Relaxin Expression From Tumor-Targeting Adenoviruses and Its Intratumoral Spread, Apoptosis Induction, and Efficacy

Joo-Hang Kim, Young-Sook Lee, Hoguen Kim, Jing-Hua Huang, A-Rum Yoon, Chae-Ok Yun

Background: **The use of oncolytic adenoviruses as cancer gene therapy is limited by their uneven penetration and distribution in tumors. We investigated whether the expression of the cell matrix–degradative protein relaxin by adenovirus could improve adenovirus distribution and penetration in tumors.** ***Methods:*** **We generated relaxin-expressing, replication-incompetent (dl-lacZ-RLX) and -competent (Ad-ΔE1B-RLX) adenoviruses by inserting a relaxin gene into the E3 adenoviral region. Controls were parental adenoviruses (dl-lacZ and Ad-ΔE1B) and phosphate-buffered saline (PBS) (vehicle). Replication-incompetent viruses, which do not lyse cells, were used to assess transduction efficiency. Viral spread in tumor spheroids, made by dissecting tumor tissue into homogeneous fragments, was assessed by reporter gene (i.e., lacZ) expression. Tumor growth inhibition was assessed by injecting adenoviruses into xenograft tumors in athymic mice (n = 8 or 9). Overall survival was assessed by the Kaplan–Meier method. Extracellular matrix was examined with Masson's trichrome staining. Therapeutic efficacy was evaluated by assessing spontaneous pulmonary metastasis in the B16BL6 melanoma mouse model and growth inhibition of orthotopically implanted hepatoma (n = 4–6). All statistical tests were two-sided.** ***Results:*** **In tumor spheroids and established solid tumors in vivo, transduction with dl-lacZ-RLX, compared with parental virus or vehicle, elicited higher transduction efficiency and viral spread throughout the tumor mass. Infection with Ad-ΔE1B-RLX, compared with parental virus, elicited greater viral persistence and spread, leading to increased survival (e.g., 100%, 95% confidence interval [CI] = 63.1% to 100%, for C33A tumor-bearing mice treated with Ad-ΔE1B-RLX, and 50%, 95% CI = 15.7% to 84.3%, for C33A tumor-bearing mice treated with Ad-ΔE1B). Infection with Ad-ΔE1B-RLX substantially decreased the collagen content of tumor tissue but not of adjacent normal tissue, compared with noninfected tissues. Intratumoral injection of Ad-ΔE1B-RLX inhibited the formation of lung metastases in mice (PBS = 268 mg of metastatic tumor per mouse and Ad-ΔE1B-RLX = 10 mg; difference = 258 mg, 95% CI = 94 to 426; *P* = .003, Mann–Whitney test). Systemic treatment with Ad-ΔE1B-RLX completely inhibited the growth of Hep1 hepatocellular carcinomas (PBS = 20.2 mg of tumor per mouse and Ad-ΔE1B-RLX = 0 mg; difference = 20.2 mg, 95% CI = 3.7 to 36.7; *P* = .004, Mann–Whitney test).** ***Conclusion:*** **Extracellular matrix degradation by relaxin expressed by adenoviruses increased viral distribution and tumor penetration, inhibited tumor growth and metastasis, and increased survival of mice. [J Natl Cancer Inst 2006;98:1482–93]**

Although the selective replication and spread of oncolytic adenoviruses within cancer cells and tissues has had some success as an anticancer treatment, its promise has been limited because of the uneven penetration and distribution of these viruses in tumor tissues. To date, ONYX-015, an oncolytic adenovirus in which the E1B 55-kDa gene has been deleted, has been administered to more than 300 cancer patients with various tumor types *(1,2)* but has had only limited success (i.e., local tumor regression rates of 0%–14%). Sauthoff et al. *(3)* demonstrated that high levels of adenoviruses persisted in xenograft tumors for at least 8 weeks after intratumoral injection of wild-type adenoviruses, but the viral distribution pattern in the tumor tissue was uneven and patchy. Harrison et al. *(4)* detected a high level of viruses in persistent viable tumors up to 100 days after the initial viral injection. Thus, despite long-term viral persistence in tumors, the limited spread of virus to cells throughout the tumor could explain the low response rates observed.

Connective tissue and the extracellular matrix appear to play a role in inhibiting viral spread in tumors. Kuriyama et al. *(5)* demonstrated that treatment of human U87, U251, or SF767 glioblastoma multiforme–derived brain tumor xenografts with collagenase, dispase, or trypsin, before the intratumoral injection of adenovirus enhanced virus-mediated gene transduction, and Maillard et al. *(6)* reported that elastase pretreatment before delivery of adenoviral vectors into rabbit iliac arteries increased viral transduction efficiency.

Relaxin is a peptide hormone that is structurally related to insulin and insulin-like growth factors *(7)*. Treatment of human lung fibroblasts and bleomycin-induced mouse lung fibrosis (i.e., the common end stage of many pneumopathies) tumors with relaxin decreases the synthesis and secretion of interstitial collagens and increases the expression of matrix metalloproteinase and procollagenase *(8)*. Relaxin is a potent inhibitor of collagen expression when collagen is overexpressed, but it does not markedly alter basal levels of collagen expression, in contrast to other collagen-modulatory cytokines, such as interferon gamma *(9)*. To further explore the barrier role of the extracellular

Affiliations of authors: Brain Korea 21 Project for Medical Sciences, Institute for Cancer Research, Yonsei Cancer Center (JHK, YSL, JHH, ARY, COY) and Department of Pathology (HK), Yonsei University College of Medicine, Seoul, Korea.

Correspondence to: Chae-Ok Yun, PhD, Yonsei Cancer Center, Yonsei University College of Medicine, 134 Shinchon-Dong, Seodaemun-Gu, Seoul, Korea (e-mail: chaeok@yumc.yonsei.ac.kr).

See "Notes" following "References."

DOI: 10.1093/jnci/djj397

matrix and connective tissue in inhibiting viral spread and penetration within tumor masses, we determined whether the expression of relaxin by adenoviruses increases their spread in tumor tissues.

Materials and Methods

Cell Lines and Cell Culture

The human embryonic kidney cell line 293 that expresses the adenoviral E1 region, the brain cancer cell lines U343 and U87MG, the cervical cancer cell line C33A, the liver cancer cell line Hep3B, and the non–small lung cancer cell line A549 were purchased from the American Type Culture Collection (ATCC, Manassas, VA). Murine B16BL6 melanoma cells (a metastatic variant of B16 melanoma cells) were obtained from Dr Y. S. Park (Yonsei University, Wonju, South Korea). All cell lines were cultured in Dulbecco's modified Eagle medium (DMEM; Gibco BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (Gibco BRL), 2 mM l-glutamine, penicillin (100 IU/mL), and streptomycin (50 μg/mL), except for Hep3B cells, which were cultured in modified Eagle medium (MEM; Gibco BRL), and B16BL6 cells, which were cultured in MEM supplemented with 5% fetal bovine serum, MEM vitamin solution (1 mM; Gibco BRL, product 11120-052), 100 mM sodium pyruvate, 10 mM of MEM nonessential amino acids solution (10 mM; Gibco BRL, product 11140-050), penicillin (500 IU/mL), and streptomycin (50 μg/mL). All cell lines were maintained at 37 °C in a humidified atmosphere at 5% CO_2 and 95% air. *Escherichia coli* was propagated in Luria Bertani medium at 37 °C.

Generation of Relaxin-Expressing Adenoviruses

To generate adenoviruses that express relaxin at the E3 region, we first excised the relaxin gene from the vector pDNR-LIB-RLX (ATCC) with the endonucleases *Sal*I and *Hin*dIII and subcloned into the vector pCA14 (Microbix, Ontario, Canada) to generate pCA14-RLX. CMV-relaxin-polA expression cassette was then excised from pCA14-RLX and cloned into the adenovirus E3 shuttle vector pSP72-E3 (*10)* that had been predigested with the endonuclease *Bam*HI to generate pSP72-E3/CMV-RLX. The newly constructed pSP72-E3/CMV-RLX shuttle vector was then linearized with *Pvu*I digestion. The replication-incompetent adenoviral vector pdl-lacZ that expresses lacZ (i.e., β-galactosidase) at E1 region of adenovirus and E1B 19-kDa– and E1B 55-kDa–deleted replication-competent adenoviral vector pAd-ΔElB were linearized with *Spe*I digestion. The linearized pSP72-E3/CMV-RLX shuttle vector was then cotransformed into *E. coli* BJ5183 with the *Spe*I-digested pdl-lacZ or pAd-ΔElB for homologous recombination *(11)* to generate pdl-lacZ-RLX and pAd-ΔE1B-RLX adenoviral vector, respectively (Fig. 1, A). E1-deleted replication-incompetent adenovirus (dl-lacZ) and E1B 19-kDa– and E1B 55-kDa–deleted replication-competent adenovirus (Ad-ΔElB) were also prepared as previously described *(12)*. All viruses were propagated in 293 cells, and the purification, titration, and quality analysis of all adenoviruses used were performed as previously described *(13)*. The number of viral particles was calculated from measurements of optical density at 260 nm, where 1 absorbency unit is equivalent to 10^{12} viral particles per milliliter, and infectious titers (plaque-forming units per milliliter) were determined by limiting dilution assay on 293 cells; the plaque-forming unit was calculated from infectious titers as follows: $T = 10^{1+d(S-0.5)}$, where d and S were the $\log_{10}$ of the dilution and the sum of ratios, respectively. The viral particle/plaque-forming unit ratios for dl-lacZ, dl-lacZ-RLX, Ad-ΔElB, and Ad-ΔElB-RLX were 57 : 1, 70 : 1, 22 : 1, and 43 : 1, respectively.

Enzyme-Linked Immunosorbent Assay for Relaxin Expression

We infected 5×10^5 U343 or C33A cells with dl-lacZ, dl-lacZ-RLX, Ad-ΔE1B, or Ad-ΔE1B-RLX at various multiplicities of infection (MOIs) in 25-T culture flasks. Forty-eight hours later, the supernatant was collected by centrifugation at 15 000*g* for 10 minutes at 4 °C, and the level of relaxin protein was assessed by enzyme-linked immunosorbent assay (Endogen, Woburn, MA). Serial dilutions of a purified recombinant human relaxin preparation with a known concentration were used to generate a standard curve.

Animal Studies

Male athymic nu/nu and C57BL/6 mice, weighing approximately 20 g at 6–8 weeks of age, were obtained from Charles River Japan Inc (Yokohama, Japan). All mice were maintained in a laminar airflow cabinet under specific pathogen-free conditions. All facilities are approved by the Association of Assessment and Accreditation of Laboratory Animal Care, and all animal experiments were conducted under the institutional guidelines established for the Animal Core Facility at Yonsei University College of Medicine.

