[CANCER RESEARCH 58, 2288–2292, June 1, 1998]

Advances in Brief

Adenovirus-mediated Wild-Type p53 Gene Transfer Down-Regulates Vascular Endothelial Growth Factor Expression and Inhibits Angiogenesis in Human Colon Cancer[1]

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Abstract

Recent studies have indicated that angiogenesis may be regulated, in part, by p53 tumor suppressor gene function. We hypothesized that wild-type p53 replacement would down-regulate vascular endothelial growth factor (VEGF) expression and inhibit angiogenesis. KM12L4 and SW620, human colon cancer cell lines with p53 mutations, were transduced with a replication-defective adenoviral vector containing the wild-type p53 gene (Ad5/CMV/p53). Reverse transcription-PCR confirmed the presence of p53 in Ad5/CMV/p53-transduced cells. Transduction of colon cancer cells with wild-type p53 decreased VEGF RNA expression compared with that of controls. The decrease in VEGF expression in SW620 cells was dose dependent, with a 49% decrease observed at a multiplicity of infection of 50, and a 71% decrease observed at a multiplicity of infection of 100. Similar effects were seen in KM12L4 cells. VEGF supernatant protein levels were significantly reduced compared with those in nontransduced controls 48 h after the introduction of wild-type p53. Ad5/CMV/p53 inhibited tumor cell-induced angiogenesis *in vivo*. Restoration of wild-type p53 expression may decrease tumor growth by inhibiting the angiogenic response. These findings may explain, in part, the bystander effect seen with p53 tumor suppressor gene therapy.

Introduction

Angiogenesis is an essential step in tumor growth and metastasis, and this process is driven by the balance of positive and negative effector molecules (1). One of these factors, VEGF,[3] is a homodimeric 34-42-kDa heparin-binding glycoprotein with potent angiogenic, mitogenic, and vascular permeability-enhancing activities specific for endothelial cells (2, 3). VEGF seems to be the angiogenic factor most closely associated with neovascularization in human colon cancer (4, 5). Recent studies have indicated that angiogenesis may be regulated, in part, by p53 tumor suppression gene function (6).

We have previously reported the utility of adenovirus-mediated p53 tumor suppressor gene therapy for colorectal cancer *in vitro* and *in vivo* (7). Although gene replacement of wild-type p53 suppresses tumor growth through the induction of apoptosis, a significant bystander effect (killing or growth arrest of nontransduced tumor cells mediated by transduced tumor cells) has been noted (8, 9). Although the mechanism of such a bystander effect has not been fully established, it is possible that p53 down-regulation of VEGF and the resultant inhibition of angiogenesis may contribute to the bystander effect. We hypothesized that the introduction of wild-type p53 into colon cancer cells harboring p53 mutations would decrease the expression of VEGF and therefore inhibit tumor-induced angiogenesis.

Materials and Methods

Cell Lines. The SW620 human colorectal carcinoma cell line, which has a mutation in the *p53* gene (codon 273, Arg→His), was obtained from the American Type Culture Collection (Rockville, MD). The KM12L4 cell line (kindly provided by Dr. Isaiah J. Fidler, M. D. Anderson Cancer Center has a transdominant mutation in the *p53* gene (codon 179, His→Arg). Cells were maintained in either RPMI 1640 (for SW620 cells) or Earl's salts medium (for KM12L4 cells) supplemented with 10% FCS, 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B (Life Technologies, Inc., Grand Island, NY). Both cell lines were incubated at 37°C in a 5% CO_2 incubator.

Recombinant Adenovirus. The construction, properties, and purification of the Ad5/CMV/p53 vector have been reported elsewhere (10). Adenovirus vector preparations were free of replication-competent adenovirus, as determined by previously described techniques (11).

Gene Delivery. Vector transductions were performed by plating 5×10^6 cells in 100-mm plates for RNA expression, ELISA, and microchamber studies or by plating 5×10^4 cells in 6-well plates (Falcon Plastics, Lincoln Park, NJ) for RT-PCR studies. Forty-eight h after plating, the cells were incubated with a purified vector in appropriate medium. To obtain transduction efficiencies of 50 and 80%, MOIs of 25 and 50 were used for the KM12L4 cell line, and MOIs of 50 and 100 were used for the SW620 cell line (7). Control groups were treated in similar fashion with PBS in place of a vector.

