Contribution of CD95 Ligand-Induced Neutrophil Infiltration to the Bystander Effect in p53 Gene Therapy for Human Cancer[1]

Toshihiko Waku,* Toshiyoshi Fujiwara,[2]* Jianghua Shao,* Takahiro Itoshima,† Takayoshi Murakami,* Masafumi Kataoka,* Shinya Gomi,* Jack A. Roth,‡ and Noriaki Tanaka*

Clinical trials of adenoviral p53 gene therapy provide the evidence that the bystander effect induced by the wild-type p53 gene transfer on adjacent tumor cells contributes to tumor progression; its mechanism, however, remains uncharacterized. We report in this work that injection of adenovirus expressing the human wild-type p53 gene (Ad5CMVp53) into established human colorectal tumors in *nu/nu* mice resulted in CD95 ligand (CD95L) overexpression, followed by a massive neutrophil infiltration. Culture supernatants of human colorectal cancer cells infected with Ad5CMVp53 exhibited a potent chemotactic activity against murine polymorphonuclear neutrophils, which could be abolished by the anti-CD95L mAb (NOK-1). In vivo cell depletion experiments indicated that neutrophils were in part responsible for the antitumor effect of the Ad5CMVp53 infection. Our data directly suggest that overexpression of CD95L by the wild-type p53 gene transfer induces neutrophil infiltration into human colorectal tumors, which may play a critical role in the bystander effect of p53 gene therapy. ***The Journal of Immunology*****, 2000, 165: 5884–5890.**

Replication-defective adenovirus-mediated p53 gene therapy for nonsmall cell lung cancer is being studied currently in clinical trials (1, 2). Although the p53 tumor suppressor gene is a key molecule responsible for apoptotic cell death, the recombinant adenovirus is not efficient enough to accomplish complete transduction of all tumor cells in vivo. The incomplete transduction efficiency may be a significant limitation of the currently available delivery system; local tumor regression, however, was observed in some patients with nonsmall cell lung cancer who had not responded to conventional therapies. The magnitude of the therapeutic response exceeded the effect expected from the transduction efficiency of viral vectors, which suggests that the wild-type p53 (wt-p53)[3] gene transfer not only induces the direct effect in the individual transduced cells, but also causes the growth suppression of bystander nontransduced cells via other mechanisms. p53 gene therapy might not need to completely transduce the tumor cells if the bystander effect contributes to tumor progression. The elucidation of the molecular mechanism of the bystander effect is thus essential.

CD95 ligand (CD95L, also called FasL/APO-1L) is a type II integral membrane protein of the TNF family that transduces an apoptotic death signal by binding to its receptor CD95, which is a type I transmembrane protein of the TNFR family. The CD95-CD95L system has been implicated in the activated T cell- or NK cell-mediated cytotoxicity, some pathologic tissue damage, and the regulation of lymphocyte homeostasis (3). CD95L is expressed in a wide range of normal tissues, such as the spleen, testis, uterus, large and small intestine, and eye as well as some malignant tumor cells, including human melanoma (4), hepatocellular carcinoma (5), colon cancer (6), and lung cancer (7), which has been proposed to contribute to their immune-privileged status by the elimination of infiltrating immune cells (8, 9). However, more recent studies have demonstrated another property of CD95L in different experimental models. Expression of CD95L on myotubes, pancreatic islet cells, or heart grafts induces neutrophilic inflammation, resulting in accelerated graft rejection in a T cell- or B cell-independent manner (10–14). Furthermore, locally produced CD95L by transfection with CD95L cDNA causes neutrophil-mediated rejection of tumor cells when s.c. injected (15). Membrane-bound CD95L can be proteolytically cleaved by metalloproteinases, thereby producing a soluble and active form of CD95L (16). It has been also reported that human soluble rCD95L is a potent chemoattractant for human and mouse polymorphonuclear neutrophils (PMNs) (17). These observations suggest that CD95L can act directly on neutrophils to induce their recruitment, indicating that CD95L may play a proinflammatory role in a specific microenvironment.

Numerous studies have shown that p53 mediates its effects by modulating the transcription of a number of cellular target genes. We previously reported the p53-mediated up-regulation of CD95 as well as CD95L in human cancer cells, although little is known about the molecular machinery underlying these processes (18). These data raised the hypothesis that increased CD95L expression

*Section of Molecular Oncology, First Department of Surgery, Okayama University Medical School, Okayama, Japan; †First Department of Surgery, Shiga University of Medical Science, Shiga, Japan; and ‡Section of Thoracic Molecular Oncology, Department of Thoracic and Cardiovascular Surgery, The University of Texas M. D. Anderson Cancer Center, Houston, TX 77030

Received for publication May 25, 2000. Accepted for publication August 21, 2000.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported in part by grants from the Ministry of Education, Science, and Culture, Japan; and by grants from the Ministry of Health and Welfare, Japan (Health Sciences Research Grants (Research on Human Genome and Gene Therapy)).

[2] Address correspondence and reprint requests to Dr. Toshiyoshi Fujiwara, First Department of Surgery, Okayama University Medical School, 2-5-1 Shikata-cho, Okayama 700-8558, Japan. E-mail address: toshi_f@med.okayama-u.ac.jp

[3] Abbreviations used in this paper: wt-p53, wild-type p53; CD95L, CD95 ligand; PMN, polymorphonuclear neutrophil.

0022-1767/00/$02.00

by p53 might induce neutrophilic infiltration, which could accelerate the destruction of neighboring tumor cells that were not transduced with wt-p53. In the present study, we show that significant CD95L accumulation and neutrophil infiltration take place in established human colorectal tumors after intratumoral injection of the adenovirus expressing the wt-p53 gene. Thus, the p53-mediated up-regulation of CD95L is likely to provide further insights for the molecular basis of the bystander effect in p53 gene therapy.

Materials and Methods

Cell culture

The human colorectal carcinoma cell lines LoVo that contain the wt-p53 and SW620 that exhibit a homogygous p53 gene mutation were maintained in monolayer cultures in 75-cm^2 tissue culture flasks. These cell lines were routinely propagated in RPMI 1640 medium supplemented with 10% FCS, 25 mM HEPES, 100 U/ml penicillin, and 100 mg/ml streptomycin. The transformed embryonic kidney cell line 293 was grown in DMEM (Life Technologies, Grand Island, NY) with high glucose (4.5 g/L) supplemented with 10% FCS, 100 U/ml penicillin, and 100 mg/ml streptomycin.

