Technical Advance

The Use of Laser Scanning Cytometry to Assess Depth of Penetration of Adenovirus p53 Gene Therapy in Human Xenograft Biopsies

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SCH58500 is an agent for gene therapy of cancer, consisting of a replication-deficient type 5 adenovirus (Ad5) expressing the human p53 tumor suppressor gene (Ad5/p53). An important question about the use of Ad5/p53 gene therapy is how to achieve the therapeutically effective delivery of an Ad5/p53 vector to the tumor. We wanted to determine the effective depth of penetration of an Ad5/p53 vector by dosing the vector in an experimental human xenograft/SCID model. To assess depth of penetration, we developed a novel methodology for scanning tissue sections by laser scanning cytometry (LSC). SCID mice were given intraperitoneal injections of either *p53^{null}* SK-OV-3 human ovarian tumor cells or *p53^{mut}* DU-145 human prostate tumor cells to establish xenograft solid tumors. Mice were then dosed once or twice at 24-hour intervals by intraperitoneal injection with SCH58500 (Ad5/p53), an adenovirus construct expressing β -galactosidase (Ad5/ β -gal), or a buffer control. Additional groups of mice received a single intraperitoneal dose of 10 mg/kg paclitaxel either alone or coadministered with Ad5/p53. Twenty-four hours after each last dose, the human solid tumor xenograft and relevant mouse tissue were removed from each mouse for the analysis of Ad5/p53 penetration. Immunohistochemistry (IHC) for β -galactosidase protein revealed a depth of penetration of between 1 and 10 cells from the tumor surface. In some mice, hepatocytes in the periportal regions of liver lobules were also positive, indicating systemic absorption of ade-

novirus from the peritoneal cavity. IHC staining for p53 and p21 proteins in SK-OV-3 solid tumor xenografts revealed similar Ad/p53 penetration. LSC was used to map and quantitate apoptosis in both tumor and liver tissue biopsies, with over 450,000 nuclei from liver tissue and 150,000 nuclei from tumor tissue being evaluated. LSC analysis demonstrated a high level of apoptosis in the tumors that had been removed from Ad5/p53-dosed mice (12.7–19.7%). This level of apoptosis was significantly higher ($P < 0.05$) than was observed for liver tissues taken from Ad5/p53-dosed mice (2.7–8.0%) or tumor tissues taken from either Ad5/ β -gal-dosed mice (3.0–6.4%) or buffer control-dosed mice (3.0–5.3%). Scan bit maps from the extensive LSC analyses confirmed that apoptosis was present to about the same depth (1–10 cells) as had been identified by IHC for β -galactosidase, p53, and p21 proteins. Paclitaxel coadministered with Ad5/p53 had no effect on Ad5 penetration into solid tumors *in vivo* as measured by IHC for p53 or p21 protein. However, the combination therapy did cause an elevation in the number of tumor cells undergoing apoptosis. (*Am J Pathol* 1999, 155:1869–1878)

p53 is a tumor suppressor gene frequently mutated in many human neoplasms.¹ The cellular roles of p53 include activation of genes that inhibit cell cycle progression, promotion of DNA repair, and induction of programmed cell death (apoptosis).² The introduction of wild-type p53 into transformed cells of a *p53^{null}* or *p53^{mut}* genotype is incompatible with the maintenance of a tumorigenic phenotype, usually inducing apoptosis (for review, see Ref. 3). However, a key issue in the introduction

Accepted for publication August 24, 1999.

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of wild-type p53 genes into neoplastic cells is the delivery vehicle or vector. One emerging approach is to deliver the gene with a type 5 adenoviral vector (Ad5/p53).⁴ To date, Ad5/p53 vectors have been used for a wide variety of preclinical proof-of-concept studies in the gene therapy of cancer³, and ongoing phase I clinical trials support their safety in human cancer patients.^{5,6}

A natural question arising from these studies concerns the efficiency of gene delivery, to provide guidance for the design of clinical protocols. For ovarian cancer, it becomes critical to determine the depth of adenovirus drug penetration into tumor nodules dispersed throughout the peritoneal cavity after single and multiple doses. Intraperitoneal human tumor xenograft models with SK-OV-3 ovarian cells (*p53^{null}*) or DU-145 prostate cells (*p53^{mut}*) were used to study this issue. Tissue was analyzed for apoptosis with a new fluorescence laser scanning cytometry (LSC) technology to perform both automated quantitative analysis and positional mapping of apoptotic architecture within thin-tissue sections. This new assay was validated using the more traditional technique of immunohistochemistry (IHC) for β -galactosidase, p53, and the p53-induced protein, p21.