RT-PCR. Total RNA extraction, reverse transcription, PCR amplification, and blot hybridization were performed by a modification of previously described techniques (12).

Northern Blot Analysis. Total RNA was extracted from 10^7 tumor cells grown in culture by using a Trizol isolation kit (Life Technologies, Inc.). Total RNA (20 μg) was fractionated on 1% denaturing formaldehyde/agarose gels, transferred to Hybond nylon membrane (Amersham Corp., Arlington Heights, IL) by capillary elution, and cross-linked with 120,000 μJ/cm^2 of UV light by using an UV Stratalinker 1800 (Stratagene, La Jolla, CA). After prehybridization, the membranes were probed for VEGF and reduced GAPDH. Each cDNA probe was purified by agarose gel electrophoresis, recovered by using a QIAEX gel extraction kit (Qiagen, Inc., Chatsworth, CA), and radiolabeled by a random primer technique with a commercially available kit that uses [α-^{32}P]dCTP (Amersham Corp.). Nylon filters were washed at 65°C with 30

Received 3/17/98; accepted 4/15/98.

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[1] This study was partially supported by a National Cancer Institute Training Grant T32-09599-08 and an American Cancer Society Oncology Fellowship (to M. B.), by gifts to the Division of Surgery from Tenneco and Exxon for its Core Laboratory Facility, by University of Texas M. D. Anderson Cancer Center Support Core Grant CA16672 from the National Cancer Institute, by Specialized Program of Research Excellence in Lung Cancer Grant P50-CA70907 from the NIH, American Cancer Society Career Development Award, The Gillson Longenbaugh Foundation, by a grant from the Mathers Foundation, and by a sponsored research agreement with Introgen Therapeutics, Inc.

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[3] The abbreviations used are: VEGF, vascular endothelial growth factor; RT-PCR, reverse transcription-PCR; MOI, multiplicity of infection; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