Recombinant adenoviruses

The recombinant adenovirus vector expressing human wt-p53 cDNA was previously constructed and characterized (19). The resultant virus was named Ad5CMVp53. The E1A-deleted adenovirus vector lacking a cDNA insert (dl312) was used as a control vector. The viral stocks were quantified by a plaque-forming assay using 293 cells and stored at −80°C.

Animal experiments

Animal experiments were conducted in accordance with the institutional animal care and use regulations. Four-week-old female BALB/c *nu/nu* mice were purchased from Shizuoka Laboratory Animal Center (Shizuoka, Japan). LoVo and SW620 suspensions (5×10^6 cells/100 μl) were s.c. inoculated into the dorsi of BALB/c *nu/nu* mice. When palpable nodules were established, tumors were injected daily with PBS (100 μl), dl312 (1×10^9 PFU/100 μl), and Ad5CMVp53 (1×10^9 PFU/100 μl) for 3 consecutive days. The tumor growth was assessed by measuring perpendicular diameters with calipers. Tumor volume was calculated from the largest (a) and smallest (b) diameter with the formula $0.5a \times b^2$. For depleting neutrophils, mice were administered 200 μg of anti-Gr-1 mAb (RB6-8C5; PharMingen, San Diego, CA) i.p. 1 day before the first injection of Ad5CMVp53 and on days 2 and 5 after the first injection. Control mice received i.p. administration of isotype-matched rat IgG2b (PharMingen).

Histological examinations

For immunohistochemistry, tumor tissues were fixed in 20% neutral buffered Formalin. Paraffin-embedded sections (4 μm thick) were deparaffinized in xylene and rehydrated through graded alcohols into PBS. Heat-induced epitope retrieval was achieved by immersion of slides in 10 mM citrate buffer (pH 6) and heating for 20 min in the autoclave. Endogenous peroxidase was blocked by 10-min incubation with 3% hydrogen peroxide in methanol. To prevent nonspecific binding, the sections were incubated in 0.25% casein (Dako, Carpenteria, CA) for 5 min at room temperature. Either mouse anti-human CD95L (Transduction Laboratories, Lexington, KY) or mouse anti-human p53 (DO-7; PharMingen) and goat anti-mouse Ig conjugated to peroxidase-labeled dextran polymer (EnVision$^+$; Dako) were mixed together and incubated for 45 min at room temperature to form Ab complexes. Then, inactivated normal mouse serum (Dako) was added to the mixture and incubated for 45 min at room temperature. Sections were then incubated with the mixture for 60 min at room temperature, treated with diaminobenzidine solution, and counterstained with hematoxylin solution. Tumor tissues were also processed for the granulocyte-specific staining that employs naphthol AS-D-chloroacetate as a substrate for esterase. Granulocytes appear to be red with this staining.

Isolation of PMNs

Thioglycolate-elicited PMNs were harvested from peritoneal exudates 4 h after an i.p. injection of 3 ml 3% thioglycolate (Difco, Detroit, MI). The purity of PMN was >85%, as assessed by Diff-Quick staining (International Reagents, Kobe, Japan) of cytospin preparations under light microscopy. PMNs were suspended in PBS containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$.

Chemotaxis assay

PMN chemotaxis was quantified using a modification of the Boyden chamber technique (20). A cell suspension containing 4×10^6 cells/ml (the total cell number loaded per well was adjusted to give equal numbers of PMNs) in PBS supplemented with 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 1 mg/ml BSA (Fraction V; Sigma, St. Louis, MO) was placed in the top wells of a 48-well microchemotaxis chamber (Neuro Probe, Bethesda, MD). A 3-μm pore-size polyvinylpyrolidone-free polycarbonate filter (Neuro Probe) separated the cells from lower wells containing culture supernatants. Recombinant human IL-8 (Genzyme, Cambridge, MA) and human CD95L (Transduction Laboratories) were used as a positive control. After incubation for 60 min at 37°C in a 5% CO_2 humidified atmosphere, the filter was removed, gently scraped off the upper face, fixed in 100% methanol, and subsequently stained with Diff-Quick stain solution. Chemotactic activity was estimated by counting the total number of PMNs migrating to the lower face of the filter in five random high power fields (×400) per well. The results were expressed as the mean number of PMNs per high power field. Each experiment was performed at least three times. For the blocking experiment, mouse anti-human CD95L (NOK-1) or control isotype-matched mouse IgG1 (PharMingen) at a concentration of 5 μg/ml was added to both upper and lower chambers.

Statistical analysis

The statistical significance of differences from control was evaluated by Student's t test. Values of $p < 0.05$ were considered significant.

Results

Effect of intratumoral injection of adenovirus-expressing wt-p53 gene

A recombinant, replication-deficient adenovirus vector carrying human wt-p53 cDNA under control of the CMV immediate early gene promoter (Ad5CMVp53) was employed to achieve efficient gene transfer into human cancer cells. When 5×10^6 SW620 human colorectal cancer cells, which are homozygous for a mutation in p53, were s.c. inoculated into *nu/nu* mice, palpable tumors appeared in 100% of mice 2 wk after tumor injection. Twenty-one days later, SW620 tumors with a diameter of 5–7 mm were treated

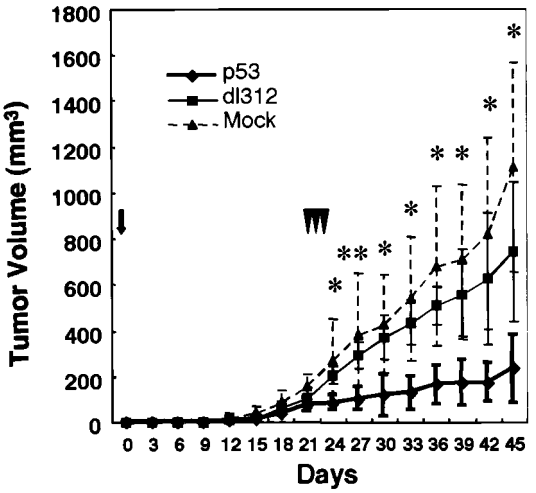

FIGURE 1. Effect of intratumoral injection of Ad5CMVp53 on the growth of SW620 human colorectal tumors. SW620 human colon cancer cells (2×10^6 cells/mouse) were s.c. inoculated into athymic BALB/c mice. On days 21, 22, and 23, mice were intratumorally injected with PBS or adenovirus vectors (1×10^9 PFU/100 μl (dl312 or Ad5CMVp53)). Two perpendicular diameters were measured and tumor volume was calculated by assuming a spherical shape with the average tumor diameter, as described in *Materials and Methods*. Data are shown as mean ± SD values of four mice in each group. There were statistically significant differences between mice treated with vehicle and Ad5CMVp53, or mice treated with dl312 and Ad5CMVp53. *, $p < 0.05$; **, $p < 0.01$. Arrow, tumor inoculation; arrowheads, intratumoral injection.