LSC is a slide-based fluorescence analytical method analogous to flow cytometry. Thus, extensive quantitation of cellular or nuclear events is possible using LSC analysis.^{7,8} In contrast to flow cytometry, the position of each fluorescent event is recorded as it is scanned on the slide, and electronic bit-map images of the scan are created. As a result, bit maps of scanned thin-tissue sections reveal the architectural context in which the fluorescent event has occurred. In tissue, LSC analysis is particularly useful for the measurement of nuclear-associated events. LSC has been used for the analysis of DNA content^{9–11} and with the immunophenotyping of malignant human biopsy tissue sections.^{12,13} Recent reports have also demonstrated the use of LSC for the measurement of nuclear-associated proteins, nuclear cyclin B1 expression,¹⁴ p53,¹⁵ and NF- κ B.¹⁶ We have developed an LSC-based method for scanning 4 to 6- μ m tissue sections for nuclear-associated apoptotic events, using terminal deoxynucleotidyltransferase (TdT) directed nick-end labeling (TUNEL) of fragmented DNA. Using this method, we analyzed human tumor xenografts and murine liver tissue from SCID mice treated with Ad5/p53 vector, Ad5/ β -gal (Ad control) vector, or vehicle (buffer control) to quantitate and map the induction of apoptosis, and we correlated these results with IHC detection of p53 protein and p21 protein expression as a measure of vector penetration. It is likely that phase II/III clinical trials will incorporate an arm comparing traditional chemotherapy with chemotherapy combined with p53 gene therapy. Therefore, it is important to study possible interactions between p53 adenovirus and chemotherapeutic drugs in preclinical models before entering the clinic. Due to the clinical importance of paclitaxel (taxol) in treating ovarian cancer in solid tumors and our previous observation that paclitaxel enhances adenovirus type 5 (Ad5) transduction efficiency,¹⁷ we decided to examine

the effect of paclitaxel on adenovirus drug penetration after intraperitoneal dosing.

Materials and Methods

Cell Lines

The SK-OV-3 and DU-145 cell lines were obtained from American Type Culture Collection (Manassas, VA). SK-OV-3 human ovarian and DU-145 human prostate tumor cell lines were cultured in Eagle's minimal essential medium (MEM) with nonessential amino acids and Earle's balanced salt solution plus 10% fetal calf serum at 37°C and 5% CO₂.

Virus Constructs and Virus Preparations

SCH585000, an *E-1*-deleted adenovirus vector (Ad/p53), was constructed using the large fragment from dl327 and a plasmid containing the 1.4-kb full-length p53 cDNA with expression driven from the human cytomegalovirus promoter.¹⁸ Recombinant virions were produced and purified as previously described.¹⁹ Viral particle concentrations were determined using anion-exchange high-pressure liquid chromatography²⁰ and $A_{260\text{nm}}$ measurement in 0.1% sodium dodecyl sulfate (w/v).²¹ SCH58000 was provided by Schering-Plough Biotechnology (Union, NJ). The β -galactosidase adenovirus vector construct was described previously¹⁸ and was provided by Canji (San Diego, CA). Viral-construct infectivity was confirmed using a flow cytometry-based adenovirus infection assay.¹⁵

Human Tumor Xenograft Models and in Vivo Administration of Vectors

The SK-OV-3 and DU-145 tumor xenograft models in SCID mice have been described previously.¹⁷ Briefly, female SCID mice were injected intraperitoneally (i.p.) with either 1×10^6 SK-OV-3 ovarian tumor cells or 2.5×10^6 DU-145 prostate tumor cells on day 0. Tumors were allowed to establish for 3 to 4 weeks. For treatment, groups of $n = 5$ mice received adenovirus constructs administered i.p. in Ad control buffer (20 mmol/L NaH₂PO₄ pH 8.0; 130 mmol/L NaCl₂; 2 mmol/L MgCl₂; 2% sucrose). After sacrifice, tumor nodules were excised for analysis. Excised tumor nodules were uniformly small to medium sized.