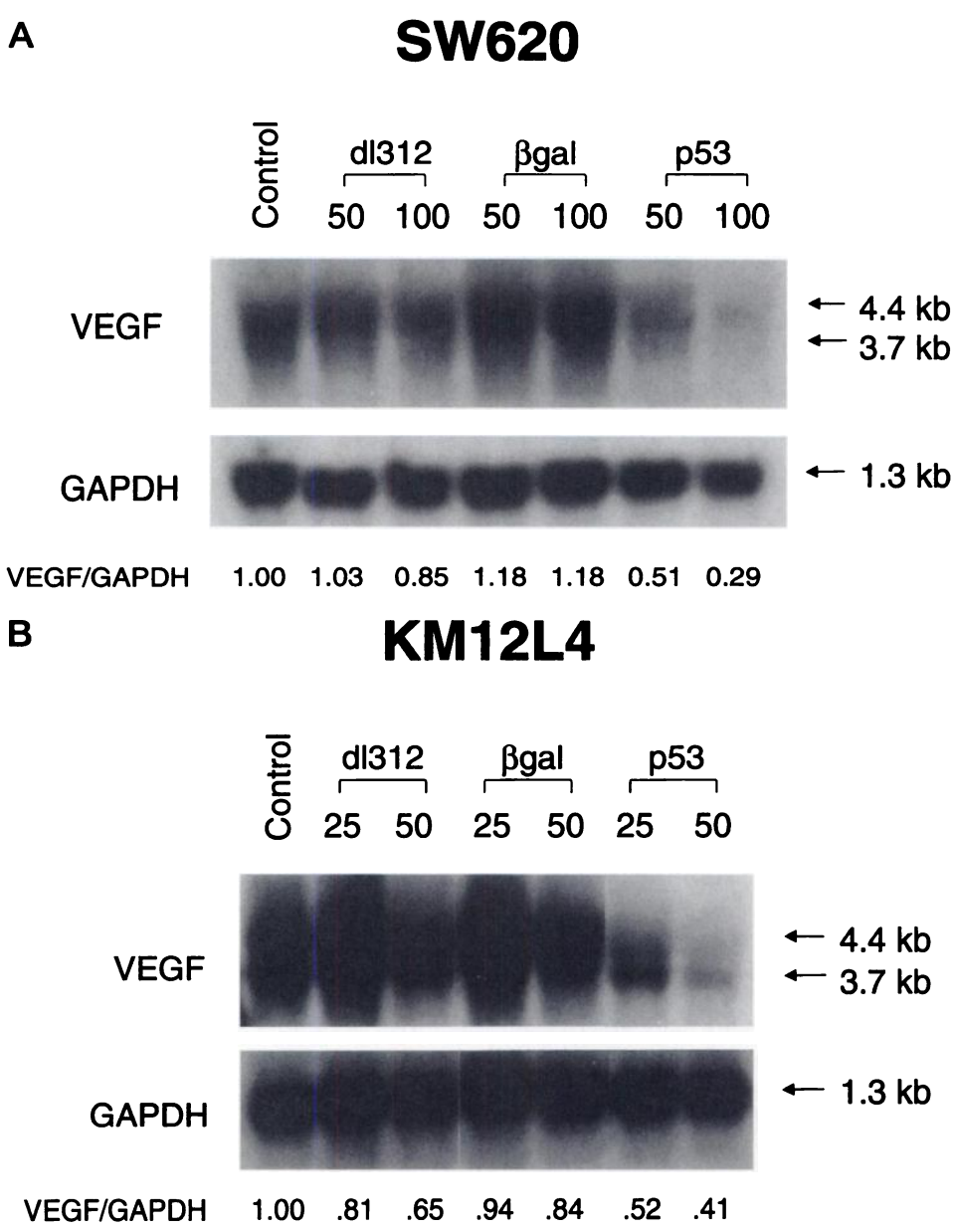

Fig. 1. Effect of wild-type p53 expression on VEGF RNA expression. *A*, Northern blot analysis of VEGF RNA expression in SW620 human colon cancer cells 48 h after transduction with Ad5/CMV/p53. Vector alone (Ad5/dl312) and vector expressing the β-galactosidase reporter gene (Ad5/CMV/β-GAL) were used for transduction at the same MOIs as controls. As an additional control, nontransduced KM12L4 cells were also examined. GAPDH transcripts were used as an internal control. Steady-state mRNA expression was quantitated by densitometry of autoradiograms. *B*, Northern blot analysis VEGF RNA expression in KM12L4 cells 48 h after transduction with Ad5/CMV/p53.

mM NaCl, 3 mM sodium citrate (pH 7.2), and 0.1% SDS (w/v). Autoradiography was then performed.

A 1.28-kb GAPDH probe (a gift from Dr. Robert Radinsky, M. D. Anderson Cancer Center) was used as an internal control. The VEGF probe, a 204-bp fragment of human VEGF cDNA, was a gift from Dr. Brygida Berse (Harvard Medical School, Boston, MA; Ref. 13). The thrombospondin (TSP-1) probe was a gift from Dr. Peter Polverini (University of Michigan, Ann Arbor, MI).

Densitometric Quantitation. VEGF and GAPDH RNA expression were quantitated by densitometry of autoradiograms using the Image Quant software program (Molecular Dynamics, Sunnyvale, CA) in the linear range of the film.

Determination of VEGF Protein Levels in Cell Supernatants. KM12L4 cells were seeded at 5×10^6 cells/100-mm tissue culture plate and grown for 24 h in DMEM supplemented with 10% fetal bovine serum. Cells were then transduced with Ad5/dl312, Ad5/CMV/β-GAL, or Ad5/CMV/p53 at MOIs of 25 and 50. Twenty-four h later, media were removed, cells were washed three times with PBS, and media were changed to 10 ml of DMEM supplemented with 1% fetal bovine serum. After 24 h, cell supernatants were collected, filtered, and stored at −80°C; concomitantly, cell pellets were harvested by trypsinization, and the cell number was determined. The amount of VEGF protein in the supernatant was determined using an ELISA kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. VEGF was expressed as picograms of VEGF protein/10^6 cells/24 h.