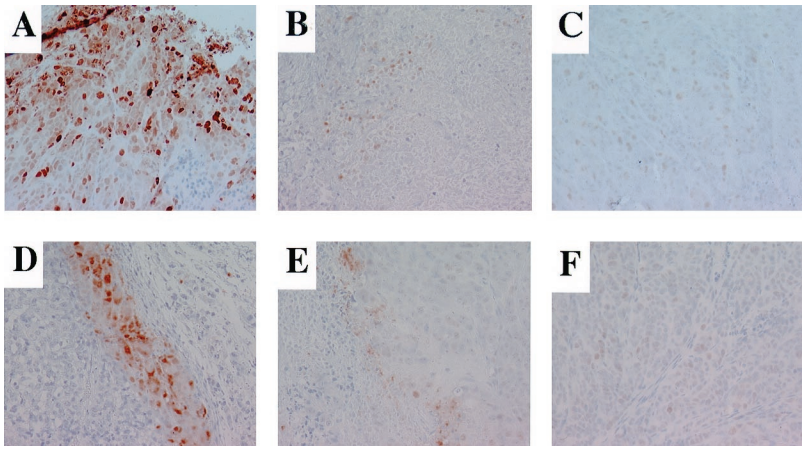

FIGURE 2. Detection of transduced p53 gene expression in LoVo (*A–C*) and SW620 (*D–F*) human colorectal tumors. BALB/c *nu/nu* mice were s.c. inoculated with 5×10^6 LoVo or SW620 cells. When the tumor diameter was between 5 and 7 mm, intratumoral injection with dl312 or Ad5CMVp53 (1×10^9 PFU/100 μl) was done daily for 3 consecutive days. Tumors were dissected, and paraffin sections were stained with anti-p53 Ab. Transduced gene expression, as evidenced by intense nuclear staining, was present in Ad5CMVp53-injected tumors 24 h after the first viral injection (*A*, LoVo; *D*, SW620), although p53-immunoreactive cells disappeared on day 7 (*B*, LoVo; *E*, SW620). No p53-positive cells were detected in dl312-injected tumors even 24 h after the first injection (*C*, LoVo; *F*, SW620). Original magnification, ×200.

daily with direct intratumoral injection of the vehicle or 1×10^9 PFUs of Ad5CMVp53 or control dl312 vector for 3 consecutive days. Ad5CMVp53-injected mice showed a significant tumor growth inhibition that started on the third day after the first injection and continued for at least 40 days, at which time approximately a 68% inhibition was seen relative to the mock-treated tumors (Fig. 1). There were no significant differences in tumor growth among the mock-treated and dl312-treated groups. We also observed the growth-inhibitory effect of Ad5CMVp53 in identical experiments using LoVo human colorectal cancer cells that contain wt-p53 (data not shown).

Assessment of in vivo gene transfer in human colorectal tumors

To determine the efficacy of transducing human colorectal cancer cells in solid tumors, we assessed the presence of p53 immunoreactivity in LoVo and SW620 tumors 24 h after the first viral injection. Immunohistochemical evaluation revealed a detectable, widespread wt-p53 protein, which was identified by intense nuclear staining, in Ad5CMVp53-injected LoVo and SW620 tumors (Fig. 2, *A* and *D*), whereas dl312-injected tumors showed no overexpression of p53 protein (Fig. 2, *C* and *F*). The geographic distribution pattern of p53-positive cells with peripheral areas of intense staining and a central portion of cellular infiltrates was noted in tumors treated with Ad5CMVp53. Seven days after the first viral injection, there was no p53 immunoreactivity in both tumors (Fig. 2, *B* and *E*). Furthermore, we detected p53 overexpression only in tumor cells, but not in infiltrating cells, suggeting that the cellular infiltrates might be a secondary event following p53 gene transduction.

Histopathological analysis of LoVo and SW620 tumors injected with Ad5CMVp53

To investigate the mechanisms for the Ad5CMVp53-mediated antitumor effect, we histopathologically analyzed LoVo and SW620 tumors injected with Ad5CMVp53 for the distribution of inflammatory and/or immune cells as well as CD95L immunoreactivity. Histological analysis at 24 h after the first injection of Ad5CMVp53 revealed massive tumor cell death and cellular infiltrates at the central portions of the tumors in which Ad5CMVp53 was injected (Fig. 3, *A*, *B*, *E*, and *F*). In contrast, tumors treated with dl312 showed neither tumor cell death nor cellular infiltrates (Fig. 3, *C*, *D*, *G*, and *H*). Intratumoral injection of physiological saline as a control also had no significant effect (data not shown). These results suggest that destruction of tumor cells was associated with a massive accumulation of inflammatory cells, and that the presence of inflammatory infiltrates might be due to transduced p53 expression rather than viral vector injection.

Immunohistochemical analysis for CD95L showed that the majority of CD95L was expressed on tumor cells in the central areas of tumors in which massive quantities of cellular infiltrates were present within 24 h after the first Ad5CMVp53 injection, although there were viable tumor cells in the peripheral areas with rare staining for CD95L (Fig. 4, *A* and *D*). At higher magnification, CD95L was shown to be expressed on the cytoplasm of tumor