Three experiments were performed to evaluate adenovirus vector penetration. The first experiment evaluated the depth of penetration of an Ad5/ β -gal construct in SK-OV-3 and DU-145 tumor-bearing mice. Each treatment dose of Ad5/ β -gal contained 1×10^{10} viral particles. Tumor tissue was analyzed for β -galactosidase activity using IHC (Figure 1). In a second experiment, SK-OV-3 tumor-bearing SCID mice were treated i.p. with Ad buffer, Ad5/ β -gal, or Ad5/p53 as either a single bolus or two consecutive doses 24 hours apart. Each dose of adenovirus construct contained 2.9×10^{10} viral particles. In a third experiment, SK-OV-3 tumor-bearing SCID mice

Figure 1. Representative tumor sections showing β -galactosidase IHC for intraperitoneal SK-OV-3 (A–C) and DU-145 tumor (D) xenografts in drug-treated SCID mice. **A:** Vehicle buffer control, magnification, $\times 200$; **B:** Ad5/ β -gal, magnification, $\times 200$; **C:** Ad5/ β -gal magnification, $\times 400$; **D:** Ad5/ β -gal magnification, $\times 400$.

received 10 mg/kg paclitaxel with the first bolus dose of buffer or Ad5/p53. In this experiment, the first dose contained 1×10^{10} virus particles of Ad5/p53; the second dose contained 2×10^{10} virus particles. Twenty-four hours after the last adenovirus dose, mice were sacrificed and tissues harvested for analysis.

C.B.17/ICR-SCID mice were purchased from Taconic Farms (Germantown, NY). All mice were maintained in a VAF-barrier facility. Animal procedures were performed in accordance with the rules set forth by the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and approved by the Schering-Plough Research Institute Animal Care and Use Committee. Paclitaxel was purchased from CalBiochem (San Diego, CA). For *in vivo* experiments, paclitaxel was dissolved in 1:1 absolute ethanol and Cremophor EL (Sigma Chemical Co., St. Louis, MO), then diluted 1:10 into 0.9% saline immediately before intraperitoneal injection.

Tissue Preparation for Immunohistochemistry and Laser Scanning Analysis

Excised tissue samples were either snap-frozen or fixed in 10% buffered formalin and processed overnight in a Miles VIP Tissue Processor (SAKura Finetek, Torrance, CA), then embedded in paraffin. Snap-frozen tissues were embedded using Tris-buffered saline medium (Triangle Biomedical Science, Durham, NC) and cut into 4-

to 6- μ m sections with a Microm HM505N cryostat (Carl Zeiss, Waldorf, Germany); paraffin-embedded tissues were cut into 5- μ m sections with a Leitz model 1512 microtomed.

Immunohistochemistry for β -Galactosidase Protein

Formalin-fixed, paraffin-embedded sections were deparaffinized and rehydrated. Endogenous peroxidase activity was blocked with 3% aqueous hydrogen peroxide for 15 minutes. Immunohistochemical staining was performed at 37°C, using a Ventana Immunostainer ES (Ventana Medical Systems, Tucson, AZ). Tissues were enzyme digested using protease 1 treatment for 4 to 8 minutes. For the detection of β -galactosidase activity, a 1:100 dilution of antibody for rabbit anti- β -galactosidase (5 Prime-3 Prime, Boulder, CO) was used. The antibody was incubated for 20 to 30 minutes at 37°C. For negative controls, primary antibody was substituted with nonimmune rabbit or mouse immunoglobulins (Vector Laboratories, Burlingame, CA) diluted to match the primary antibody protein concentration. The Ventana DAB detection system was used to detect specific antibody binding. The slides were counterstained with hematoxylin, dehydrated, cleared, and coverslipped using Permount (Fisher Scientific).

p53 and p21 Immunohistochemistry

Formalin-fixed, paraffin-embedded sections were deparaffinized and rehydrated. Sections were treated with ethanol:acetic acid (2:1) at -20°C for 10 minutes, and antigens were retrieved by microwaving in 0.01 mol/L citric acid buffer (pH 6.0) for 10 minutes. Endogenous peroxidase activity was quenched by incubating the slides with 1.5% hydrogen peroxide in cold ethanol for 10 minutes. The slides were then blocked for nonspecific binding, using normal rabbit serum (NovoCastra, Newcasttle, UK). The primary antibodies used were mouse anti-human p53 (NovoCastra) and mouse anti-human p21 (PharMingen, San Diego, CA). Both primary antibodies were incubated on slides for 1 hour at 23°C . For negative controls, primary antibodies were substituted with nonimmune mouse immunoglobulin (PharMingen) diluted to match the primary antibody protein concentration. Slides were then incubated for 30 minutes at 23°C , using biotinylated rabbit anti-mouse antibody diluted 1:500 in Dulbecco's modified phosphate buffered saline (DPBS; NovoCastra). Specific antibody binding was detected using ABC reagent (avidin/biotinylated horseradish peroxidase; NovoCastra) combined with diaminobenzidine (DAB) chromogen (Vector) for color development. The slides were then counterstained with hematoxylin (Sigma), washed in water once, washed in 95% ethanol three times for 30 seconds per wash, washed twice for 1 minute per wash with 100% ethanol, and then washed twice for 1 minute per wash, using Clear Rite (Richard-Allen, Kalamazoo, MI). Coverslips were added to the slides with Permount (Fisher Scientific, Pittsburgh, PA).

