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

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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.

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

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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 homogenous p53 gene mutation were maintained in non-aqueous culture in 75 cm² 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 × 10⁶ 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 × 10⁹ PFU/100 μl), and Ad5CMVP53 (1 × 10⁹ PFU/100 μl) for 5 consecutive days. The tumor growth was assessed by measuring perpendicularly diameters with calipers. Tumor volume was calculated from the largest (a) and smallest (b) diameter with the formula 0.5a × b². 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 high glucose (4.5 g/L) supplemented with 10% FCS, 100 U/ml penicillin, and 100 mg/ml streptomycin.

PMN chemotaxis was quantified using a modification of the Boyden chamber technique (20). A cell suspension containing 4 × 10⁶ cells/ml (the total cell number loaded per well was adjusted to give equal numbers of PMNs) in PBS supplemented with 1 mM CaCl₂, 1 mM MgCl₂, 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 polycarbonate filter was added between lower and upper 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 × 10⁶ 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 with 10% FCS, 100 U/ml penicillin, and 100 mg/ml streptomycin. 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 PMNs was >85%, as assessed by Diff-Quick staining (International Reagents, Kobe, Japan) of cytospin preparations under light microscopy.

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 PMNs 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 CaCl₂ and 1 mM MgCl₂.

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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 PMNs 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 CaCl₂ and 1 mM MgCl₂.
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, suggesting 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.
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. 5B, 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. 6A).

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. 6B). 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 (IgG1). 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.
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 apoptotic 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 accelerated 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 dI312-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 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, dI312 (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.
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 syngeneic 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.

![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.](http://www.jimmunol.org/)
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 effect in p53 gene therapy for cancer.

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