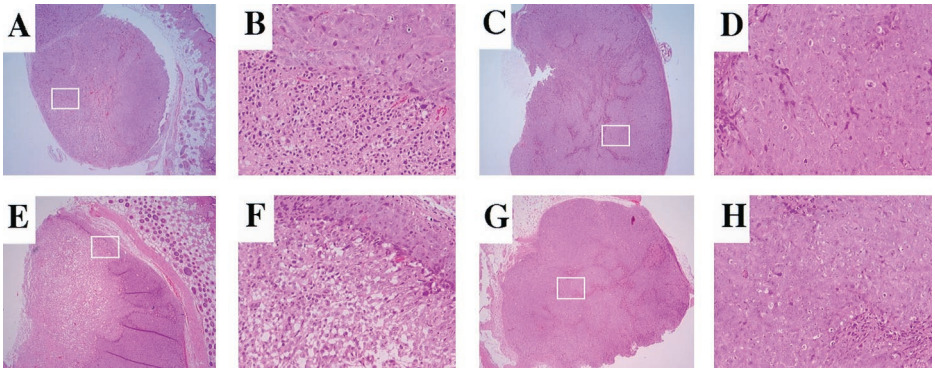

FIGURE 3. Histological examination of LoVo (*A–D*) and SW620 (*E–H*) human colorectal tumors s.c. implanted into BALB/c *nu/nu* mice. Mice were treated with either dl312 or Ad5CMVp53, as described in the legend for Fig. 2. Tumors were dissected 24 h after the first viral injection, and paraffin sections were stained with hematoxylin and eosin. The *right column* shows higher magnification of the areas boxed in the *left column*. Massive inflammatory infiltration was demonstrated at a central portion in Ad5CMVp53-injected tumors (*A* and *B*, LoVo; *E* and *F*, SW620), whereas few cellular infiltrates were present in tumors treated with dl312 (*C* and *D*, LoVo; *G* and *H*, SW620). Original magnification, *A*, *C*, *E*, and *G*, ×25; *B*, *D*, *F*, and *H*, ×200.

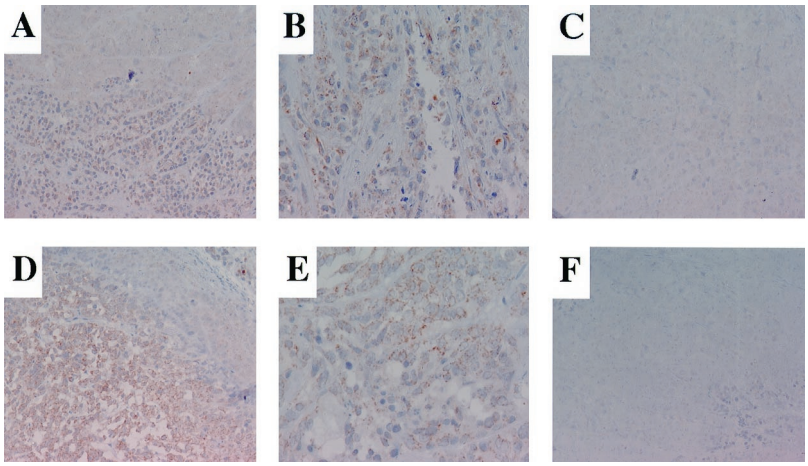

FIGURE 4. Immunohistochemical analysis for CD95L in LoVo (*A–C*) and SW620 (*D–F*) tumors. Paraffin tumor sections obtained 24 h after the first viral injection were stained with Ab against CD95L. CD95L immunoreactivity was detected in tumor cells in the central areas of Ad5CMVp53-injected tumors that were occupied by massive quantities of cellular infiltrates (*A*, LoVo; *D*, SW620; ×200). High power magnification shows intense staining in the cytoplasm of tumor cells (*B*, LoVo; *E*, SW620; ×400). In contrast, no intense cytoplasmic staining was observed in tumors injected with the control dl312 vector (*C*, LoVo; *F*, SW620; ×200).

cells, while infiltrating cells completely lacked staining (Fig. 4, *B* and *E*). Tumors in mice treated with dl312 vector had no CD95L-overexpressing cells (Fig. 4, *C* and *F*). To characterize the infiltrating cells, we next performed esterase staining, which can specifically detect neutrophils, on the section of SW620 tumors injected with Ad5CMVp53. As shown in Fig. 5*B*, PMNs were identified morphologically as well as by esterase staining. These results indicate that p53-mediated CD95L expression may act on neutrophils, inducing their recruitment into solid tumors and thereby causing in part antitumor effect of the wt-p53 gene transfer.

CD95L is responsible for p53-mediated chemotactic activity

To determine whether CD95L could be attributed to Ad5CMVp53-induced mouse neutrophil migration, cell migration was evaluated by a modified Boyden chamber assay with a nitrocellulose filter. Culture supernatants of LoVo and SW620 cells infected with Ad5CMVp53 exhibited the chemotactic activity against thioglycolate-elicited PMNs obtained from BALB/c *nu/nu* mice in a dose-dependent manner. Supernatants of dl312-infected cells were also chemotactic, presumably because of some factors produced by viral infection; the magnitude of PMN migration induced by AdCMVp53-infected cells, however, was significantly greater than that generated by dl312-infected cells as well as by the classic chemoattractant IL-8 (Fig. 6*A*).

To directly confirm the role of CD95L on p53-mediated PMN migration, anti-CD95L mAb (NOK-1) or isotype-matched control mAb (IgG1) was added to both upper and lower chambers in the cell migration assay. The antagonistic NOK-1 Ab, but not a control IgG1, significantly inhibited the Ad5CMVp53 ability of inducing neutrophil migration (Fig. 6*B*). These results suggest that the wt-p53-induced CD95L is indeed capable of stimulating neutrophil migration.

Contribution of p53-induced neutrophil accumulation to the antitumor effect

To further define the antitumor effect of PMNs accumulated by Ad5CMVp53, in vivo neutrophil depletion experiments were performed by using anti-Gr-1 mAb (RB6-8C5) or isotype-matched control mAb (IgG2b). The growth suppression of SW620 tumors injected with Ad5CMVp53 was partially but significantly reduced in RB6-8C5-treated mice compared with that in mock-treated or IgG2b-treated mice (Fig. 7). Pretreatment with IgG2b had no effect on the antitumor effect of Ad5CMVp53. These results suggest that the wt-p53 gene transfer could not only induce the direct effect in the individual transduced cell, but could also cause the growth suppression of bystander, nontransduced cells via neutrophil accumulation.

Discussion

A large body of evidence supports the possibility that the bystander effect in p53 gene therapy may have contributed to the tumor regression observed in clinical trials. Exploration of the mechanisms of the bystander effect is not only biologically important but also relevant in terms of therapeutic implications. In this study, we found that p53-induced CD95L expression is endowed with chemotactic properties toward murine neutrophils, which can partially mediate the antitumor activity of the wt-p53 gene transfer. Although it has been reported that neutrophils expressing CD95 underwent apoptosis in response to CD95L (21), our results suggest that CD95L expression induced by intratumoral injection of Ad5CMVp53 promotes apoptosis in bystander, nontransduced tumor cells through neutrophil accumulation rather than inducing apoptosis in infiltrating cells to lead to immune suppression.