Method of LSC Analysis for Apoptosis

Sections from snap-frozen SK-OV-3 tumor tissue and murine liver tissue were used for the first experiment. Sections from formalin-fixed, paraffin-embedded SK-OV-3 tumor tissue and murine liver tissue were used for the second experiment. Serial sections of 4 to 6 μm were used. Frozen tissue sections were pre-fixed by incubation in a solution of 10% buffered formalin at 4°C for 10 minutes and washed in DPBS. Prefixed frozen and formalin-fixed deparaffinized tissue sections were fixed by incubation in a solution of ethanol:acetic acid (2:1) at 20°C for 10 minutes and then washing for 5 minutes in DPBS. Apoptosis was detected with TUNEL and fluorescein-labeled dUTP (Boehringer-Mannheim, Indianapolis, IN) per kit instructions. For each set of serial sections, a TdT-negative control slide was used to set background nonspecific binding of fluorescein-dUTP. After TUNEL labeling, slides were incubated twice at 37°C for 10 minutes each, using TNT containing 1% bovine serum albumin. Slides were stained for nuclear localization by incubation at 23°C for 30 minutes, using 0.001% propidium iodide solution in DPBS-ethylenediaminetetraacetic acid/Triton X-100 solution containing a 1:7 dilution of RNase (Sigma). Slides were washed twice with DPBS and mounted using anti-fade medium.

LSC analysis was performed using the CompuCyte (Cambridge, MA) brand LSC with analysis by WinCyte

2.1 PC-based software. A detailed description of LSC methodology has been previously published.^{8, 15, 22} The desired area of analysis was located visually using epifluorescent visual microscopy on the instrument, and the scan areas were set. Slides were scanned using a 20 $\times$ objective and an Ar laser operating at 5 mW and using a 488-nm line. The focal plane was adjusted to the rear charged-coupled device camera, ie, the plane of the laser line. To avoid detector saturation, fluorescein and propidium iodide detector gain voltages were set in the LSC menu so that a maximum of 75% saturation was achieved for the brightest maximum pixel (max pixel) events scanned. Individual nuclei were detected using the orange- to long-red filter/detector cube configuration. Individual nuclei were contoured using long-red fluorescence and not exceeding a 100-pixel minimum-area threshold, centered on a scanned max pixel event. In general, this method contoured about 50% of a typical cell from the nucleus out. Contouring extensively outside the nuclear area, ie, greater than 67% of the cell, was kept to a minimum to avoid the contouring of multiple nuclei as a single event. Area versus max pixel and CompuSort (CompuCyte) relocation was used to help discriminate inadvertent multiple-nuclei contouring. TUNEL-positive events were detected using the fluorescein filter/detector configuration.

Quantitation of Apoptosis

The strategy for quantitative analysis was to analyze a single representative tumor and a single representative liver section for each mouse in a treatment group. For the Ad5/p53 study, tissues from four mice per group were used; in the Ad5/p53+ paclitaxel study, tissues from five mice per group were used. For LSC studies, only tissue from the SK-OV-3 human tumor xenograft model was used. For each tumor or liver section, at least four serial sections were made for analysis, with sections about 10 μm apart. The first section was used as the TUNEL-nonspecific binding (NSB) control in which the enzyme TdT was left out, but the fluorescein-dUTP label was included. This control section was used to establish the background green fluorescence within the scanned tissue. The analytical gate to be used to define a positive apoptotic event was then set at between 0.5 and 1.0 log-order above the mean max pixel for the TUNEL-NSB-scanned population. After analysis of the control section, the remaining serial sections for each tissue were analyzed for quantitation. TUNEL-positive nuclei and total nuclei scanned for all serial scans of a single tissue were summed, and a total percentage of TUNEL-positive nuclei was calculated for the single tissue. Weight-average percentages were used to calculate the group mean and percentage coefficient of variance. Statistical analysis was performed on the weight-average percentages by the single-tailed Student's *t*-test.