Preparation of Tumor Spheroids and the Spreading and Penetration of Relaxin-Expressing Replication-Incompetent Adenovirus dl-lacZ-RLX in Spheroids

Xenografts of U343, U87MG, C33A, or A549 cells were established by subcutaneously injecting 1×10^7 cells into the abdomen of 6- to 8-week-old male nude mice (Charles River Japan Inc). When a tumor reached a volume of 150–200 mm^3 (3–4 weeks), tumor tissue was surgically excised, blood and necrotic tissue were removed from the tissue, and tumor fragments 1–2 mm in diameter were dissected with sterile 21-gauge needles. These explants (i.e., 30–50 explants) were plated individually on 1.45% agarose-coated 48-well plates and cultured in DMEM supplemented with 10% fetal bovine serum. Cultures received fresh medium once every 2 weeks. For our experiments, we used only those explants that became spherical (i.e., spheroids) and that were approximately 2 mm in diameter.

For transduction of tumor spheroids, 100 μL of culture medium was removed from each tumor spheroid culture dish by aspiration, and 50 μL of adenovirus dl-lacZ or dl-lacZ-RLX in culture medium (i.e., 1×10^8, 1×10^9, or 1×10^{10} viral particles) was added. Three days later, β-galactosidase expression in each tumor spheroid was assessed by use of 5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside (Life Technologies, Rockville, MD), as described previously *(14)*. Each experiment was carried out three to four times with three replicates in each group, and the results were similar in all experiments. Data from a representative experiment are shown.

Spreading and Penetration of dl-lacZ-RLX In Vivo

To determine the extent of viral spread and tumor penetration, subcutaneous xenografts of U343, U87MG, C33A, Hep3B, and A549 cells were established by injecting 1×10^7 cells into the abdomen of 6- to 8-week-old male nude mice (123 mice total). When tumors reached a volume of 100–200 mm^3, mice were randomly assigned to receive either dl-lacZ or dl-lacZ-RLX adenovirus from 5×10^9 to 5×10^{10} viral particles per 50 μL that was intratumorally injected into the tumors three times, every other day. Three days after the last injection of virus, animals were killed by decapitation, and tumors were excised to determine viral distribution by assessing β-galactosidase expression with 5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside *(15)*. For better quantitation of β-galactosidase gene activity in tumor tissue, entire tumors were homogenized in lysis buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, and 0.1% Nonidet P-40, at pH 7.0) with a homogenizer (ART Labortecnik, Müllheim, Germany). The supernatant was collected by centrifugation at 15 000*g* for 10 minutes at 4 °C. The β-galactosidase activity was measured in the supernatant by use of an enhanced β-galactosidase assay kit (Gene Therapy Systems Inc, San Diego, CA) and then normalized to the total protein content of the extract. The protein content of the extracts was measured with a BCA protein assay kit (Pierce Biotechnology, Rockford, IL). Transduction efficiency was expressed as total cellular β-galactosidase activity per total protein content of each tumor.

Plaque Development Assay

Monolayers of Hep3B cells were infected with adenoviruses at an MOI of 1×10^{-4} in six-well plates. After a 4-hour incubation at 37 °C, the infected cells were overlayed with 4 mL of 0.7% Ultra-Pure agarose (Invitrogen, Carlsbad, CA) in DMEM with 5% fetal bovine serum. After 4–16 days of incubation, the agarose overlay was removed after soaking with 10% trichoroacetic acid for 30 minutes, and the remaining cells were stained with 0.5% crystal violet in 50% methanol. The number of plaques on any given day was plotted as the percentage of plaques on the final day of the assay.

Terminal Deoxynucleotidyltransferase-Mediated Deoxyuridine Triphosphate Nick End Labeling Assay

Apoptosis was examined by use of the terminal deoxynucleotidyltransferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) assay. U343, U87MG, C33A, Hep3B, and A549 cells were first plated onto chamber slides at a density of 5×10^4 cells per chamber. Twenty-four hours later, cells were infected with dl-lacZ or dl-lacZ-RLX adenovirus at an MOI of 5–10 or with Ad-ΔE1B or Ad-ΔE1B-RLX adenovirus at an MOI of 0.1–5 for 48 hours. Apoptotic cells were identified by detecting cleaved deoxyribonucleic acid by use of the in situ ApopTag kit (Chemicon International, Temecula, CA) according to the manufacturer's instructions. The apoptotic cells were visually identified in 10 selected fields and photographed at a magnification of ×400. More than 2000 cells were counted to calculate the percentage of TUNEL-positive cells (apoptotic cell ratio). To detect apoptotic cells in vivo, formalin-fixed tissue sections were prepared and processed as described previously *(10)*. Briefly, tumor sections (8 μm thick) were treated with proteinase K (20 μg/mL) for 15 minutes, and then endogenous peroxidase was blocked by treating sections with 3% hydrogen peroxide in phosphate-buffered saline (PBS) for 10 minutes. Sections were then incubated with terminal deoxynucleotidyltransferase buffer for 1 hour at 37 °C and were treated with an anti-digoxignenin antibody conjugate for 30 minutes. Standard avidin–biotin immunohistochemical techniques were used to cleave deoxyribonucleic acid according to the manufacturer's recommendations (Chemicon International). Diaminobenzidine was used as a chromogen, and 0.5% methyl green was used as counterstain.

Flow Cytometry Analysis

Development of apoptosis and cell nuclear damage were determined by dual staining with annexin-V–fluorescein isothiocyanate and propidium iodide and analyzed by flow cytometry. Cells were infected with dl-lacZ or dl-lacZ-RLX adenovirus at an MOI of 20–50 or with Ad-ΔE1B or Ad-ΔE1B-RLX adenovirus at an MOI of 0.5–20. As a positive control for the induction of apoptosis, cells were treated with 1 μM camptothecin. After 2 days of infection or treatment with camptothecin, cells were processed according to manufacturer's instruction in the ApoAlert Annexin V–FITC apoptosis kit (Clontech, Palo Alto, CA). Apoptosis and nuclear cell damage were quantified on a fluorescence-activated cell sorter (FACS) (Becton Dickinson, Sunnyvale, CA), and data from 10 000 events were collected for further analysis.

Antitumor Effects of Relaxin-Expressing Adenovirus in Human Xenograft Models

Xenograft tumors were implanted in the abdomens of 5- to 6-week-old male nude mice by subcutaneous injection of 1×10^7 U343, U87MG, C33A, Hep3B, or A549 cancer cells in 100 μL of Hanks' balanced salt solution (Gibco BRL). When tumors reached a volume of 60–80 mm^3, mice were randomly assigned to one of three groups to receive PBS, Ad-ΔE1B, or Ad-ΔE1B-RLX (eight or nine mice per group). The first day of treatment was designated as day 1. Adenovirus or PBS was administered intratumorally (for C33A tumors, 1×10^{10} viral particles per tumor in 50 μL of PBS, and for U343, U87MG, Hep3B, or A549 tumors, 5×10^{10} viral particles per tumor in 50 μL of PBS) on days 1, 3, and 5. Tumor growth delay was assessed by taking measurements every 2 or 3 days. Tumor volume was calculated by the following formula: volume = $0.523LW^2$, where L is length and W is width. Tumor responses to each treatment were compared by use of the Mann–Whitney test. The percentage of surviving mice was determined by monitoring the death of mice (for U87MG tumors) or tumor growth–related events (tumor size of >1000 mm^3 for A549 xenograft tumors or tumor size of >2000 mm^3 for U343, C33A, and Hep3B xenograft tumors) over a period of 80 days. Various endpoints were used for each tumor type because growth characteristics of each tumor model were different.

Evaluation of Tumor Xenograft by Histology and Immunohistochemistry

Tumor tissue was fixed in 10% formalin and embedded in paraffin (Wax-it, Vancouver, Canada), and 3-μm sections were cut. Representative sections were stained with hematoxylin–eosin or with Masson's trichrome and then examined by light microscopy. We used reagents supplied by DAKO ARK (Dako, Carpinteria, CA) to

detect adenovirus antigens, as described previously *(16)*. The same paraffin slides were also used to identify apoptotic cells in tumors according to the instructions in the ApopTag kit for detecting cleaved deoxyribonucleic acids in situ with the TUNEL method.

Murine B16BL6 Spontaneous Lung Metastasis Model

A spontaneous metastasis model was used to examine the effect of increased relaxin gene expression on tumor metastasis. Briefly, 2×10^5 B16BL6 cells per mouse were administered subcutaneously into the right hind footpad of a 6-week-old male C57BL/6 mouse (Charles River Korea, Seoul, Korea). When the tumor reached a volume of approximately 200 mm^3, mice were randomly assigned to one of three groups to receive PBS, Ad-ΔE1B, or Ad-ΔE1B-RLX (five mice per group). The first day of treatment was designated as day 1, and then 5×10^{10} viral particles per tumor in 50 μL of PBS, mixed with Lipofectamin and Plus solution (Gibco BRL) at a 2 (2 μL) : 6 (6 μL) ratio or PBS was injected directly into the tumor on days 1, 3, and 5. On day 7, primary tumors were surgically removed by amputating the right hind leg below the knee under mild anesthesia. On day 25 after primary tumor removal, the weight of metastatic tumor lesions in the lungs of the mice was assessed.

Orthotopic Hepatocellular Carcinoma Model in Athymic Mice

To determine the effects of systemic injection of relaxin-expressing, replication-competent adenovirus Ad-ΔE1B-RLX on hepatic tumor growth, 1×10^5 Hep1 human hepatoma cells in 50 μL of Hanks' balanced salt solution were injected directly into the left lobe of the liver of athymic nude mice. Seven days after tumor cell implantation, mice were randomly assigned to receive systemic treatment with PBS (5 mice) or with 2×10^{10} Ad-ΔE1B (four mice) or Ad-ΔE1B-RLX (six mice) viral particles in 100 μL of PBS three times every other day by tail-vein injection. At 42 days after the tumor challenge, mice were killed by decapitation, and livers were excised. To determine the tumor burden of the excised livers, tumors were then carefully removed from the liver and weighed for comparison.

Toxicology Studies

Because human adenoviruses replicate only in human cells, toxicology studies with adenoviruses are hampered by the availability of animal models; thus, we assessed toxicity in a nonpermissive host, 8-week-old male C58BL/6 mice. For blood analysis, at 3 days after intravenous (tail vein) or intratumoral administration at a dose of 2×10^{10} dl-lacZ, dl-lacZ-RLX, Ad-ΔElB, or Ad-ΔElB-RLX adenoviral particles, blood samples were obtained by cardiac puncture and assayed for the aspartate aminotransferase (AST), alanine aminotransferase (ALT), and serum levels of T-bilirubin to assess liver function. In addition, liver tissues were fixed in 10% neutral buffered formalin for 24 hours, and 5-μm tissue sections were cut and stained with hematoxylin and eosin. Examination for tissue toxicity or damage was carried out by an experienced mouse pathologist who was blinded to this study.