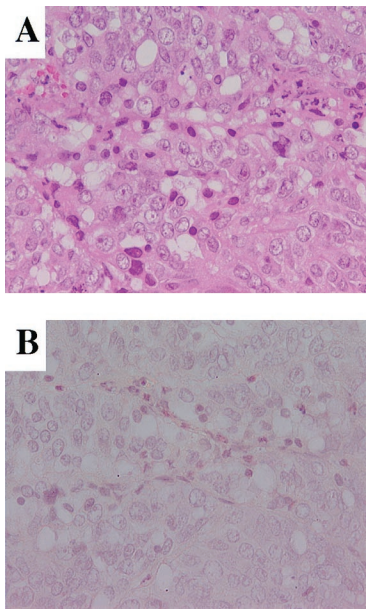

FIGURE 5. Neutrophil-specific esterase staining of SW620 tumors treated with intratumoral Ad5CMVp53 administration. Paraffin tumor sections obtained 24 h after the first viral injection underwent either hematoxylin/eosin (*A*) or esterase staining (*B*). The cellular infiltrates observed in *A* were identified as neutrophils by esterase staining in *B*. Magnification, ×400.

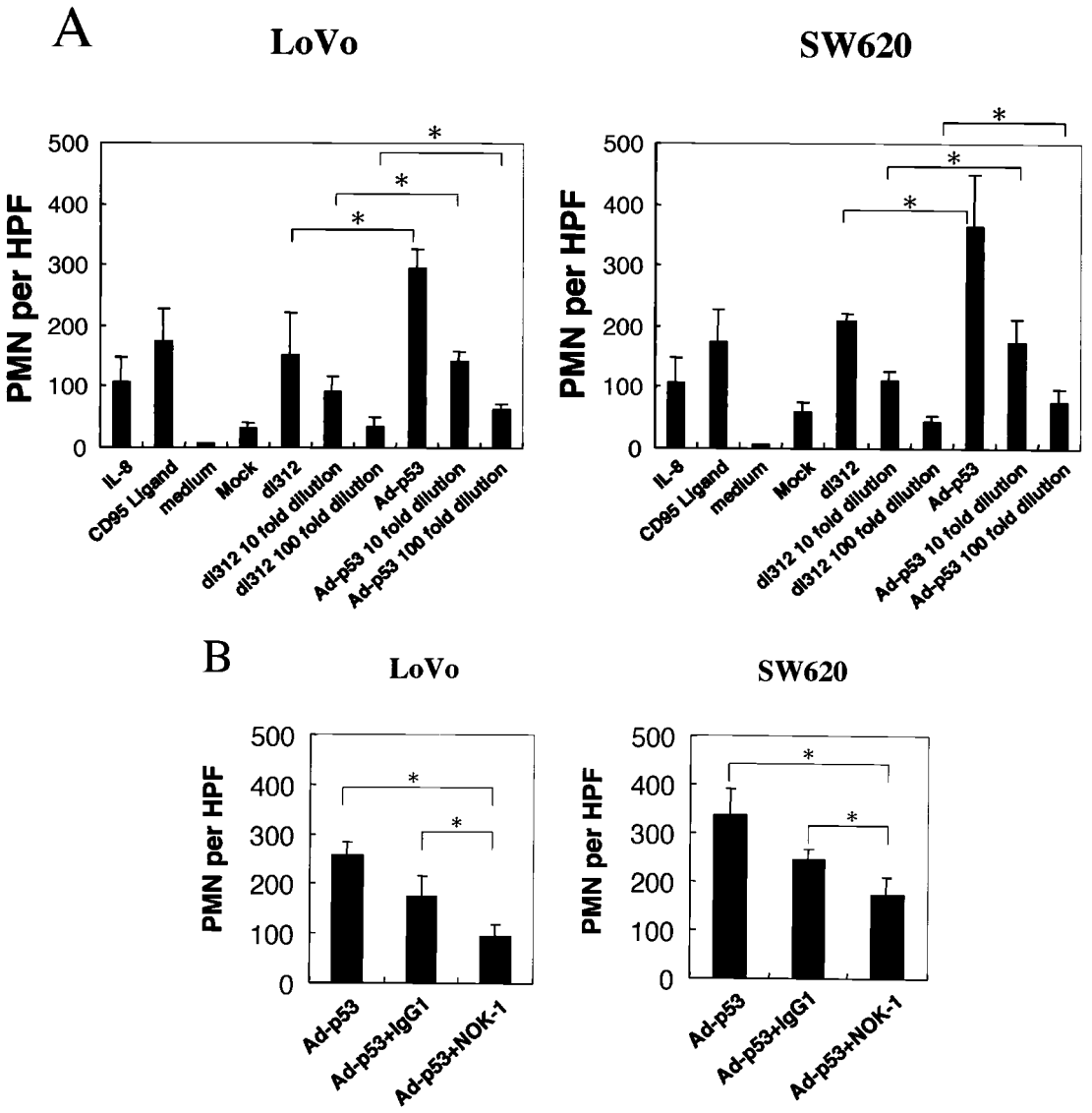

FIGURE 6. *A*, Effect of culture supernatants of tumor cells infected with Ad5CMVp53 on murine PMN migration. Monolayer cultures of LoVo and SW620 cells were infected with mock, dl312 (50 multiplicity of infection (MOI)), or Ad5CMVp53 (50 MOI), and incubated for an additional 24 h. Supernatants and their dilutions were assessed for chemotactic activity against murine PMNs by a modified Boyden chamber assay. Recombinant human IL-8 (20 nM) and human CD95L (1 nM) were used as a positive control. Medium alone was used as a negative control. Data are shown as mean ± SD of three independent experiments. *, $p < 0.01$. *B*, Effect of anti-CD95L mAb (NOK-1) on murine PMN migration induced by supernatants of Ad5CMVp53-infected tumor cells. Control isotype-matched mouse IgG1 or mouse anti-human CD95L mAb (NOK-1) at a concentration of 5 μg/ml was added to both upper and lower chambers. Migration of PMNs was significantly inhibited in the presence of anti-CD95L mAb. Data are expressed as mean ± SD of three independent experiments. *, $p < 0.01$. HPF, high power field.