Angiogenesis by Tumor Cells *in Vivo*. *In vivo* angiogenesis was assayed by the dorsal air sac method (14). Briefly, 1×10^7 cultured SW620 cells or SW620 cells transduced with Ad5/RSV/luc or Ad5/CMV/p53 at MOIs of 50 were suspended in PBS and packed into round-shaped cellulose ester membrane chambers with a diameter of 14 mm (pore size, 0.45 μm; Millipore, Bedford, MA) 36 h after transduction. Cell viability at a MOI of 50 remained >80% for the duration of the assay (7). The chambers were then implanted into a dorsal air sac of a nude mouse. Five mice were used in each of the three groups. The mice were killed on day 5, and the s.c. region overlying the chamber in each mouse was photographed and scanned into an image analyzer (BioScan, Version 4.10; Optimas, Bothell, WA). Vessel diameters were measured (10/mouse, 5 mice/group).

Statistical Analysis. Descriptive statistics such as mean and SD were reported to summarize the study results. Graphical assessment was performed to check the validity of parametric tests. A two-sample *t*-test was performed to compare VEGF protein secretion in cell lines treated under various conditions. The *in vivo* antiangiogenesis effect of p53 transduction was analyzed by the nested ANOVA in which the vessel diameter was treated as the dependent variable, the group was treated as a fixed effect covariate, and the mouse was treated as a random effect covariate. All *P*s reported were based on two-sided tests. Bonferroni adjustment was applied for multiple comparisons to maintain the overall significance level.

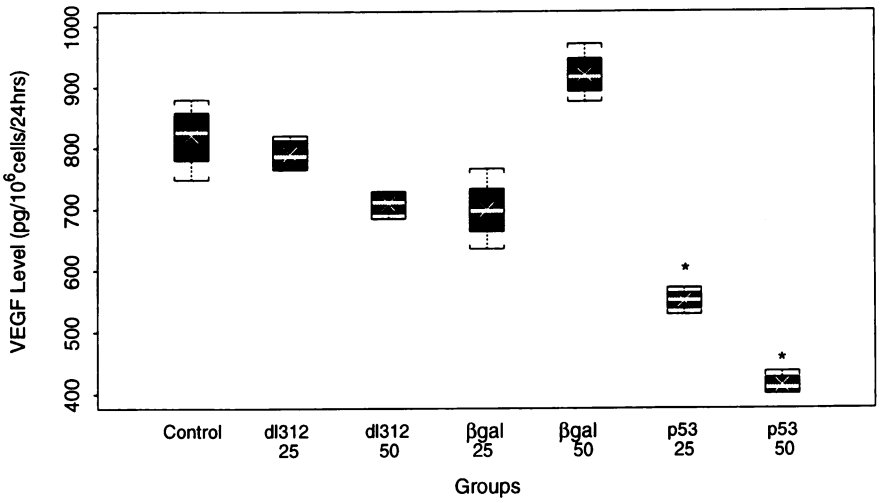

Fig. 2. Box plot of the effect of wild-type p53 expression on supernatant VEGF protein levels. The *shaded box* indicates the distribution of the middle 50% of the data. The median is marked by a *highlighted bar inside the center box*, and the mean is marked by an *X*. The *lower* and *upper brackets* extend to the minimum and maximum of the data when there are no outliers. When outliers exist, the brackets extend to 1.5 × interquartile range beyond the lower and upper quartiles. Outliers are marked by *horizontal bars outside the brackets*. The mean ± SD supernatant VEGF protein was significantly reduced in the p53-treated cells (*) compared with that in nontransduced controls ($P < 0.001$).

Results

Successful p53 Expression in Virally Transduced Cells. To confirm p53 expression in virally transduced cells, total RNA was extracted from KM12L4 and SW620 cells 24 h after transduction with Ad5/dl312, Ad5/CMV/β-GAL, or Ad5/CMV/p53 at a MOI of 100. RT-PCR was performed with either the CMV3 + RN3 primers specific for viral p53 mRNA or human GAPDH primers as positive controls. Vector-specific p53 mRNA was present only in KM12L4 and SW620 cells transduced with Ad5/CMV/p53 (data not shown).