Statistical Analysis

Data were expressed as the mean with its 95% confidence interval (CI). Statistical comparisons were made with Stat View software (Abacus Concepts, Inc, Berkeley, CA) and the Mann–Whitney test (nonparametric rank sum test). *P* values of less than .05 were considered statistically significant. All statistical tests were two-sided. Survival was assessed with the Kaplan–Meier method, and results were compared with a log-rank test (SPSS software, version 12.0, SPSS Inc, Chicago, IL). Differences in survival were considered statistically significant when *P* values were less than .05.

Results

Transduction Efficiency and Viral Spread of Relaxin-Expressing Adenovirus

We first examined the expression of relaxin in cultured C33A and U343 cells after their infection with adenovirus dl-lacZ-RLX or Ad-ΔE1B-RLX. Infection with Ad-ΔE1B-RLX induced higher levels of relaxin expression than transduction with dl-lacZ-RLX at all MOIs examined (Fig. 1, B).

Tumor tissue spheroids were then used to evaluate the transduction efficiency and tissue penetration of relaxin-expressing adenoviruses. Tumor tissue spheroids provide a useful three-dimensional model resembling tumor tissue for assessing viral spread and a convenient means of maintaining primary tumor cells in vitro. Spheroids transduced with dl-lacZ-RLX expressed β-galactosidase more strongly on their peripheral surface than spheroids transduced with dl-lacZ (Fig. 1, C and Supplementary Fig. 1, A [available at: http://jncicancerspectrum.oxfordjournals.org/jnci/content/vol98/issue20]). Cross sectioning of spheroids transduced with dl-lacZ demonstrated that expression of β-galactosidase was limited to a few outer layers of cells. Increasing the adenoviral concentration up to 1×10^{10} viral particles per spheroid or analyzing gene transfer at later time points did not enhance the transduction of tumor cells beyond the rim of the spheroids (data not shown). In contrast, β-galactosidase expression in spheroids transduced with dl-lacZ-RLX was observed throughout the entire spheroid. Thus, relaxin-expressing adenovirus dl-lacZ-RLX was transduced and spread to the core of the spheroid more efficiently than the control vector dl-lacZ.

To determine whether the enhanced transduction efficiency and viral spread of dl-lacZ-RLX observed in tumor spheroids would lead to an increase in lacZ gene delivery to tumor mass in vivo, we used xenograft tumors in mice. In all xenograft models examined, only a few β-galactopyranoside–positive cells were observed in dl-lacZ–transduced tumors, but cells throughout dl-lacZ-RLX–transduced tumors strongly expressed β-galactosidase (Fig. 1, D and Supplementary Fig. 1, B [available at: http://jncicancerspectrum.oxfordjournals.org/jnci/content/vol98/issue20]). In particular, intense β-galactosidase expression was observed throughout the U87MG and C33A tumor xenografts transduced with dl-lacZ-RLX, indicating extensive viral spread and penetration in these xenograft models.

To further evaluate β-galactosidase expression in vivo, U343 and A549 xenograft tumors were intratumorally injected one, three, and five times with adenovirus dl-lacZ or dl-lacZ-RLX, and β-galactosidase activity was assessed. Tumors transduced with adenovirus dl-lacZ-RLX contained higher levels of β-galactosidase activity than tumors infected with the control virus dl-lacZ, in both U343 and A549 xenograft models (Fig. 1, E). We also observed higher gene transfer efficiency in tumors injected with adenovirus five times than in tumors injected one or three times. Thus, relaxin expression appeared to enhance transgene expression and viral spread in solid tumors in vitro and in vivo.

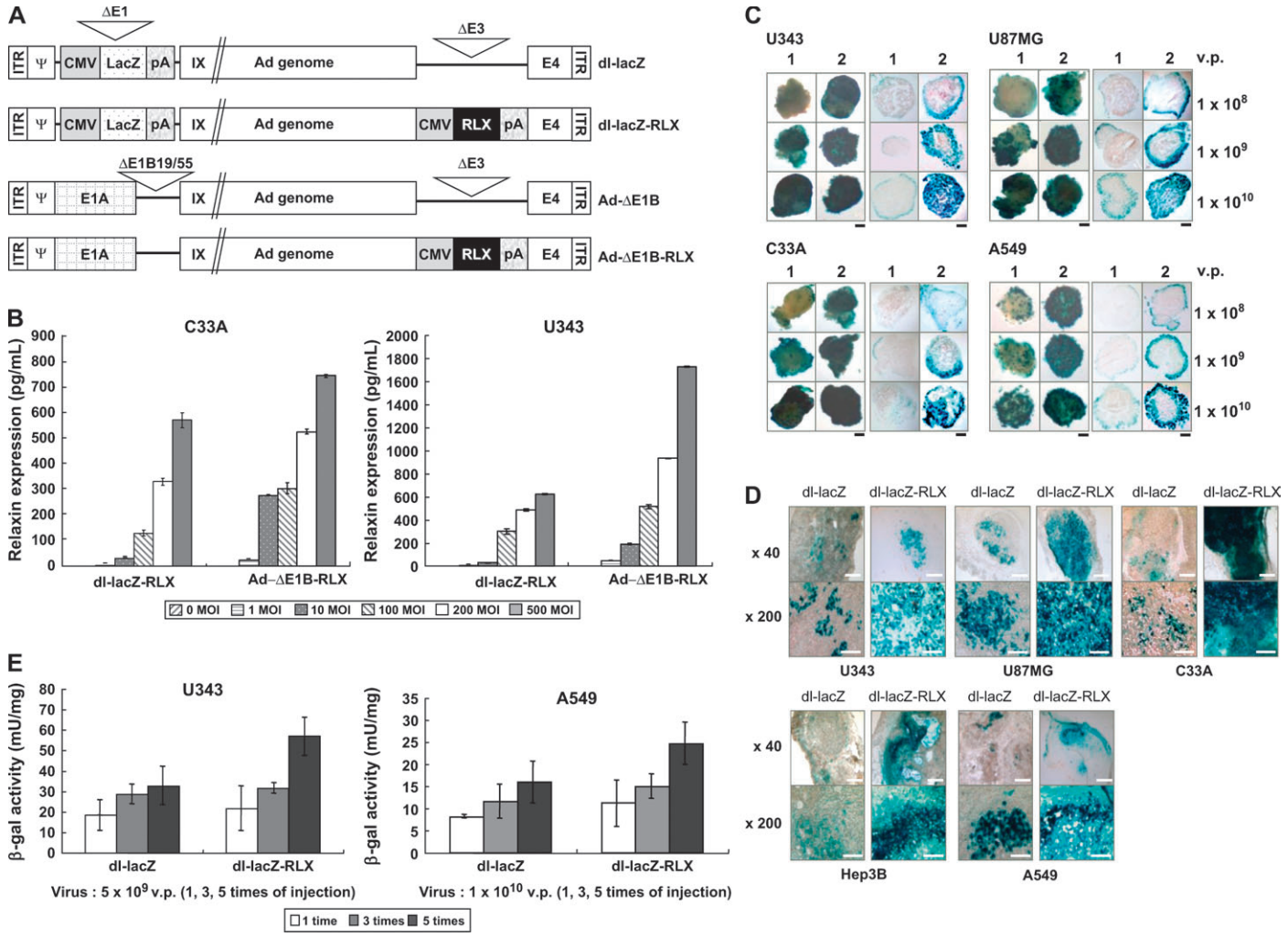

Fig. 1. Characterization of relaxin-expressing adenoviruses. **A**) Schematic representations of the adenoviral vectors used in this study. All indicated adenoviral vectors were derived from full-length adenovirus genomes cloned and manipulated in *Escherichia coli* as bacterial plasmids. Replication-incompetent adenovirus that does not express relaxin, dl-lacZ, has the whole E1 region deleted and expresses the reporter gene lacZ (β-galactosidase protein) under the control of the constitutive cytomegalovirus (CMV) promoter inserted into the E1 region. Replication-competent adenovirus that does not express relaxin, Ad-ΔE1B, contains a normal E1A gene but lacks E1B 19-kDa and E1B 55-kDa genes. The replication-incompetent relaxin-expressing adenovirus dl-lacZ-RLX and the replication-competent relaxin-expressing adenovirus Ad-ΔE1B-RLX carry a relaxin gene driven by the CMV promoter that was inserted into the E3 region. ΔE1 and ΔE3 denote the deletion of E1 and E3 genes, respectively. ITR = inverted terminal repeat; Ψ = packaging signal; pA = polyA sequence; IX = protein IX; and RLX = relaxin. **B**) Quantitative enzyme-linked immunosorbent assay results showing relaxin expression mediated by adenoviruses dl-lacZ-RLX and Ad-ΔE1B-RLX at multiplicities of infection (MOIs) between 0 and 500. Relaxin concentration was measured in the culture supernatant 48 hours after adenoviral infection with conventional enzyme-linked immunosorbent assay kits. Data are representative of three independent experiments with each point performed in quadruplicate. **Error bars** indicate 95% confidence intervals for quadruplicate data points. **C**) Transduction efficiency and viral spread of relaxin-expressing adenovirus in tumor spheroids. We prepared tumor spheroids from the indicated xenograft tumors in mice. Spheroids were transduced with adenoviruses dl-lacZ, which did not express relaxin (**columns 1**), or dl-lacZ-RLX, which expressed relaxin (**columns 2**), at 1×10^8, 1×10^9, and 1×10^{10} viral particles per 50 μL. Three days after infection, spheroids were processed to visualize β-galactosidase expression (**blue**) and then sectioned. Viral penetration and transduction efficiency in the central areas of the spheroids were then assessed by light microscopy. In each panel, **columns to the left** show whole spheroids (stereoscopic light microscope; original magnification = ×38) and **columns to the right** show sections through the spheroid midpoint (original magnification = ×40). **Scale bars** are 400 μm. **D**) Transduction efficiency and viral spread of relaxin-expressing adenovirus in U343, U87MG, C33A, Hep3B, and A549 xenografts. We established xenografts in nude mice and then injected them intratumorally with dl-lacZ or dl-lacZ-RLX adenovirus (at 5×10^9 to 5×10^{10} viral particles per 50 μL) three times every other day. Three days after the last injection of adenovirus, tumors were harvested and processed to visualize β-galactosidase expression (**blue**). **Scale bars** are 400 μm at ×40 and 100 μm at ×200 magnifications (Original magnifications = ×40 and ×200, respectively). **E**) Quantitation of the β-galactosidase expression in tumor tissue treated one, three, or five times with adenoviruses at 5×10^9 virus particles (v.p.) or at 1×10^{10} v.p. that do not (dl-lacZ) or do (dl-lacZ-RLX) express relaxin. The transduction efficiency was expressed as total cellular β-galactosidase activity (in milliunits) per total protein content (in milligrams). Data are the means and 95% confidence intervals of three independent experiments, with each point performed in triplicate.