LSC Bit Maps

From each scan an x-y positional bit-map of total nuclei was generated to image the tissue; TUNEL-positive nu-

clei were then false-color-imaged with Adobe Photoshop (Adobe Systems, San Jose, CA) and overlaid onto the total nuclei to create a comprehensive bit map showing the localization of apoptosis within the tissue analyzed. Nuclei with green fluorescence more than 0.5 log above the TdT-negative control were false colored red on the bit maps to best contrast against nonapoptotic nuclei (light blue).

Results

Tumors from Ad5/ β -gal-treated mice were first analyzed for vector penetration by β -galactosidase protein IHC. Figure 1 shows IHC staining (brown) for the presence of β -galactosidase protein in representative SK-OV-3 xenograft tissue (Figure 1, B and C) and DU-145 xenograft tissue (Figure 1D) from Ad5/ β -gal-treated mice, but not from buffer control treatment (Figure 1A). The two-dimensional penetration of the β -gal adenovirus vector shows staining limited usually to the first 1 to 10 cells from the edge of the tumor. In some mice, the hepatocytes in the periportal regions of liver lobules were also positive, indicating systemic absorption of adenovirus from the peritoneal cavity (data not shown).

Because the SK-OV-3 tumor cell is a p53^{null} phenotype, we were able to perform IHC staining for the presence of human p53 protein to assess depth of penetration of the Ad5/p53 vector. As shown in Figure 2 (C and D), tumor sections from Ad5/p53-treated mice showed strong staining for p53 protein at the edge of the tumor section, with expression limited to about 10 cells in depth. p21 is a downstream p53-regulated protein involved in the progression of cell cycle. IHC staining of serial sections for p21 protein revealed very strong staining along the tumor edge, with the same depth of penetration as seen by β -galactosidase and p53 IHC (Figure 2, F and G). SK-OV-3 tumor cells are p21^{wt}, and therefore light background staining for p21 was detected in central regions of the tumor. Simultaneous administration of paclitaxel did not change the depth of adenovirus particle penetration into tumor nodules for p53 protein expression (Figure 2E) or p21 protein expression (Figure 2H).

LSC methodology was developed to detect apoptotic cell nuclei for quantification and to confirm depth of penetration of the functional endpoint (apoptosis) of p53 protein expression as a result of Ad5/p53 vector delivery. As described in Materials and Methods, individual cells within the tissue were contoured using a low concentration of propidium iodide to locate each nucleus in the orange-long-red wavelengths. In control experiments, we have determined that, at the gain settings used for the fluorescein (green) detector configuration, no spectral overlap from 488-nm excitation of propidium iodide is detected.

Our first goal with LSC analysis was to correlate the fluorescence TUNEL assay with the IHC observations for p53 and p21 proteins. Additional tissue sections were also analyzed using IHC Apoptag (Oncor, Gaithersburg, MD) to correlate the LSC-based TUNEL assay (data not shown). We observed that apoptotic nuclei could be

easily discriminated from nonapoptotic nuclei by fluorescein intensity and using an analytical gate set by the NSB control as described in Materials and Methods. In Figure 3, representative tissue scans of tumors taken from a buffer control-treated mouse (A), an Ad5/ β -gal-treated mouse (B), and an Ad5/p53-treated mouse (C–E) demonstrate the depth of penetration for apoptosis. Ad5/p53 treatment resulted in significant fluorescence from nick-end labeling of DNA ends (red color dots) associated with nuclei located on the edge of the tumor (D), with penetration of between 1 and 10 cells into the tumor. Apoptotic nuclei were minimal, diffuse, and nonlocalized in tumor tissues taken from buffer control and Ad5/ β -gal-treated mouse. Excised tumor nodules were small to medium sized, and no significant necrosis was observed. Serial sections from the same Ad5/p53-treated tumor are shown in Figure 3, C–E. Sections shown in panels C and E were taken from either end of the tumor, but were sectioned within 10 cell layers of the tumor surface. For these sections, more internal apoptotic nuclei are observed, although there is a bias towards the edge of the section as well. The section in Figure 3D was taken from the mid-section of the tumor, such that the internal cells are greater than 10 cell layers from either end of the tumor; for this section few apoptotic nuclei are observed in the tumor center.