Intratumoral injection of Ad5CMVp53 led to significant suppression of the growth of human colorectal tumors s.c. transplanted in *nu/nu* mice (Fig. 1). Although histologic examination demonstrated that rapid apoptotic tumor cell death occurred after the wt-p53 transduction (data not shown), as we previously reported in a variety of tumor models (18, 22–25), our findings that notable cellular infiltrates were evident at the site of Ad5CMVp53 injection in both LoVo and SW620 tumors (Fig. 3) suggest that these infiltrating cells might be involved as effector cells in the antitumor effect. The absence of cellular infiltrates in the peripheral portion of the tumor, in which most of the cells were still viable, also supports this hypothesis; these infiltrates, however, were unlikely to be cytotoxic T lymphocytes, as the antitumor effect was observed in T cell-deficient *nu/nu* mice. Consistently, specific esterase staining indicated that most of inflammatory infiltrates are murine neutrophils (Fig. 5). Immunohistochemical analysis showed that CD95L was detected on apototic tumor cells in the area occupied by murine PMNs (Fig. 4). These observations suggest that Ad5CMVp53-induced CD95L expression resulted in the accelerated infiltration of neutrophils, thereby leading to the massive destruction of tumor tissues. This scenario could be supported by recent studies showing that soluble CD95L is chemotactic for murine and human PMNs (17) and showing that tumors expressing CD95L are made to regress by accerelated PMN infiltration (15, 26, 27).

Host immune interactions with the adenovirus have been known; the presence of the inflammatory infiltrates, however, is not due exclusively to the administration of adenovirus vectors, because dl312-injected tumors showed neither CD95L overexpression nor PMN infiltration. Other possibilities, such as inflammatory responses induced by dead tumor cells, seem unlikely in view of the fact that a massive cellular infiltration could be observed as

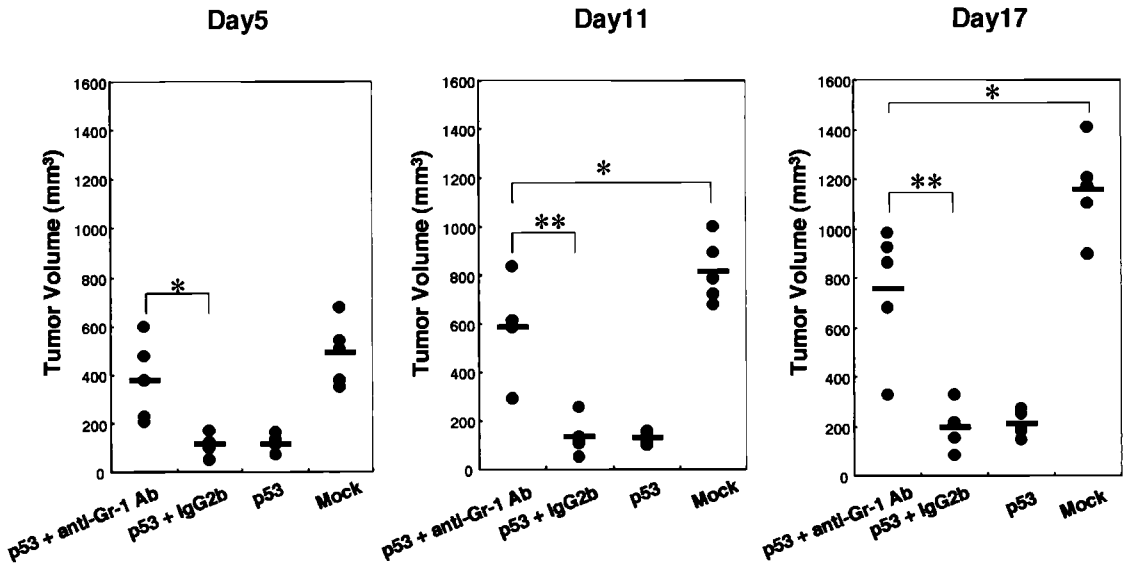

FIGURE 7. Contribution of neutrophil accumulation to Ad5CMVp53-mediated antitumor effect. SW620 tumors were treated daily with intratumoral injections of Ad5CMVp53 or PBS for 3 consecutive days, as described in the legend for Fig. 1. Mice were administered 200 μg of anti-Gr-1 mAb (RB6-8C5) i.p. to deplete neutrophils 1 day before the first injection of Ad5CMVp53 and on days 2 and 5 after the first injection. Control mice received i.p. administration of isotype-matched rat IgG2b or PBS. Tumor volume was calculated at 5, 11, and 17 days after the first Ad5CMVp53 injection, as described in *Materials and Methods*. Closed circles represent tumor volumes in individual mice. The horizontal lines represent the mean. *, $p < 0.05$; **, $p < 0.01$.

early as 24 h after the first Ad5CMVp53 injection (Fig. 3). It has been reported that caspases released by apoptotic cells could process and activate IL-1β, thus inducing inflammation (28); our preliminary experiments, however, demonstrated that IL-1β could be detected neither in supernatants nor in cell lysates of tumor cells after Ad5CMV-p53 infection in vitro (data not shown). We reported that levels of CD95L mRNA can be greatly elevated, reaching their maximum at 24 h after Ad5CMVp53 infection, followed by a rapid decrease (18). Recently, it was demonstrated that soluble CD95L acts as a direct chemoattractant against murine PMNs in vitro (17). Indeed, our modified Boyden chamber assay showed that supernatants obtained from Ad5CMVp53-infected tumor cells induced murine PMN chemotaxis, which could be abolished by adding neutralizing anti-CD95L Ab (Fig. 6). Thus, although the molecular mechanisms governing soluble CD95L-dependent chemotactic activity are presently unknown, Ad5CMVp53-induced CD95L secretion exhibits potent chemotactic properties toward murine neutrophils.

The phagocytosis of apoptotic cells by macrophages has been shown to produce human proinflammatory cytokine IL-8, which is a common chemoattractant active on neutrophils (29). Furthermore, in murine systems, macrophages ingesting apoptotic cells produced macrophage-inflammatory protein-2, a murine IL-8 homologue, which was associated with transient infiltration of neutrophils (30). These observations support the additional hypothesis that other chemoattractants such as IL-8 and macrophage-inflammatory protein-2 secreted from surrounding cells including macrophages might play a role on neutrophil infiltration in vivo. Further studies will be necessary to confirm these possibilities. Interestingly, supernatants obtained from dl312-infected tumor cells display some chemoattractant activity in vitro compared with those from mock-infected tumor cells (Fig. 6*A*), while we did not observe detectable PMN migration in dl312-injected tumors in vivo (Fig. 3). Other chemotactic factors could be produced by tumor cells treated with dl312; those levels of expression, however, might be below the in vivo threshold, at which stimuli for neutrophil infiltration are generated (10).