Cytopathic Effect and Plaque Formation of Relaxin-Expressing Adenovirus

To achieve a clinically significant therapeutic outcome, replicating adenoviral vectors must be able to be distributed efficiently throughout the tumor mass and to overcome the barrier imposed by the extracellular matrix. In an attempt to overcome limited tumor mass penetration by oncolytic adenovirus, we generated an E1B-deleted oncolytic adenovirus (termed Ad-ΔE1B-RLX) that expresses relaxin. We first examined Ad-ΔE1B-RLX in an in vitro cytopathic effect assay to determine the effect of relaxin expression on de novo adenoviral replication. Adenovirus Ad-ΔE1B-RLX elicited a greater cytopathic effect than Ad-ΔE1B, indicating that relaxin expression does not inhibit viral replication (data not shown).

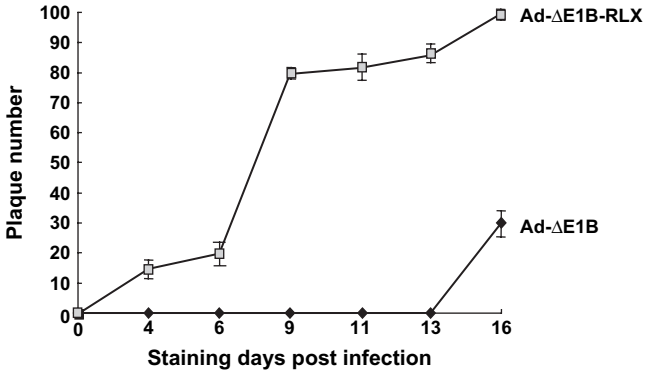

Fig. 2. Plaque-forming ability of relaxin-expressing oncolytic adenovirus. Hep3B cells were infected with control adenovirus Ad-ΔE1B or relaxin-expressing adenovirus Ad-ΔE1B-RLX. After 4 hours of adsorption, plates were overlaid with agarose and incubated. Four, 6, 9, 11, 13, and 16 days after infection, cells were visualized by staining with crystal violet; the number of plaques formed was counted. Data are representative of three independent experiments with each point performed in triplicate. **Error bars** are 95% confidence intervals.

To visualize the effect of relaxin expression on the cytopathic ability and viral spread into surrounding cells, we used a plaque-forming assay (Fig. 2). Adenovirus Ad-ΔE1B, which does not express relaxin, generated plaques 16 days after infection, whereas the relaxin-expressing adenovirus Ad-ΔE1B-RLX generated plaques only 4 days after infection, demonstrating that the expression of relaxin is associated with the induction of early viral release and accelerated cell-to-cell spread of viruses.

Relaxin and Apoptosis

We used a terminal TUNEL assay to determine whether the increased cell killing of a relaxin-expressing adenovirus was mediated by the induction of apoptosis. Ad-ΔE1B-RLX induced a statistically significantly higher percentage of all cells examined to undergo apoptosis than Ad-ΔE1B ($P = .025$ for U343, $P = .012$ for U87MG, $P = .036$ for C33A, $P = .028$ for Hep3B, and $P = .021$ for A549) (Fig. 3). More specifically, there were 69.7% (95% CI = 65.0 to 74.4), 77.0% (95% CI = 61.2 to 92.8), 79.8% (95% CI = 61.8 to 97.8), 69.7% (95% CI = 56.0 to 83.4), and 75.2% (95% CI = 74.1 to 76.3) of TUNEL-positive cells among Ad-ΔE1B-RLX–treated U343, U87MG, C33A, Hep3B, and A549 cells, respectively, whereas there were only 32.5% (95% CI = 21.5 to 43.5), 16.5% (95% CI = 11.9 to 21.1), 45.2% (95% CI = 38.5 to 51.9), 38.5% (95% CI = 36.2 to 40.8), and 34.8% (95% CI = 24.9 to 44.7), respectively, of TUNEL-positive, Ad-ΔE1B–treated cells.

The induction of apoptosis by relaxin-expressing adenoviruses was also evaluated with flow cytometry in several cell lines (Table 1). Healthy cells are negative for annexin-V and propidium iodide, whereas early apoptotic cells are positive for annexin-V and negative for propidium iodide. When U343 cells

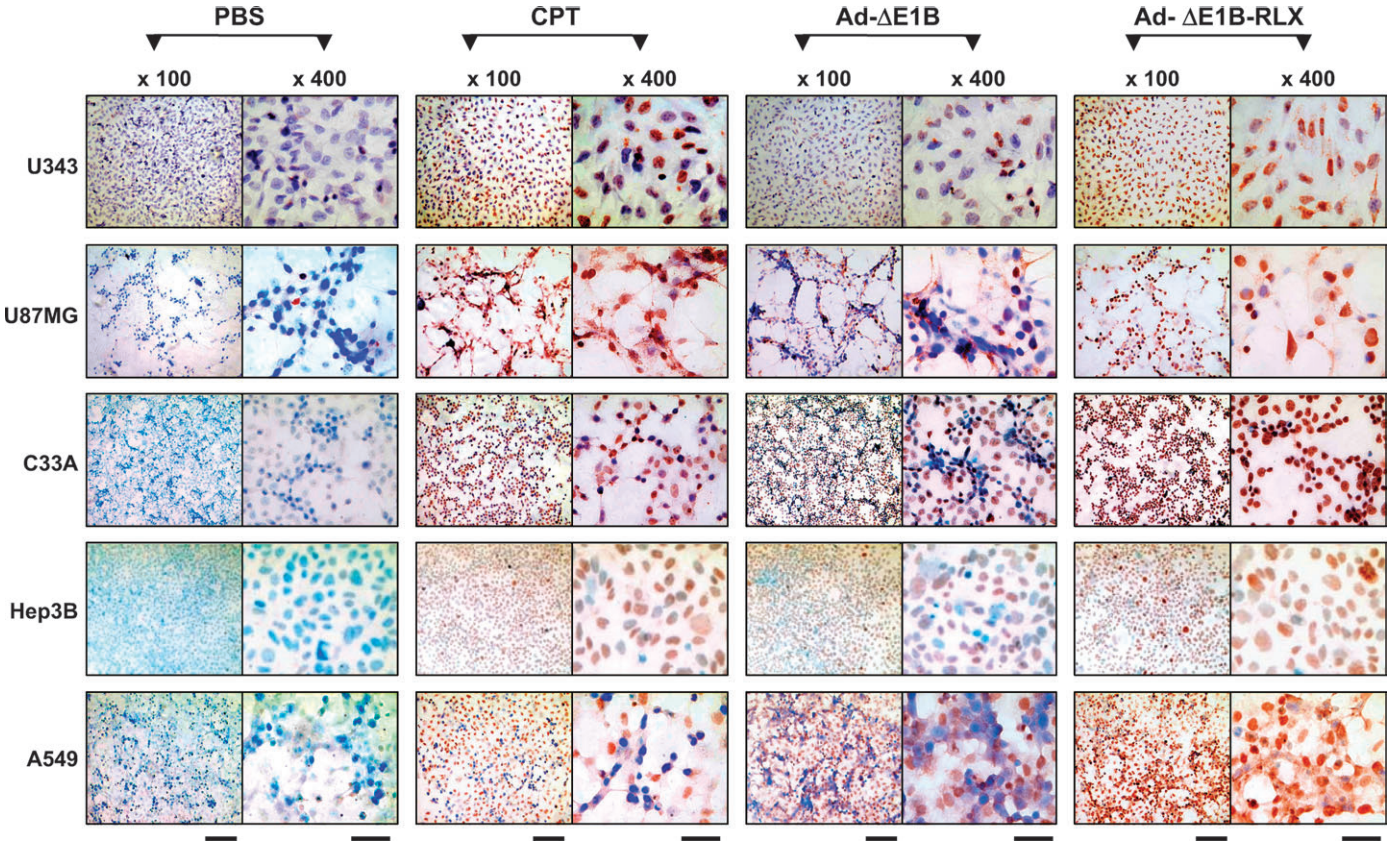

Fig. 3. Relaxin and apoptosis. An in vitro terminal deoxynucleotidyltransferase-mediated deoxyuridine triphosphate nick end labeling assay was used to assess apoptosis. U343, U87MG, C33A, Hep3B, and A549 cells were treated with phosphate-buffered saline (PBS) or with 1 μM camptothecin (CPT) or infected with control adenovirus Ad-ΔE1B or relaxing-expressing adenovirus Ad-ΔE1B-RLX at a multiplicity of infection of 0.1–5. Forty-eight hours after treatment, apoptotic cells were detected by labeling with 3,3′-diaminobenzidine and terminal deoxynucleotidyltransferase and treated with anti-digoxignenin antibody conjugate to visualize positive cells. Slides were then counterstained with methyl green. DNA strand breakage is indicated by **brown staining**. A representative field from three independent experiments is shown. (Original magnifications = ×100 and ×400). **Scale bars** are 50 μm for ×40 and 100 μm for ×400.