The effect of paclitaxel coadministration with Ad5/p53 on tumor apoptosis is shown in representative tumor section scans in Figure 4. The administration of paclitaxel i.p. resulted in increased numbers of apoptotic nuclei on the edge of the tumor (Figure 4B) relative to buffer control (Figure 4A). The characteristic edge-restricted apoptosis for Ad5/p53 is shown in Figure 4C. However, the combination of paclitaxel + Ad5/p53 resulted in many more apoptotic nuclei present on the edge of the tumor, as well as increased apoptosis in more central regions (Figure 4D). This effect was consistent across all mice in the study.

The total apoptotic nuclei for each treatment group of SK-OV-3 tumor-bearing mice were enumerated using LSC as described in Materials and Methods. Figure 5 summarizes the results of the study that compared single and two-bolus doses of Ad5/p53 to vector and buffer controls. About 60,000 total liver tissue nuclei/group and 20,000 total tumor tissue nuclei/group were scanned for the quantitative analysis (Figure 5B). The total number of nuclei scanned was dependent on the amount of tissue recovered, the contouring efficiency of the LSC scan, and the type of tissue being scanned. There was a significantly higher ($P < 0.05$) percentage of apoptotic nuclei in the tumors of mice treated with either a single dose or two bolus doses of Ad5/p53 vector, compared with either buffer control or Ad5/ β -gal control treatment groups. Ad5/p53-treated tumors had between 12.7 and 19.7% apoptotic nuclei present, whereas both buffer control- and Ad5/ β -gal-treated tumors had between 3.0 and 5.3% and 3.0 and 6.4% apoptotic nuclei, respectively. Tumor tissue was observed to have higher background apoptosis compared with liver tissue. The trend for both Ad5/ β -gal and Ad5/p53 treatment appeared to be toward higher apoptosis compared with buffer treatment in liver sec-

Figure 2. Representative tumor sections showing p53 protein (A–E) and p21 protein (F–H) IHC for intraperitoneal SK-OV3 tumor xenografts in drug-treated SCID mice. **A:** Paclitaxel + Ad5/ β -gal magnification $\times 200$; **B:** paclitaxel + Ad5/ β -gal magnification, $\times 400$; **C:** Ad5/p53 magnification, $\times 200$; **D:** Ad5/p53 magnification, $\times 400$; **E:** paclitaxel + Ad5/p53 magnification, $\times 400$; **F:** Ad5/p53 magnification, $\times 200$; **G:** Ad5/p53 magnification, $\times 400$; **H:** paclitaxel + Ad5/p53 magnification, $\times 400$.

Figure 3. LSC for apoptosis depth of penetration with Ad5/p53 therapy: representative 4 to 6- μ m tissue sections stained for DNA fragmentation using TUNEL. The laser scanning method is described in detail in Materials and Methods. TUNEL-positive nuclei (red dots) are displayed against TUNEL-negative nuclei (blue dots). Scans displayed are tissues taken from the Ad5/p53 study. **A:** Buffer control; **B:** Ad5/ β -gal vector treatment; **C-E:** Ad5/p53 treatment. **C** through **E** are from the same tumor tissue; **C** and **E** are the top and bottom sections of the tumor, whereas **D** is taken from about midpoint in the tumor.

tions. This was consistent with previous observations of adenovirus-mediated hepatotoxicity in SCID mice.²³

The same enumeration method was used for the study of paclitaxel coadministration (Figure 6). Tumors taken from mice treated with paclitaxel coadministered with a single bolus dose of Ad5/p53 had 42.0% apoptotic nuclei, whereas treatment with paclitaxel alone (15.1%) or Ad5/p53 alone (27.6%) had lower percentages of apo-

Figure 4. LSC for apoptosis: depth of penetration with paclitaxel and paclitaxel + Ad5/p53 therapy. TUNEL-stained tissue scans are from the second study. **A:** Buffer control; **B:** single dose, 10 mpk paclitaxel i.p. only; **C:** Ad5/p53 treatment; **D:** Ad5/p53 treatment with single-dose paclitaxel coadministration.

Figure 5. Quantitation of apoptosis by LSC for Ad5/p53 therapy. Four liver biopsy tissues and four tumors, one each per mouse, were scanned by LSC for each treatment group as described in Materials and Methods. **A:** Group mean percentage apoptotic nuclei with either one or two-bolus doses of buffer control, Ad5/ β -gal vector, or Ad5/p53. Error bars represent the maximal percent coefficient of variance of the weighted means. **B:** Total number of nuclei scanned for each treatment group.

ptotic nuclei. The effect of paclitaxel + Ad5/p53 was significantly different from the taxol group ($P < 0.05$) but not significantly different from the Ad5/p53 group. Tumors taken from mice treated with paclitaxel alone, Ad5/p53 alone, or the paclitaxel + Ad5/p53 combination were all significantly higher in apoptosis relative to the buffer control.

