suppression in an orthotopic breast cancer xenograft model. Our data are consistent with that reported previously using p202 delivery systems other than adenoviral vector, *i.e.*, polymer and liposome (4, 5). The efficacy of systemic Ad-p202 treatment is encouraging because it shows that Ad-p202 had overcome immunological (nude mice possess immune response, albeit much reduced), physiological, and structural barriers inside and outside the blood vessels to reach tumor cells and unloads the p202 therapeutic gene (20). This result is the first demonstration of efficacy by systemic treatment of p202. It is possible that the systemic Ad-p202 treatment may affect normal tissues the same way it affects tumor tissues. One way to minimize the potential cytotoxicity of the p202 effect on normal tissues is to develop a tumor-specific p202 expression system using a breast cancer-specific promoter to direct p202 expression. This effort is currently in progress. Although toxicity, if any, associated with Ad-p202 treatment remains to be determined, our results nevertheless raise the possibility of using p202-based gene therapy in systemic cancer treatment. In Ad-p202-treated tumors, we also found a reduced level of an angiogenic marker, vascular endothelial growth factor (data not shown). This observation is consistent with the ability of p202 to inhibit angiogenesis (4).

In addition to prostate (data not shown) and breast cancer xenograft models (this study and Ref. 5), the fact that Ad-p202 treatment resulted in an antitumor effect on a pancreatic cancer xenograft model (data not shown; Ref. 4) suggests a general application of p202-based gene therapy in cancer treatment. In addition, because p202 sensitizes cells to TNF-α-induced apoptosis (this study and Ref. 5), our data further support the possible use of Ad-p202/TNF-α combined therapy to achieve better efficacy, especially for cancer cells that are resistant to TNF-α therapy. Experiments are under way to test this possibility in animal models. Taken together, the data we present here strongly suggest that Ad-p202 is a potent therapeutic agent suitable for further development in cancer gene therapy.

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