Table 1. Relaxin and apoptosis

Assay	Cell line	Treatment			
		PBS	CPT	Ad-ΔE1B or dl-lacZ	Ad-ΔE1B-RXL or dl-lacZ-RLX
TUNEL assay	U343	10.5 (5.4 to 15.6)	53.5 (47.0 to 60.0)	32.5 (21.5 to 43.5)	69.7 (65.0 to 74.4)
	U87MG	2.5 (1.5 to 3.5)	83.0 (57.3 to 100)	16.5 (11.9 to 21.1)	77.0 (61.2 to 92.8)
	C33A	5.65 (2.8 to 8.5)	60.1 (37.4 to 82.8)	45.2 (38.5 to 51.9)	79.8 (61.8 to 97.8)
	Hep3B	1.65 (1.1 to 2.2)	71.2 (57.4 to 85.0)	38.5 (36.2 to 40.8)	69.7 (56.0 to 83.4)
	A549	3.5 (2.8 to 4.2)	37.5 (32.8 to 42.2)	34.8 (24.9 to 44.7)	75.2 (74.1 to 76.3)
FACS analysis†	U343	1.15 (0.8 to 1.5)	32.15 (25.7 to 38.6)	24.15 (18.1 to 30.2)	32.70 (25.2 to 40.2)
	U87MG	2.54 (1.5 to 3.6)	70.35 (63.3 to 77.4)	7.04 (6.4 to 7.7)	18.28 (14.6 to 22.0)
	C33A	2.93 (2.1 to 3.8)	57.10 (52.0 to 62.2)	21.99 (19.4 to 24.6)	33.03 (28.5 to 37.6)
	Hep3B	4.97 (4.2 to 5.7)	13.00 (11.6 to 14.4)	16.58 (12.4 to 20.8)	37.87 (28.4 to 47.3)
	A549	0.53 (0.2 to 0.8)	4.48 (2.9 to 6.1)	2.05 (1.7 to 2.4)	13.12 (9.2 to 17.0)
TUNEL assay‡	U343	1.5 (0 to 4.2)	74.7 (63.8 to 85.5)	15.1 (8.6 to 21.6)	46.3 (44.1 to 48.6)
	U87MG	1.9 (0.6 to 3.3)	40.0 (28.5 to 51.5)	15.0 (12.9 to 17.1)	44.4 (38.1 to 50.8)
	C33A	3.6 (1.5 to 5.8)	70.5 (56.6 to 84.5)	18.6 (9.8 to 27.3)	54.7 (43.1 to 66.4)
	Hep3B	3.3 (2.0 to 4.7)	75.9 (73.1 to 78.6)	23.6 (15.5 to 31.7)	45.3 (38.8 to 51.7)
	A549	3.0 (1.1 to 4.9)	69.8 (62.5 to 77.1)	7.8 (5.7 to 10.0)	52.1 (40.1 to 64.1)
FACS analysis§	U343	4.11 (2.5 to 5.7)	19.41 (13.4 to 25.4)	3.93 (1.6 to 6.3)	12.84 (10.3 to 15.4)
	U87MG	3.61 (1.8 to 5.4)	9.34 (6.5 to 12.2)	4.84 (2.9 to 6.8)	8.33 (7.1 to 9.6)
	C33A	4.47 (2.8 to 6.1)	22.50 (14.6 to 30.4)	12.50 (8.8 to 16.2)	16.57 (12.1 to 21.0)
	Hep3B	11.76 (9.4 to 14.1)	23.54 (17.4 to 29.7)	5.74 (4.7 to 6.8)	13.41 (10.2 to 16.6)
	A549	5.01 (3.3 to 6.7)	14.76 (13.1 to 16.4)	5.03 (2.5 to 7.6)	11.47 (9.5 to 13.4)

*TUNEL analysis for apoptotic cells. Cells were infected with Ad-ΔE1B or Ad-ΔE1B-RLX adenoviruses or treated with CPT or PBS and then subjected to TUNEL analysis. The number of apoptotic cells per 2000 cells in each micrograph in Fig. 3 was counted. Data are the mean percentage of brown-stained TUNEL-positive apoptotic cells (95% confidence interval [CI]) in triplicate samples. CPT = camptothecin; PBS = phosphate-buffered saline; TUNEL = terminal deoxynucleotidyl-transferase-mediated dUTP nick end labeling.

†Analysis of annexin-V and propidium iodide fluorescence by flow cytometry (FACS = fluorescence-activated cell sorter). Cells were infected with Ad-ΔE1B or Ad-ΔE1B-RLX adenoviruses or treated with CPT or PBS. Cell pellets were resuspended in 100 μL of binding buffer and then reacted with annexin-V (3 μL) and propidium iodide (2 μL) at room temperature. The stained cells were then analyzed with a FACS. Data are the percentage of annexin-V-positive and propidium iodide-negative cells (i.e., cells in early apoptosis) (95% CI). Results are representative of three independent experiments with triplicate samples for each condition.

‡TUNEL analysis of cells transduced with dl-lacZ or dl-lacZ-RLX at a multiplicity of infection of 5–10 or treated with PBS or CPT. Data are the mean percentage of apoptotic cells induced by each treatment (95% CI). Results are representative of three independent experiments, with each point being the mean of triplicate samples.

§Analysis of annexin-V and propidium iodide fluorescence of the cells transduced with adenoviruses dl-lacZ or dl-lacZ-RLX or treated with PBS or CPT. Data are the mean percentage of annexin-V-positive and propidium iodide-negative cells (i.e., cells in early apoptosis) (95% CI). Results are representative of three independent experiments, with each point being the mean of triplicate samples.

were evaluated, 32.15 % (95% CI = 25.7 to 38.6) of cells were apoptotic after treatment with camptothecin, 32.70% (95% CI = 25.2 to 40.2) of cells were apoptotic after infection with the relaxin-expressing adenovirus Ad-ΔE1B-RLX, but only 24.15% (95% CI = 18.1 to 30.2) of cells were apoptotic after infection with Ad-ΔE1B, which does not express relaxin. These same patterns were observed in the rest of the cancer cell lines (U87MG, C33A, Hep3B, and A549) evaluated. Thus, relaxin expressed from adenoviruses was associated with increased levels of apoptosis and is consistent with the results of the cytopathic effect and plaque development assays.

To further confirm the role of relaxin as an apoptosis inducer, we also performed TUNEL and flow cytometry analyses with the relaxin-expressing, replication-incompetent adenovirus dl-lacZ-RLX. Two days after adenovirus transduction, the mean percentage of TUNEL-positive apoptotic cells increased by 2.7% (95% CI = 0% to 5.8%) in untreated cultures, by 16.0% (95% CI = 5.7% to 31.7%) in dl-lacZ–transduced cultures, and by 48.6% (95% CI = 38.1% to 66.4%) in dl-lacZ-RLX–transduced cultures (Table 1). In flow cytometry analyses, the percentage of cells in early apoptosis (i.e., cells positive for annexin-V and negative for propidium iodide) was also higher when cells were transduced with dl-lacZ-RLX than with dl-lacZ (Table 1). In another series of experiments, in which A549 or U343 cells were transfected with expression plasmids that did (pSP72ΔE3-RLX) or did not (pSP72ΔE3) express relaxin, instead of with adenoviruses, we found that expression of relaxin statistically significantly increased the percentage of cells in the sub-G_1 population, which reflects the population of apoptotic cells (Supplementary Fig. 2, available at: http://jncicancerspectrum.oxfordjournals.org/jnci/content/vol98/issue20). These data demonstrate, for the first time to our knowledge, that the expression of relaxin by adenoviruses could induce apoptosis.

Enhanced Antitumor Effect of Relaxin-Expressing Oncolytic Adenovirus

The relaxin-expressing oncolytic adenovirus Ad-ΔE1B-RLX was next examined for its ability to suppress the growth of human xenograft tumors when administered intratumorally. The growth of all xenograft tumors treated with relaxin-expressing adenovirus Ad-ΔE1B-RLX was substantially delayed compared with that of tumors treated with PBS or with Ad-ΔE1B, which does not express relaxin (Fig. 4, A). For example, by day 32 after treatment, tumors of C33A tumor-bearing mice treated with PBS reached an average tumor volume of 2252 mm^3 (95% CI = 1980 to 2524 mm^3), those treated with Ad-ΔE1B reached 917 mm^3 (difference = 1335 mm^3, 95% CI = 725 to 2449 mm^3; P = .007), and those treated with Ad-ΔE1B-RLX reached 77 mm^3 (difference = 2175 mm^3, 95% CI = 1184 to 3159 mm^3; P<.001). In addition, at day 45 after treatment, complete tumor regression was observed in 25% of the mice with Ad-ΔE1B–treated tumors and in 50% of those with Ad-ΔE1B-RLX–treated tumors. The tumor growth suppression that was observed in the C33A