Discussion

The efficiency of adenoviral vectors for the delivery of therapeutic genes is a critical question for the emerging field of molecular medicine. The depth of penetration from bolus systemic administration of such vectors needs to be well studied and carefully enumerated to develop rational paradigms to guide clinical studies in gene therapy using adenoviral delivery vectors. In this study, we chose to use tumor-bearing, immune-deficient mouse models as the vehicle in which to test the efficiency of delivery of adenoviral vectors.

Figure 6. Effect of paclitaxel on Ad5/p53 induction of apoptosis. Five tumors, one per mouse, for each treatment group were scanned using LSC. For each group, 50,000 total nuclei were scanned. Group mean percentages of apoptotic nuclei are shown; error bars represent the maximal percent coefficient of variance of the weighted means.

IHC for β -galactosidase protein showed that the administration of Ad5/ β -gal i.p. resulted in a depth of penetration from the edge of a tumor of about 10 cells. This penetration was observed to occur in both p53^{null} and p53^{mut} tumors taken from the treated SCID mice. IHC staining for p53 protein in p53^{null} tumors taken from Ad5/p53 (SCH58500)-treated SCID mice also showed intense staining for p53 protein penetrating from the edge of the tumor into about 10 cells. In some mice, hepatocytes in the periportal regions of liver lobules were also found to be positive, indicating some systemic absorption of the adenoviral vector from the peritoneal cavity. We were also able to use IHC staining for p21 protein to correlate the same degree of penetration in Ad5/p53 vector-treated p53^{null} tumors, and we also demonstrated similar penetration in Ad5/p53 vector-treated p53^{null} tumors. Comparative evaluation of IHC photomicrographs from the tumors of Ad5/p53-treated mice and paclitaxel + Ad5/p53-treated mice did not show a significant increase in depth of adenovirus particle penetration into tumor nodules with coadministration of paclitaxel as measured by β -galactosidase expression, p53 protein, or p21 protein.

The difficulty in using immunohistochemical assay is the subjective nature of interpreting staining and the labor intensiveness of enumeration. To better evaluate vector depth of penetration and quantification of delivery, we developed staining and scanning methodology for murine tissues, using the CompuCyte LSC. We used a low propidium iodide solution (0.001%) to locate and contour nuclei in tissue as individual events. Then, with a separate filter/detector configuration for fluorescein, located apoptotic nuclei by the TUNEL method for detection of DNA fragmentation. Apoptotic nuclei could then be overlaid onto the total nuclei scanned, and either bit maps were generated to show where within the tissue apopto-

sis was occurring, or nuclei were enumerated at high numbers to generate statistically meaningful data. As the LSC generated fluorescence data similar to the flow cytometer, the intensity of fluorescence could be manipulated by changing detector sensitivity. Fluorescence detector gains were adjusted to correlate LSC data with IHC data for depth of penetration. The gains used for the study were set between 30 and 40% of the possible sensitivity for the detectors, suggesting that significantly increased analytical sensitivity may be derived in future studies.

Using this approach the LSC was able to generate objective bit maps to look for areas of high apoptotic nuclear fluorescence from nick-end labeling. We observed that tumor sections taken between 1 and 10 cell layers into the tumor had much higher numbers of highly fluorescent nuclei than deeper sections and that penetration from the edge was usually limited as well. Where apoptosis was observed more internally, using LSC rather than IHC staining, the fluorescence intensity was also observed to be lower than that associated with nuclei near the edge of the tumor. These data confirm that the Ad5/p53 vector delivery to the tumor resulted in the induction of apoptosis in the areas of the tumor consistent with expression of p53 protein encoded by SCH58500. These areas of expression were also consistent for general vector delivery (Ad/ β -gal) and for the expression of p21, a p53-regulated downstream protein involved in cell cycle regulation. Data from the IHC assays suggested that two consecutive bolus doses of Ad5/p53 resulted in more p53- and p21-positive cells than with a single dose. Using LSC, we observed that within a 5 to 10 cell layer of depth from the tumor surface in representative sections, more apoptotic nuclei were observable with the two consecutive doses of Ad5/p53. However, this anecdotal observation did not withstand the quantitative LSC analysis. This underscores the utility of using LSC in the quantitative format for rigorous tissue analysis. Because of the uniformity of excised tumor nodules, we could not determine whether a correlation existed between tumor size and depth of construct penetration.