Decrease in VEGF mRNA Due to Expression of Wild-Type p53. VEGF mRNA levels were determined in virally transduced cells. Transduction of colon cancer cells with wild-type p53 decreased VEGF mRNA expression compared with that of controls. The decrease in VEGF expression in SW620 cells was dose dependent, with a 49% decrease observed at a MOI of 50, and a 71% decrease observed at a MOI of 100 at 48 h (Fig. 1*A*). Minimal effect on VEGF expression was seen in controls transduced with a vector only. Similar effects were seen in the KM12L4 cell line (Fig. 1*B*). Forty-eight h after transduction with Ad5/CMV/p53, VEGF RNA expression in KM12L4 cells was decreased by 48% at a MOI of 25 and by 59% at a MOI of 50 compared with that of the nontransduced control. These experiments were repeated twice with similar results. In contrast, thrombospondin mRNA, a wild-type p53-regulated inhibitor of angiogenesis, was not detected in either control vector or Ad/CMV/p53-transduced SW620 or KM12L4 cells (data not shown).

Significant Reduction in Supernatant VEGF Protein Levels after Introduction of Wild-Type p53. To determine the VEGF protein levels in the medium of cells transduced with adenoviral vectors, cell supernatants were collected 48 h after transduction with Ad5/dl312, Ad5/CMV/β-GAL, or Ad5/CMV/p53 at MOIs of 25 and 50. Nontransduced cells were used as an additional control. Cell counts were used to correct for differences in cell number between groups. The mean ± SD supernatant VEGF protein was 820.5 ± 55.0 pg/10^6 cells/24 h in the control group. A significant reduction in VEGF secretion was found in the p53-treated cells [550.5 ± 18.2 and 413.1 ± 16.2 pg/10^6 cells/24 h for MOIs of 25 and 50, respectively ($P < 0.001$; Fig. 2)]. No significant change of VEGF levels was found between control and vector-only transduced cells. These experiments were repeated twice with similar results.

Ad5/CMV/p53 Inhibition of Tumor Cell-induced Angiogenesis *in Vivo*. *In vivo* angiogenesis was assessed by photographing the s.c. neovascularization overlying a semipermeable membrane chamber containing SW620 cells (Fig. 3). The mean blood vessel diameters, as measured by image analysis, for the nontransduced controls, vector-transduced controls (Ad5/RSV/luc), and Ad5/CMV/p53-transduced mice were 24.6, 28.2, and 9.6 μm, respectively. Blood vessel diameters were significantly reduced in mice implanted with Ad5/CMV/p53-transduced tumor cells compared with those of controls ($P < 0.001$; Fig. 4).

Discussion

Although tumors 1–2 mm in diameter can receive all nutrients by diffusion, additional growth depends on the development of an adequate blood supply through angiogenesis (1). The induction of angiogenesis is mediated by several factors released by both tumor and host cells. VEGF seems to be the angiogenic factor most closely associated with neovascularization in colon cancer (4, 15).

Several studies have implicated the p53 tumor suppressor gene in the regulation of angiogenesis (6, 16–18). Loss of tumor suppressor genes may contribute to a more angiogenic phenotype by decreasing the production of factors that inhibit angiogenesis. For example, loss of wild-type p53 results in a dramatic decrease in the secretion of the angiogenesis inhibitor thrombospondin in fibroblasts (6, 16) and that of an anonymous angiogenesis inhibitor in human glioblastoma cells. We examined the production of thrombospondin in our model of colon cancer. No thrombospondin mRNA was detected in either cell line by Northern blot analysis before or after treatment with Ad5/CMV/p53. Other studies have suggested that the p53 protein may be involved in the regulation of VEGF expression. Transfection of mutant p53 into NIH3T3 cells potentiates protein kinase C induction of VEGF (17), whereas transfection of wild-type p53 into several tumor and transformed cell lines decreases VEGF expression (18).