Neutrophils are considered to be partially responsible for the antitumor effect of Ad5CMVp53, because in vivo depletion of neutrophils by anti-Gr-1 mAb partially inhibited the response (Fig. 7). It has been reported that the local inflammation elicited by CD95L-expressing tumor cells induced bystander rejection of parental tumor cells (15), suggesting that Ad5CMVp53-induced PMN migration via CD95L up-regulation may be a major mechanism of the bystander effect in p53 gene therapy. However, how neutrophils exhibit cytotoxic activity against tumor cells remains to be determined. PMNs have been reported to interact directly with CD95L-expressing tumor cells to mediate their destruction (15, 26). Furthermore, Chen et al. demonstrated that CD95L-induced neutrophil cytotoxicity was dependent on p38 mitogen-activated protein kinase function (26). In our system, whether murine neutrophils specifically lyse the wt-p53-expressing human tumor cells can hardly be examined because overexpression of the wt-p53 gene itself has a direct proapoptotic effect against tumor cells. The precise mechanism must be identified in further studies using different model systems.

Recently, Dewey et al. reported that adenovirus-mediated conditional cytotoxic gene therapy successfully inhibited the syngenic glioma growth, but the inflammatory infiltrate also induced secondary demyelination (31). Neutrophilic inflammation induced by Ad5CMVp53 injection has the potential to affect normal surrounding tissues; no apparent histopathological changes, however, were observed at the neighboring and distant organs in our murine models (data not shown). The widespread presence of immunoreactive transgene expression throughout the brain was noted in their system (31), whereas transduced p53 as well as CD95L expression were localized at the injection sites presumably because of limited distribution of Ad5CMVp53 in our experiments. These observations have important implications for the safety evaluation of intratumoral administration of Ad5CMVp53, although we cannot rule out the possible involvement of activated T cells or NK cells at the site of Ad5CMVp53 injection when immunocompetent mice are used.

Our studies provide the first evidence that overexpression of the wt-p53 gene induces transient CD95L expression with a proinflammatory function, leading to neutrophil-mediated tumor cell destruction. We previously reported that wt-p53 gene transfer can attenuate tumor cell-induced neovascularization in vivo and suppress the growth of bystander tumor cells (32, 33). Cell-to-cell transfer of phosphorylated ganciclovir via gap junctions between the herpes simplex virus-thymidine kinase-transduced tumor cells and neighboring unmodified cells is known to play an important role in the bystander effect (31); other mechanisms such as immune reaction or blood vessel destruction, however, have been proposed to explain the bystander effect in conditional cytotoxic gene therapy. Our data presented in this work thus offer a rational basis for the mechanism of the bystander effct in p53 gene therapy for cancer.

Acknowledgments

We thank Dr. Hideo Yagita for providing mouse anti-human CD95L (NOK-1) Ab; Yoshiko Takata for technical support; and Sharon S. Voss and Julie Starr for preparing the manuscript.