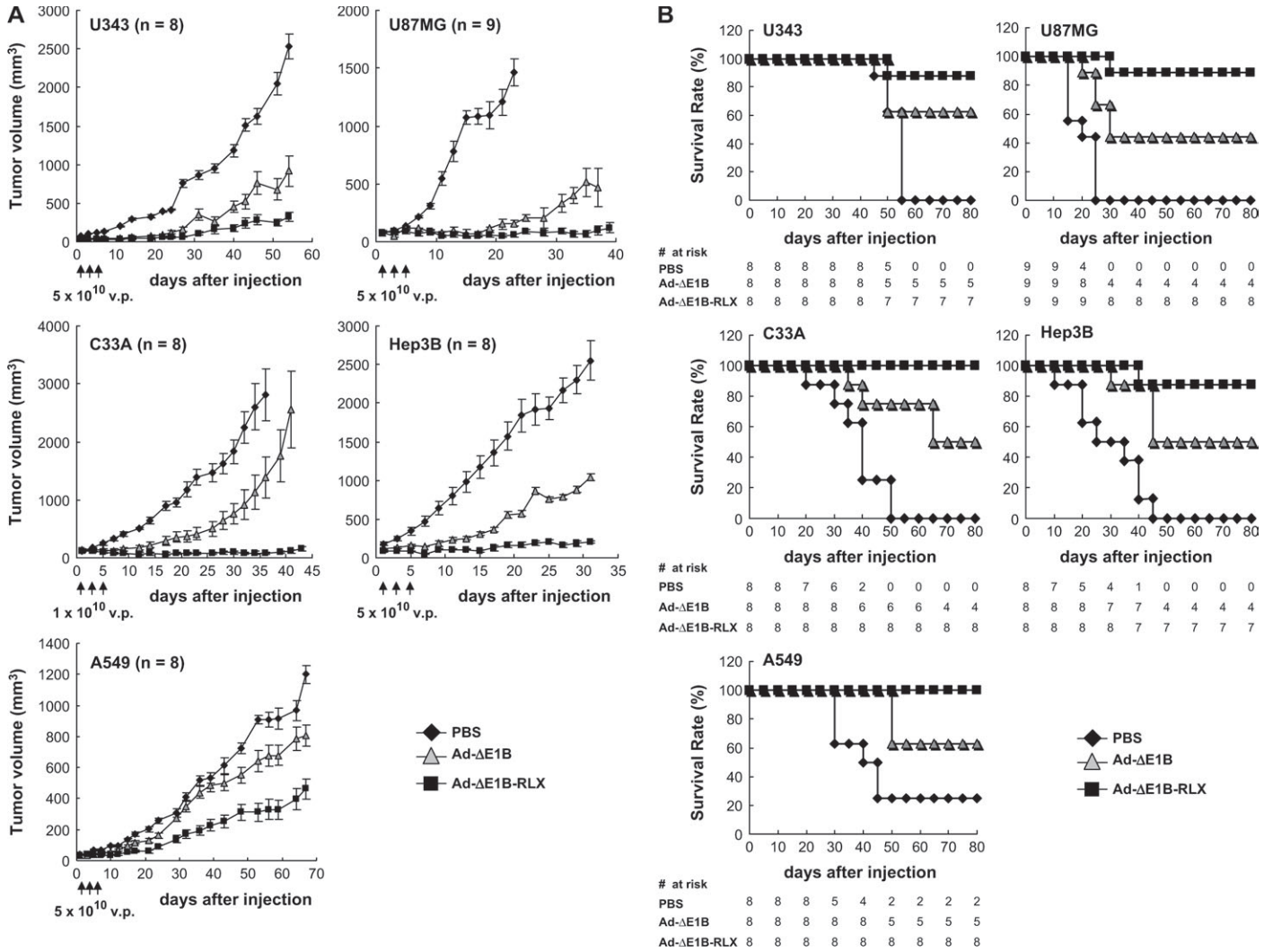

Fig. 4. Relaxin-expressing adenovirus Ad-ΔE1B-RLX and the growth of established tumors and survival of mice. U343, U87MG, C33A, Hep3B, or A549 tumors that were growing subcutaneously in the abdomen of nude mice were injected intratumorally with phosphate-buffered saline (PBS), control adenovirus Ad-ΔE1B, or relaxin-expressing adenovirus Ad-ΔE1B-RLX. **A**) Tumor volume was monitored regularly after treatment. **Arrows** indicate when treatment was given (1×10^{10} viral particles per 50 μL for C33A and 5×10^{10} viral particles per 50 μL for U343, U87MG, Hep3B, and A549 per treatment). Data are the means and 95% confidence intervals (CIs) (n = 8 animals per group with U343, C33A, Hep3B, or A549 tumors and n = 9 for U87MG tumors). **B**) Overall survival rate. The percentage of surviving mice was determined by monitoring the death of mice over a period of 80 days after treatment. For mice bearing U343 tumors that were treated with PBS, survival probability was 0% (95% CI = 0% to 36.9%); for mice treated with Ad-ΔE1B, survival was 62.5% (95% CI = 24.5% to 91.5%); and for mice treated with Ad-ΔE1B-RLX, survival was 87.5% (95% CI = 47.3% to 99.7%). For mice bearing U87MG tumors that were treated with PBS, survival probability was 0% (95% CI = 0% to 33.6%); for mice treated with Ad-ΔE1B, survival was 44.4% (95% CI = 13.7% to 78.8%); and for mice treated with Ad-ΔE1B-RLX, survival was 88.9% (95% CI = 51.8% to 99.7%). For mice bearing C33A tumors treated with PBS, survival probability was 0% (95% CI = 0% to 36.9%); for mice treated with Ad-ΔE1B, survival was 50% (95% CI = 15.7% to 84.3%); and for mice treated with Ad-ΔE1B-RLX, survival was 100% (95% CI = 63.1% to 100%). For mice bearing Hep3B tumors treated with PBS, survival probability was 0% (95% CI = 0% to 36.9%); for mice treated with Ad-ΔE1B, survival was 50% (95% CI = 15.7% to 84.3%); and for mice treated with Ad-ΔE1B-RLX, survival was 87.5% (95% CI = 47.3% to 99.7%). For mice bearing A549 tumors treated with PBS, survival probability was 25% (95% CI = 0.3% to 52.7%); for mice treated with Ad-ΔE1B, survival was 62.5% (95% CI = 24.5% to 91.5%); and for mice treated with Ad-ΔE1B-RLX, survival was 100% (95% CI = 63.1% to 100%). Ad-ΔE1B-RLX–treated mice survived statistically significantly longer ($P<.001$ for U87MG, $P = .002$ for U343, $P<.001$ for C33A, $P<.001$ for Hep3B, $P<.001$ for A549) than mice treated with Ad-ΔE1B or PBS. Survival was assessed by the Kaplan–Meier method, and the results were compared with a log-rank test (SPSS software). All statistical tests were two-sided.

xenograft tumor model was also observed in all other xenograft (U343, U87MG, Hep3B, and A549) models tested.

We also performed survival analyses for PBS-, Ad-ΔE1B–, and Ad-ΔE1B-RLX–treated tumor-bearing mice (Fig. 4, B). For C33A tumor-bearing mice, 80 days after the beginning of the treatment, 100% (95% CI of survival probability = 63.1% to 100%) of the animals treated with Ad-ΔE1B-RLX were still viable, whereas only 50% (95% CI of survival probability = 15.7% to 84.3%) of Ad-ΔE1B–treated mice were viable. In all xenograft models examined, tumor-bearing mice treated with Ad-ΔE1B-RLX survived longer than those treated with Ad-ΔE1B.

In Vivo TUNEL, Histologic, and Immunohistochemical Characterization

The antitumor activity of intratumorally administered relaxin-expressing oncolytic adenovirus was further investigated by histologic examination. Tumors were harvested from mice in each treatment group 7 days after the third of three sequential (every other day) adenovirus treatments. Most of the tumor mass remaining after treatment with the relaxin-expressing adenovirus Ad-ΔE1B-RLX was necrotic as shown by hematoxylin–eosin staining, whereas necrotic lesions were detected only

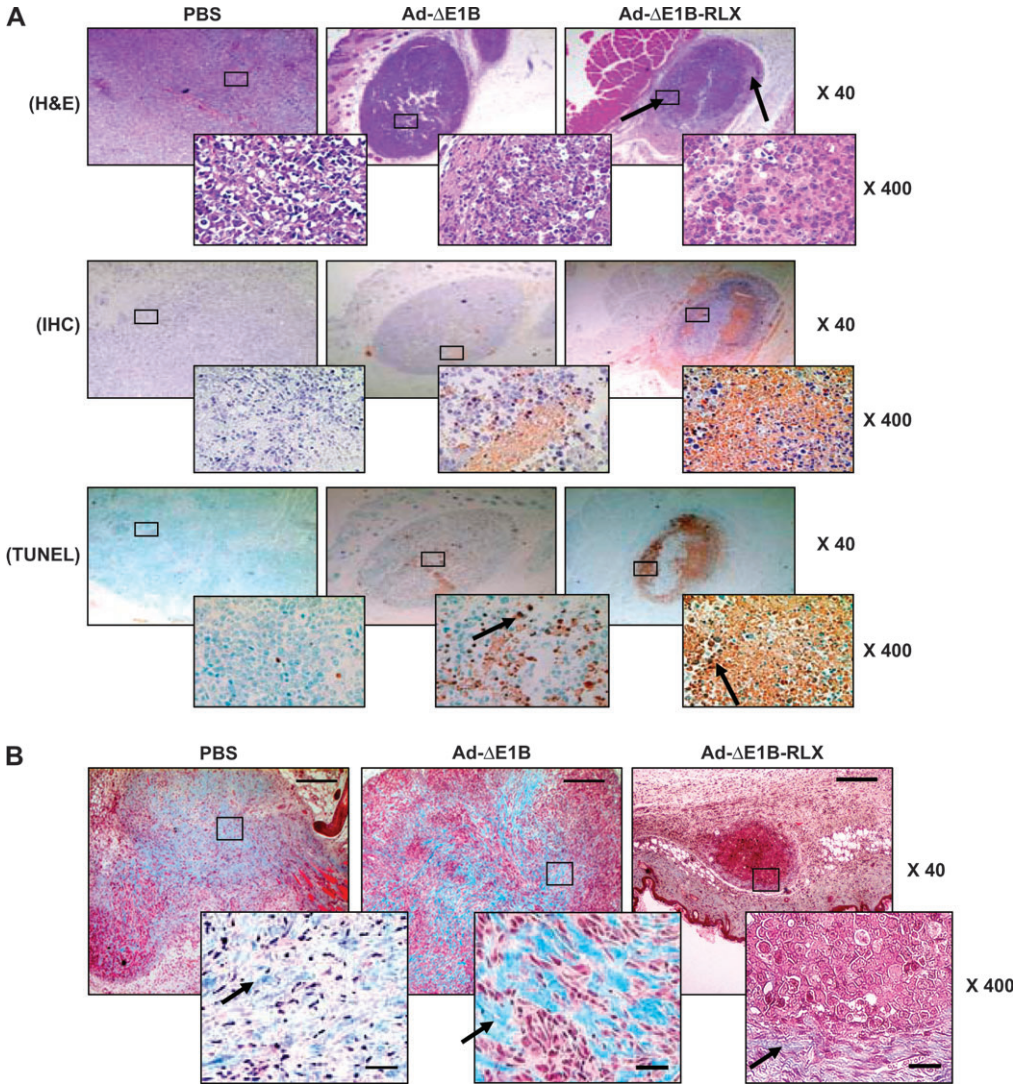

Fig. 5. Analysis of apoptosis and extracellular matrix of tumor tissues. C33A tumors were treated with phosphate-buffered saline (PBS), control adenovirus Ad-ΔE1B, or relaxin-expressing adenovirus Ad-ΔE1B-RLX, sectioned, and processed as indicated. **A**) Tumor sections were stained with hematoxylin–eosin (H&E) after treatment with PBS, Ad-ΔE1B, or Ad-ΔE1B-RLX. Necrotic cells in the periphery of the tumor treated with Ad-ΔE1B-RLX are indicated by **arrows**. Immunohistochemical (IHC) staining against adenoviral hexon protein was used to localize adenovirus in tumor tissue (**brown**). Terminal deoxynucleotidyltransferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) staining was used to identify apoptotic cells in tumor tissue, and then tissue was counterstained with methyl green. **Arrows** indicate apoptotic cells. **B**) Masson's trichrome staining of extracellular matrix (**blue**) in the tumor tissue sections. Tumors treated with Ad-ΔE1B-RLX were smaller than those treated with PBS or Ad-ΔE1B. **Arrows** indicate extensive bundles of collagen (**blue**). (Original magnification = ×40 and ×400, as indicated) **Scale bars** are 400 μm for ×40 and 40 μm for ×400.

in the central region of tumors treated with the control adenovirus Ad-ΔE1B (Fig. 5, A). Necrotic lesions in the tumors treated with Ad-ΔE1B-RLX were found mainly on the periphery of the tumor section but not at the center of tumor, where the blood supply is low or nonexistent. These observations indicate that necrosis was most likely the result of injected virus.

We also found more adenoviral particles in wider areas of Ad-ΔE1B-RLX–treated tumors, most frequently in the peripheral tumor area, than in Ad-ΔE1B–treated tumors. Furthermore, the level of apoptosis (as determined by the percentage of TUNEL-positive cells) was higher in Ad-ΔE1B-RLX–treated tumor tissue (40.2%, 95% CI = 31.1% to 49.2%; $P = .023$) than in PBS- or Ad-ΔE1B–treated tumor tissue (0.7%, 95% CI = 0% to 1.5%, and 8.1%, 95% CI = 1.6% to 14.7%, respectively). Apoptotic cells were also found mainly in the periphery of the tumor, corresponding to the area of necrosis and adenovirus particles detected. Thus, relaxin appeared to be able to induce apoptosis in vivo as well as in vitro.