This relationship between p53 and VEGF also seems to be important *in vivo*. An association between mutant p53, increased microvessel counts, and increased VEGF expression has been shown by immunohistochemical staining of human colon cancer specimens (19). A similar correlation was found in non-small cell lung carcinoma tumors (20).

Although the exact mechanism by which p53 down-regulates VEGF is unknown, there is evidence that the regulation may be at the level of RNA transcription. Using a 2.6-kb promoter-luciferase construct, Mukhopadhyay *et al.* (18) have analyzed the effect of wild-type p53 or mutant p53 on this promoter by cotransfection assays. Wild-

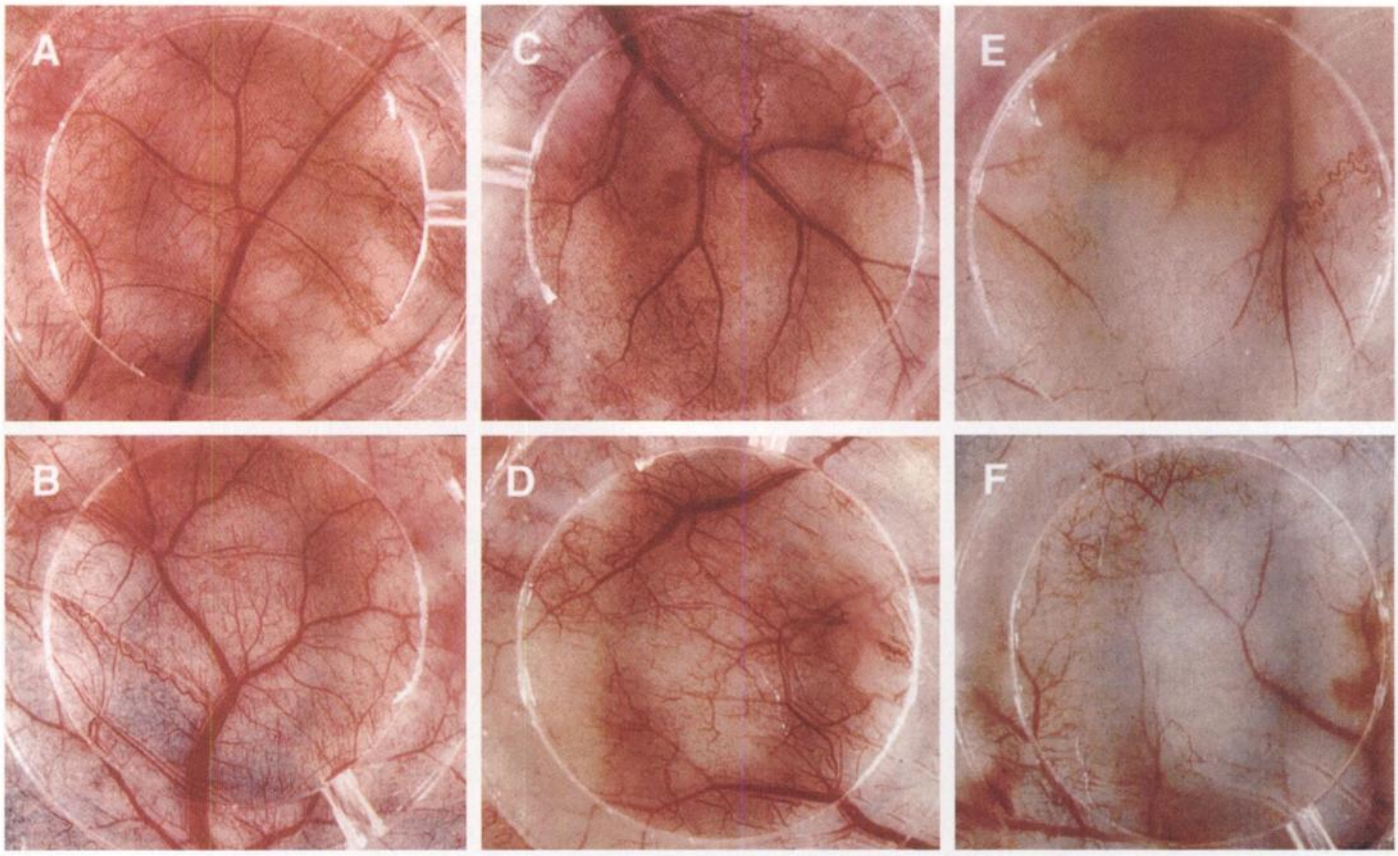

Fig. 3. Effect of wild-type p53 on SW620 cell-induced angiogenesis. *In vivo* angiogenesis was assessed with a semipermeable membrane chamber implanted in the dorsal air sac of nude mice. The chamber allowed the diffusion of soluble growth factors, such as VEGF, but not of cells. After 5 days, mice were killed, and the s.c. region overlying the chamber was assessed for neovascularization by image analysis. Images from two mice in each treatment group are shown. *A* and *B*, control cells; *C* and *D*, Ad5/RSV/luc (control vector)-treated cells; *E* and *F*, Ad5/CMV/p53-treated cells.

type p53 inhibited VEGF promoter activity in a dose-dependent manner in both U87 and 293 cells. When 0.5 μg of wild-type p53 plasmid DNA was used, the promoter activity was reduced by 70% compared with an empty vector control.