References

1. Swisher, S. G., J. A. Roth, J. Nemunaitis, D. D. Lawrence, B. L. Kemp, C. H. Carrasco, D. G. Connors, A. K. El-Naggar, F. Fossella, B. S. Glisson, et al. 1999. Adenovirus-mediated p53 gene transfer in advanced non-small-cell lung cancer. *J. Natl. Cancer Inst. 91:763.*
2. Nemunaitis, J., S. G. Swisher, T. Timmons, D. Connors, M. Mack, L. Doerksen, D. Weill, J. Wait, D. D. Lawrence, B. L. Kemp, et al. 2000. Adenovirus-mediated p53 gene transfer in sequence with cisplatin to tumors of patients with non-small-cell lung cancer. *J. Clin. Oncol. 18:609.*
3. Nagata, S., and P. Golstein. 1995. The Fas death factor. *Science 267:1449.*
4. Hahne, M., D. Rismolde, M. Schroter, P. Romero, M. Screier, L. E. French, P. Schneider, T. Bornand, A. Fontana, D. Lienard, et al. 1996. Melanoma cell expression of Fas (Apo-1/CD95) ligand: implications for tumor immune escape. *Science 274:1363.*
5. Strand, S., W. J. Hofmann, H. Hug, M. Muller, G. Otto, D. Strand, S. M. Mariani, W. Stremel, P. H. Krammer, and P. R. Galle. 1996. Lymphocyte apoptosis induced by CD95 (APO-1/Fas) ligand-expressing tumor cells: a mechanism of immune evasion? *Nat. Med. 2:1361.*
6. O'Connell, J., G. C. O'Sullivan, J. K. Collins, and F. Shanahan. 1996. The Fas counterattack: Fas-mediated T cell killing by colon cancer cells expressing Fas ligand. *J. Exp. Med. 184:1075.*
7. Niehans, G. A., T. Brunner, S. P. Prizelle, J. C. Liston, C. T. Salerno, D. J. Knapp, D. R. Green, and R. A. Kratzke. 1997. Human lung carcinomas express Fas ligand. *Cancer Res. 57:1007.*
8. Bellgrau, D., D. Gold, H. Selawry, J. Moore, A. Franzusoff, and R. C. Duke. 1995. A role for CD95 ligand in preventing graft rejection. *Nature 377:630.*
9. Griffith, T. S., T. Brunner, S. M. Fletcher, D. R. Green, and T. A. Ferguson. 1995. Fas ligand-induced apoptosis as a mechanism of immune privilege. *Science 270: 1189.*
10. Kang, S.-M., D. B. Schneider, Z. Lin, D. Hanahan, D. A. Dichek, P. G. Strock, and S. Baekkeskov. 1997. Fas ligand expression in islets of Langerhans does not confer immune privilege and instead targets them for rapid destruction. *Nat. Med. 3:738.*
11. Kang, S.-M., A. Hofmann, D. Le, M. L. Springer, P. G. Stock, and H. M. Blau. 1997. Immune response and myoblasts that express Fas ligand. *Science 278: 1322.*
12. Allison, J., H. M. Georgiou, A. Strasser, and D. L. Vaux. 1997. Transgenic expression of CD95 ligand on islet β cells induces a granulocytic infiltration, but does not confer immune privilege upon islet allografts. *Proc. Natl. Acad. Sci. USA 94:3943.*
13. Chervonsky, A. V., Y. Wang, F. S. Wong, I. Visintin, R. A. Flavell, C. A. Janeway, Jr., and L. A. Matis. 1997. The role of Fas in autoimmune diabetes. *Cell 89:17.*
14. Takeuchi, T., T. Ueki, H. Nishimatsu, T. Kajiwara, T. Ishida, K. Jishage, O. Ueda, H. Suzuki, B. Li, N. Moriyama, and T. Kitamura. 1999. Accelerated rejection of Fas ligand-expressing heart grafts. *J. Immunol. 162:518.*
15. Seino, K., N. Kayagaki, K. Okumura, and H. Yagita. 1997. Antitumor effect of locally produced CD95 ligand. *Nat. Med. 3:165.*
16. Kayagaki, N., A. Kawasaki, T. Ebata, H. Ohmoto, S. Ikeda, S. Inoue, K. Yoshino, K. Okumura, and H. Yagita. 1995. Metalloproteinase-mediated release of human Fas ligand. *J. Exp. Med. 182:1777.*
17. Seino, K., K. Iwabuchi, N. Kayagaki, R. Miyata, I. Nagaoka, A. Matuzawa, K. Fukao, H. Yagita, and K. Okumura. 1998. Cutting edge: chemotactic activity of soluble Fas ligand against phagocytes. *J. Immunol. 161:4484.*
18. Fukazawa, T., T. Fujiwara, Y. Morimoto, J. Shao, M. Nishizaki, Y. Kadowaki, A. Hizuta, L. B. Owen-Schaub, J. A. Roth, and N. Tanaka. 1999. Differential involvement of the CD95 (Fas/APO-1) receptor/ligand system on apoptosis induced by the wild-type p53 gene transfer in human cancer cells. *Oncogene 18: 2189.*
19. Zhang, W.-W., X. Fang, W. Mazur, B. A. French, R. N. Georges, and J. A. Roth. 1994. High-efficiency gene transfer and high-level expression of wild-type p53 in human lung cancer cells mediated by recombinant adenovirus. *Cancer Gene Ther. 1:1.*
20. Rot, A. 1991. Chemotactic potency of recombinant human neutrophil attractant/activation protein-1 (interleukin-8) for polymorphonuclear leukocytes of different species. *Cytokine 3:21.*
21. Iwai, K., T. Miyawaki, T. Takizawa, A. Konno, K. Ohta, A. Yachie, H. Seki, and N. Taniguchi. 1994. Differential expression of *bcl*-2 and susceptibility to anti-Fas-mediated cell death in peripheral blood lymphocytes, monocytes, and neutrophils. *Blood 84:1201.*
22. Fujiwara, T., E. A. Grimm, T. Mukhopadyhay, D. W. Cai, L. B. Owen-Schaub, and J. A. Roth. 1993. A retroviral wild-type p53 expression vector penetrates human lung cancer spheroids and inhibits growth by inducing apoptosis. *Cancer Res. 53:4129.*
23. Kagawa, S., T. Fujiwara, A. Hizuta, T. Yasuda, W.-W. Zhang, J. A. Roth, and N. Tanaka. 1997. p53 expression overcomes $p21^{WAF1/CIP1}$-mediated G_1 arrest and induces apoptosis in human cancer cells. *Oncogene 15:1903.*
24. Ogawa, N., T. Fujiwara, S. Kagawa, M. Nishizaki, Y. Morimoto, T. Tanida, A. Hizuta, T. Yasuda, J. A. Roth, and N. Tanaka. 1997. Nobel combination therapy for human colon cancer with adenovirus-mediated wild-type p53 gene transfer and DNA-damaging chemotherapeutic agent. *Int. J. Cancer 73:367.*
25. Shao, J., T. Fujiwara, Y. Kadowaki, T. Fukazawa, T. Waku, T. Itoshima, T. Yamatsuji, M. Nishizaki, J. A. Roth, and N. Tanaka. 2000. Overexpression of the wild-type p53 gene inhibits NF-κB activity and synergizes with aspirin to induce apoptosis in human colon cancer cells. *Oncogene 19:726.*
26. Chen, J.-J., Y. Sun, and G. J. Nabel. 1998. Regulation of the proinflammatory effect of Fas ligand (CD95L). *Science 282:1714.*
27. Arai, H., D. Gordon, E. G. Nabel, and G. J. Nabel. 1997. Gene transfer of Fas ligand induces tumor regression in vivo. *Proc. Natl. Acad. Sci. USA 94:13862.*
28. Miwa, K., M. Asano, R. Horai, Y. Iwakura, S. Nagata, and T. Suda. 1998. Caspase 1-independent IL-1β release and inflammation induced by the apoptosis inducer Fas ligand. *Nat. Med. 11:1287.*
29. Kurosaka, K., N. Watanabe, and Y. Kobayashi. 1998. Production of proinflammatory cytokines by phorbol myristate acetate-treated THP-1 cells and monocyte-derived macrophages after phagocytosis of apoptotic CTLL-2 cells. *J. Immunol. 161:6245.*
30. Uchimura, E., N. Watanabe, O. Niwa, M. Muto, and Y. Kobayashi. 2000. Transient infiltration of neutrophils into the thymus in association with apoptosis induced by whole-body X-irradiation. *J. Leukocyte Biol. 67:780.*
31. Dewey, R. A., G. Morrissey, C. M. Cowsill, D. Stone, F. Bolognani, N. J. F. Dodd, T. D. Southgate, D. Klatzmann, H. Lassmann, M. G. Castro, and P. R. Lowenstein. 1999. Chronic brain inflammation and persistent herpes simplex virus 1 thymidine kinase expression in survivors of syngeneic glioma treated by adenovirus-mediated gene therapy: implications for clinical trials. *Nat. Med. 5:1256.*
32. Bouvet, M., L. M. Ellis, M. Nishizaki, T. Fujiwara, W. Liu, C. D. Bucana, B. Fang, J. J. Lee, and J. A. Roth. 1998. Adenovirus-mediated wild-type p53 gene transfer down-regulates vascular endothelial growth factor expression and inhibits angiogenesis in human colon cancer. *Cancer Res. 58:2288.*
33. Nishizaki, M., T. Fujiwara, T. Tanida, A. Hizuta, H. Nishimori, T. Tokino, Y. Nakamura, M. Bouvet, J. A. Roth, and N. Tanaka. 1999. Recombinant adenovirus expressing wild-type p53 is antiangiogenic: a proposed mechanism for bystander effect. *Clin. Cancer Res. 5:1015.*