Relaxin-Expressing Oncolytic Adenovirus and Collagen Content in Tumors

Sections from xenograft tumors in nude mice treated with PBS, Ad-ΔE1B, or Ad-ΔE1B-RLX were stained with Biebrich's scarlet acid fuchsin (Masson's trichrome stain). Control tumors treated with PBS or Ad-ΔE1B had a high content of collagen, but tumors treated with relaxin-expressing Ad-ΔE1B-RLX appeared to lack collagen, indicating that relaxin expression was associated with a reduction in the expression of collagen (Fig. 5, B). Tumors treated with Ad-ΔE1B-RLX were encapsulated with connective tissue, with collagen found only in the boundary area between tumor and normal tissue. This result closely parallels and supports the finding of increased viral spread in the tumor mass and enhanced antitumor effect of a relaxin-expressing oncolytic adenovirus.

Inhibition of Tumor Metastasis by Relaxin-Expressing Oncolytic Adenovirus

Because relaxin-induced matrix metalloproteinase expression has the potential to increase tumor cell metastasis, we evaluated the effect of relaxin-expressing oncolytic adenovirus on tumor metastasis by use of the B16BL6 spontaneous tumor metastasis model. The volume of B16BL6 lung metastases was statistically significantly reduced from that in PBS-treated mice (268 mg) by treatment with Ad-ΔE1B (48 mg; difference = 220 mg, 95% CI = 56 to 387 mg; $P = .009$) or Ad-ΔE1B-RLX (10 mg; difference = 258 mg, 95% CI = 94 to 426 mg; $P = .003$)

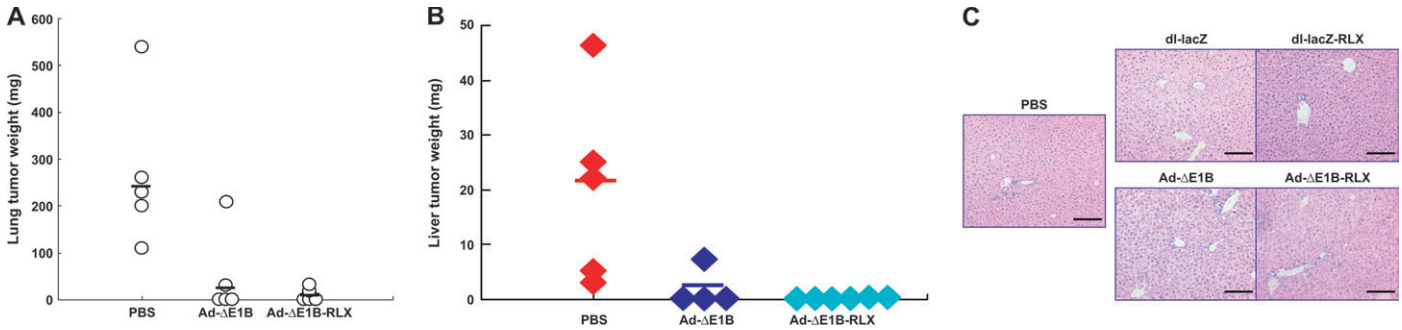

Fig. 6. Therapeutic efficacy of relaxin-expressing adenovirus Ad-ΔE1B-RLX on spontaneous pulmonary metastasis and orthotopic hepatoma model. **A**) Ad-ΔE1B-RLX and spontaneous lung metastasis. B16BL6 tumor-bearing mice were treated with phosphate-buffered saline (PBS), Ad-ΔE1B, or Ad-ΔE1B-RLX three times every other day, and then the primary tumors were surgically removed. Twenty-five days later, the total weight of pulmonary metastatic lesions from each mouse was determined. Each **point** represents the tumor burden for one mouse (five mice per group), and the mean weight of metastatic lesions for each group, compared with that of PBS treatment (268 mg), is shown with a **line** (for Ad-ΔE1B, 48 mg, 95% confidence interval [CI] = 0 to 161 mg, $P = .009$ versus PBS; for Ad-ΔE1B-RLX, 10 mg, 95% CI = 0 to 27.55 mg, $P = .003$ versus PBS; and for Ad-ΔE1B-RLX versus Ad-ΔE1B, $P = .408$). **B**) Ad-ΔE1B-RLX and orthotopic hepatic tumor growth by systemic administration. Seven days after the implantation of 1×10^5 Hep1 human hepatic cancer cells into the liver, mice were randomly assigned to treatment with PBS (n = 5 mice), Ad-ΔE1B (n = 4 mice), or Ad-ΔE1B-RLX (n = 6 mice) three times every other day by tail-vein injection. Livers were then isolated 42 days after Hep1-cell implant. Each **point** represents the tumor burden for one mouse, and the mean weight of tumor lesions for each group is shown with a **line**. $P = .004$ for Ad-ΔE1B-RLX versus PBS. **C**) Histologic analysis of liver tissue from mice treated with adenovirus dl-lacZ, dl-lacZ-RLX, Ad-ΔElB, or Ad-ΔElB-RLX intravenously (each with 2×10^{10} viral particles in 100 μL). Hematoxylin–eosin staining. **Scale bar** is100 μm.

(Fig. 6, A). The average relative tumor burden in the lung from mice treated with Ad-ΔE1B was reduced by 82% and that from mice treated with Ad-ΔE1B-RLX was reduced 96%. Moreover, in three of five mice treated with either Ad-ΔE1B or Ad-ΔE1B-RLX, the formation of metastatic lesions was completely inhibited. Thus, in this model system, the intratumoral injection of relaxin-expressing oncolytic adenovirus into the primary tumor site appeared to reduce, not promote, the formation of metastatic lesions in the lung.

Therapeutic Efficacy of Systemic Treatment With Relaxin-Expressing Oncolytic Adenovirus

To evaluate the effect of systemically delivered relaxin-expressing oncolytic adenovirus on tumor growth, we used the Hep1 orthotopic hepatoma model. Systemic treatment of mice bearing Hep1 tumors with control Ad-ΔE1B (1.8 mg of tumor per mouse; difference = 18.4 mg, 95% CI = 0.5 to 36.7 mg; $P = .007$) or relaxin-expressing Ad-ΔE1B-RLX (0 mg; difference = 20.2 mg, 95% CI = 3.7 to 36.7 mg; $P = .004$) resulted in a marked inhibition of tumor growth compared with PBS-treated controls (20.2 mg) (Fig. 6, B). In fact, only one of four mice in the Ad-ΔE1B group had any liver tumor, and none of the six animals in the Ad-ΔE1B-RLX–treated group had any visible macroscopic tumor lesions. Therefore, systemically delivered relaxin-expressing Ad-ΔE1B-RLX adenovirus appears to inhibit hepatic tumor growth.

Relaxin-Expressing Oncolytic Adenovirus and Toxicity

In preliminary analyses, we assessed potential side effects of relaxin-expressing adenoviruses by monitoring the level of liver transaminases in blood and animal body weights. Intravenous injection of 2×10^{10} adenoviral particles resulted in moderate increase in AST and ALT levels in one of three mice treated with dl-lacZ or Ad-ΔElB and modest increase in T-bilirubin levels in one, two, and one of three mice treated with dl-lacZ, dl-lacZ-RLX, and Ad-ΔElB-RLX, respectively (Table 2). Moreover, adenoviruses injected intratumorally at the same dose did not increase the level of AST, ALT, or T-bilirubin (data not shown). Furthermore, no specific abnormality in general appearance and microscopic examination was observed. These results indicate that relaxin-expressing adenoviruses do not cause evident toxicity. Low-level periportal–sinusoidal lymphocytic accumulation was observed histologically in the livers of mice treated systemically with any one of the four adenoviruses tested (Fig. 6, C). No substantial weight loss was observed in any mice in response to any adenovirus challenge. Thus, neither relaxin-expressing adenoviruses, dl-lacZ-RLX or Ad-ΔElB-RLX, administered by intravenous (i.e., systemic) or intratumoral injection caused evident

Table 2. Evaluation of toxicologic profile after intravenous administration of PBS, dl-lacZ, dl-lacZ-RLX, Ad-ΔElB, or Ad-ΔElB-RLX*

	PBS			dl-lacZ			dl-lacZ-RLX			Ad-ΔE1B			Ad-ΔE1B-RLX		
Test (unit)	m1	m2	m3	m1	m2	m3	m1	m2	m3	m1	m2	m3	m1	m2	m3
AST (U/L)	158	148	NT	71	200	329	37	21	15	517	1	2	71	19	125
ALT (U/L)	1	25	15	88	27	457	55	78	69	25	19	80	139	56	25
T-bilirubin (mg/dL)	0.9	4	NT	1	1.1	5.3	4.3	0.8	4.5	0.3	0.7	1.3	0.5	0.5	4.3
Conclusion of chemistry test	−	−	−	−	−	+	−	−	−	+	−	−	−	−	

*Each of the three mice (m1, m2, and m3) weighed 20–21 g. PBS = phosphate-buffered saline; AST = aspartate aminotransferase; ALT = alanine aminotransferase; NT = not tested; − = normal; + = abnormal liver function. The microscopic examinations for all organs were normal. The general appearance for all mice tested was normal.

toxicity, compared with their corresponding control adenoviruses, dl-lacZ and Ad-ΔElB.

Discussion

The pressing challenge for contemporary gene therapy is to deliver an effective level of therapeutic genes to cancer cells throughout a tumor in vivo. The extracellular matrix provides one barrier to adenoviral dispersion in a tumor. To overcome this barrier, we inserted the gene for relaxin, an enzyme that degrades the extracellular matrix, into adenoviruses. By using these relaxin-expressing adenoviruses, we have demonstrated that relaxin expression increased the dispersion of the virus from cell to cell within the tumor and the induction of tumor cell apoptosis. Thus, the expression of relaxin by replicating adenoviruses increased the antitumor activity of these adenoviruses in all five tumor xenograft models examined, including the inhibition of tumor growth and metastases. This study is, to our knowledge, the first to report that the use of relaxin achieved a therapeutic benefit with increased distribution of viral vectors in tumor tissues. We have also demonstrated the ability of relaxin expression by replication-incompetent adenoviruses to increase transduction efficiency and by oncolytic adenoviruses to increase the survival of mice.