In contrast to the IHC observations for p53 and p21 proteins, the LSC measurement of apoptosis revealed an effect of combination therapy (paclitaxel + Ad5/p53). The bit maps from the paclitaxel coadministration study showed that there were more apoptotic nuclei around the edge of tumor tissues with the combination treatment. Paclitaxel administration alone resulted in detectable apoptotic nuclei within the tumor interior, whereas Ad5/p53 administration alone was restricted to the edge of the tumor. The combination treatment appeared to also increase the number of apoptotic nuclei in the interior of the tumor above the effect of paclitaxel alone. Quantitation of the scanned tissues also revealed an increase in the percentage of tumor cells undergoing apoptosis from 15 to 28% in mice dosed with either drug alone to 42% in mice dosed with both drugs simultaneously. A possible mechanism to explain the combined effect may be derived from reported *in vitro* studies that described increased transduction efficiency of tumor cells by recom-

binant adenovirus vector in the presence of low nanomolar concentrations of paclitaxel.¹⁷

The advantage to automated scanning of tissue is the large sampling number that can be generated for statistical analysis. Using the scan paradigms developed, we scanned over 450,000 liver nuclei and 150,000 tumor nuclei from all treatment groups of SK-OV-3 tumor-bearing mice from the Ad5/p53 study and 200,000 tumor nuclei from the paclitaxel + Ad5/p53 study. From these results, we were able to statistically resolve an Ad5/p53 treatment effect in tumors of the treated mice. We were able to differentiate the Ad5/p53 treatment effect from vector-dependent effects (Ad5/ β -gal), in the tumor tissue and in the liver tissue of treated animals. The vector effect of Ad5 in mouse liver tissue has been documented.²³ In the paclitaxel coadministration study, enumeration clearly showed that the combination of paclitaxel + Ad5/p53 resulted in a significant increase in the number of apoptotic nuclei relative to the regimen of paclitaxel alone.

The ability of the LSC instrumentation and methods to break Ad5/p53 treatment from vector control effect and to differentiate a paclitaxel + Ad5/p53 effect from paclitaxel alone demonstrates the value of this approach to the bioanalytical community. LSC is a rapidly emerging field with great potential for use in clinical tissue analysis. We have previously demonstrated that LSC can be used for assessing intracellular and nuclear p53 protein from cell culture.¹⁵ In combination with the TUNEL method described here, we have recently developed a single-laser method for determining colocalization of p53 nuclear protein and TUNEL and also quantitation, using either snap-frozen or formalin-fixed, paraffin-embedded clinical tumor biopsies. Colocalization of nuclear p53 and apoptosis in tissues treated by several different routes of administration have been observed. In addition, the use of a second laser line at 633 nm is currently being explored to expand the endpoint multiplex to an additional one or two signals. Thus, the LSC appears to have a great application in multiple endpoint analysis of tissue for localization studies and for high-throughput quantitation. In combination with existing conventional histological and immunohistological analyses, it can provide important multiple endpoint analyses for clinically derived tissue biopsies.

Conclusions

The intraperitoneal administration of Ad5/p53 resulted in the penetration of the vector to between 1 and 10 cells from the edge of tumors located in the peritoneum of mice. This localization was measured by multiple parameters and two different analytical techniques. IHC demonstrated the limited "edge-in" penetration with β -galactosidase activity from an Ad5/ β -gal vector construct and the expression of p53 protein and p21 protein from the Ad5/p53 vector. LSC was developed for scanning tissues and used to map and quantitate the amount of penetration of apoptosis with TUNEL. The LSC data confirmed that Ad5/p53 administration resulted in the limited pene-

tration of apoptosis from the edge of the tumor, but that such apoptosis was significantly higher in the Ad5/p53-treated tumors than either buffer or Ad5/ β -gal vector control-treated tumors. Finally, LSC analysis was used to demonstrate that there was a significant increase in intratumor apoptosis with paclitaxel + Ad5/p53 treatment *versus* paclitaxel treatment alone.

Acknowledgments

We thank Ms Bin Shi for technical assistance and Mr. David Small and Mr. Peter Carleen (CompuCyte) for assistance with the Laser Scanning Cytometer.

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