The results of our study indicate that reintroduction of the wild-type *p53* gene into human colon cancer cells causes a dose-dependent decrease in VEGF RNA expression and VEGF protein secretion. This reduction in VEGF secretion is associated with an inhibition of neovascularization *in vivo*. These findings provide further evidence that the *p53* gene plays an important role in control of angiogenesis.

Although gene replacement of wild-type p53 suppresses tumor growth through the induction of apoptosis (7), a significant bystander effect (killing or growth arrest of nontransduced tumor cells mediated by transduced tumor cells) has been noted (8, 9). Although the mechanism of such a bystander effect has not been fully established, it is possible that p53 down-regulation of VEGF and the resultant inhibition of angiogenesis may contribute to a more generalized

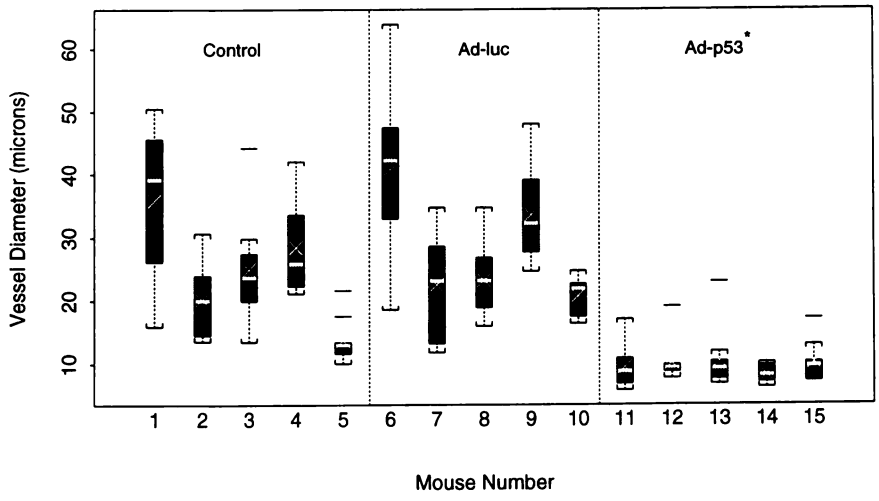

Fig. 4. Box plot of the mean blood vessel diameter in mice implanted with p53-treated tumor cells compared with controls. Image analysis was used to measure 10 vessel diameters from each mouse (5 mice/group). The *shaded box* indicates the distribution of the middle 50% of the data. The median is marked by a *highlighted bar inside the center box*, and the mean is marked by an *X*. The *lower* and *upper brackets* extend to the minimum and maximum of the data when there are no outliers. When outliers exist, the brackets extend to 1.5 × interquartile range beyond the lower and upper quartiles. Outliers are marked by *horizontal bars outside the brackets*. Blood vessel diameters were significantly reduced in mice implanted with p53-treated tumor cells (*) compared with those of controls ($P < 0.001$).

inhibition of tumor growth after transduction of a fraction of tumor cells with wild-type p53.

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