An important issue in replicating virus-based cancer gene therapy that has received little attention is the limited distribution that such viruses have been able to achieve in solid tumors *(17)*. To infect cells that are located far from the site of viral inoculation, replication-competent adenovirus must transverse the extracellular space and connective tissue between many cells to reach such uninfected cells. To increase the distribution of replicating virus within tumors, we explored the use of relaxin, an enzyme that degrades the cell matrix, to increase viral spread and tumor tissue penetration. We found that relaxin-expressing adenoviruses (dl-lacZ-RLX) penetrated into the core of spheroids composed of tumor cells but that adenoviruses that did not express relaxin (dl-lacZ) were restricted to a few outer layers of cells. This same effect was observed in xenograft tumors in mice. Moreover, the enhanced efficiency of gene transfer and viral spread by use of dl-lacZ-RLX and dl-GFP-RLX was also evident in primary human tumors from patients with breast cancer or patients with colon cancer (Supplementary Fig. 1, B and Supplementary Fig. 3; available at: http://jncicancerspectrum.oxfordjournals.org/jnci/content/vol98/issue20). Thus, relaxin expression increased viral spread and tumor tissue penetration both in vitro and in vivo.

The mechanism by which relaxin increases the ability of adenoviruses to infect tumors or spheroids appears to be related to its degradation of the extracellular matrix. We observed that the gene transfer efficiency of relaxin-expressing adenoviruses was greater than that of non–relaxin-expressing adenoviruses. Because tumor spheroids were cultured with viruses for 3 days, residual relaxin-expressing adenoviruses had enough time to infect cells progressively closer to the core of the spheroid. In vivo, repeated treatments of established xenografts with adenoviruses provided enough time for relaxin to reduce the amount of extracellular matrix in tumor tissue to allow the penetration and spread of adenoviruses into the tumor. In both tumors and spheroids, previous exposure to relaxin-expressing replication-incompetent dl-lacZ-RLX adenoviruses appeared to make the tumor environment more favorable to subsequent viral infections.

To elucidate how relaxin increased the potency of adenoviruses, we first investigated the induction of apoptosis by use of TUNEL and FACS analyses. Relaxin-expressing adenoviruses induced more tumor cells to become apoptotic than non–relaxin-expressing control adenoviruses. Relaxin expression mediated by other adenoviruses, such as green fluorescent protein–expressing replication-incompetent adenovirus (dl-GFP-RLX) and human telomerase reverse transcriptase (TERT) promoter–controlled replication-competent adenovirus (Ad-TERT-Δ19-RLX), also increased the number of apoptotic cells (data not shown). Furthermore, plasmid-mediated relaxin expression also increased the size of the sub-G_1 population in cultures of U343 and A549 cells, indicating that relaxin may have a direct role in inducing apoptosis (Supplementary Fig. 2).

A limitation of this study is that the mechanism by which relaxin induces apoptosis is not clearly understood. Relaxin activates the mitogen-activated protein kinase pathway *(18)* and causes the release of nitric oxide *(19)*, both of which could play a role. The receptors for relaxin, LGR7 and LGR8, have also been identified *(20,21)*. LGR7 and LGR8 are heterotrimeric G-protein–coupled receptors that mediate the action of relaxin through the cyclic adenosine 3′,5′-monophosphate–dependent pathway. The possibility cannot be ruled out that the extracellular matrix–degradative and –remodeling functions of relaxin also play a role in the induction of apoptosis by changing the number and availability of receptors, as well as rearranging cytoskeletal elements in the cell and thus triggering apoptosis. Alternatively, induction of apoptosis by relaxin and/or E1A gene product of replicating adenovirus could also accelerate viral spread. Apoptotic bodies typically contain organelles and/or nuclear DNA fragments, and these bodies can play a role in dispersing adenovirus throughout the intercellular spaces when apoptotic cells are rapidly phagocytosed by neighboring cells *(22)*. Support that induced apoptosis increases viral spread is provided by our previous reports *(12,16)* that adenoviruses, in which the E1B 19-kDa gene is deleted, potently enhanced apoptosis, increased cytolytic potency, and accelerated plaque development. The relaxin-expressing adenovirus that we used is an E1B 19-kDa and E1B 55-kDa double-deleted replicating adenovirus-expressing relaxin that thus induces relaxin- and E1A-mediated apoptosis and direct viral cytolysis, all of which ultimately appeared to lead to an increased antitumor effect.

Relaxin has been shown to induce the expression of matrix metalloproteinases, which may increase the metastatic potential. To investigate the effect of relaxin expression on metastases, we used the B16BL6 mouse melanoma model. Intratumoral administration of Ad-ΔE1B-RLX inhibited the formation of pulmonary metastases of B16BL6 melanoma cells in mice. We cannot rule out the possibility that the observed reduction in metastatic potential was also a result of a direct inhibitory effect of the relaxin-expressing oncolytic adenovirus on the growth of the primary tumor rather than on the metastatic potential. The effect of relaxin on metastasis should be further and rigorously investigated with other metastatic models. Furthermore, systemic delivery of relaxin-expressing oncolytic adenovirus also completely inhibited the growth of orthotopically implanted Hep1 hepatocellular carcinomas. Thus, relaxin-expressing oncolytic adenovirus appeared to elicit a potent antitumor effect against solid tumors and metastatic tumors when injected locally or systemically. Moreover, we would expect the therapeutic advantage of treatment with relaxin-expressing oncolytic adenovirus that we demonstrated in a murine model of spontaneous metastasis to be

enhanced in humans because human tumor cells are more permissive to adenoviral infection and replication than murine cells.

The pressing challenge for contemporary gene therapy is to deliver enough therapeutic genes to enough cancer cells in vivo. The use of relaxin expression from replicating adenoviruses increased the ability of adenovirus to disperse from one cell to another cell and increased the induction of apoptosis in all five tumor xenograft models examined. Thus, the expression of relaxin may increase the efficacy of virus-mediated cancer gene therapy.

References

(1) Kirn D. Clinical research results with dl1520 (Onyx-015), a replication-selective adenovirus for the treatment of cancer: what have we learned? Gene Ther 2001;8:89–98.

(2) Kirn D, Martuza RL, Zwiebel J. Replication-selective virotherapy for cancer: biological principles, risk management and future directions. Nat Med 2001;7:781–7.

(3) Sauthoff H, Hu J, Maca C, Goldman M, Heitner S, Yee H, et al. Intratumoral spread of wild-type adenovirus is limited after local injection of human xenograft tumors: virus persists and spreads systemically at late time points. Hum Gene Ther 2003;14:425–33.

(4) Harrison D, Sauthoff H, Heitner S, Jagirdar J, Rom WN, Hay JG. Wild-type adenovirus decreases tumor xenograft growth, but despite viral persistence complete tumor responses are rarely achieved—deletion of the viral E1b-19-kD gene increases the viral oncolytic effect. Hum Gene Ther 2001;12:1323–32.

(5) Kuriyama N, Kuriyama H, Julin CM, Lamborn K, Israel MA. Pretreatment with protease is a useful experimental strategy for enhancing adenovirus-mediated cancer gene therapy. Hum Gene Ther 2000;11:2219–30.

(6) Maillard L, Ziol M, Tahlil O, Le Feuvre C, Feldman LJ, Branellec D, et al. Pretreatment with elastase improves the efficiency of percutaneous adenovirus-mediated gene transfer to the arterial media. Gene Ther 1998;5:1023–30.

(7) Blundell TL, Humbel RE. Hormone families: pancreatic hormones and homologous growth factors. Nature 1980;287:781–7.

(8) Unemori EN, Pickford LB, Salles AL, Piercy CE, Grove BH, Erikson ME, et al. Relaxin induces an extracellular matrix-degrading phenotype in human lung fibroblasts in vitro and inhibits lung fibrosis in a murine model in vivo. J Clin Invest 1996;98:2739–45.

(9) Amento EP, Bhan AK, McCullagh KG, Krane SM. Influences of gamma interferon on synovial fibroblast-like cells. Ia induction and inhibition of collagen synthesis. J Clin Invest 1985;76:837–48.

(10) Yun CO, Kim E, Koo T, Kim H, Lee YS, Kim JH. ADP-overexpressing adenovirus elicits enhanced cytopathic effect by induction of apoptosis. Cancer Gene Ther 2005;12:61–71.

(11) Chartier C, Degryse E, Gantzer M, Dieterle A, Pavirani A, Mehtali M. Efficient generation of recombinant adenovirus vectors by homologous recombination in Escherichia coli. J Virol 1996;70:4805–10.

(12) Kim J, Cho JY, Kim JH, Jung KC, Yun CO. Evaluation of E1B gene-attenuated replicating adenoviruses for cancer gene therapy. Cancer Gene Ther 2002;9:725–36.

(13) Lee H, Kim J, Lee B, Chang JW, Ahn J, Park JO, et al. Oncolytic potential of E1B 55 kDa-deleted YKL-1 recombinant adenovirus: correlation with p53 functional status. Int J Cancer 2000;88:454–63.

(14) Yun CO, Yoon AR, Yoo JY, Kim H, Kim M, Ha T, et al. Coxsackie and adenovirus receptor binding ablation reduces adenovirus liver tropism and toxicity. Hum Gene Ther 2005;16:248–61.

(15) Yun CO, Cho EA, Song JJ, Kang DB, Kim E, Sohn JH, et al. dl-VSVG-LacZ, a vesicular stomatitis virus glycoprotein epitope-incorporated adenovirus, exhibits marked enhancement in gene transduction efficiency. Hum Gene Ther 2003;14:1643–52.

(16) Yoon AR, Kim JH, Lee YS, Kim H, Yoo JY, Sohn JH, et al. Markedly enhanced cytolysis by E1B-19kD-deleted oncolytic adenovirus in combination with cisplatin. Hum Gene Ther 2006;17:379–90.

(17) Vile RG, Russell SJ, Lemoine NR. Cancer gene therapy: hard lessons and new courses. Gene Ther 2000;7:2–8.

(18) Zhang Q, Liu SH, Erikson M, Lewis M, Unemori E. Relaxin activates the MAP kinase pathway in human endometrial stromal cells. J Cell Biochem 2002;85:536–44.

(19) Bani D, Masini E, Bello MG, Bigazzi M, Sacchi TB. Relaxin activates the L-arginine-nitric oxide pathway in human breast cancer cells. Cancer Res 1995;55:5272–5.

(20) Hsu SY, Nakabayashi K, Nishi S, Kumagai J, Kudo M, Sherwood OD, et al. Activation of orphan receptors by the hormone relaxin. Science 2002;295:671–4.

(21) Kumagai J, Hsu SY, Matsumi H, Roh JS, Fu P, Wade JD, et al. INSL3/Leydig insulin-like peptide activates the LGR8 receptor important in testis descent. J Biol Chem 2002;277:31283–6.

(22) Mi J, Li ZY, Ni S, Steinwaerder D, Lieber A. Induced apoptosis supports spread of adenovirus vectors in tumors. Hum Gene Ther 2001;12:1343–52.

Notes

J.-H. Kim and Y.-S. Lee contributed equally to the work.

This work was supported by grants from the Ministry of Commerce, Industry, and Energy, Republic of Korea (990-14-05-00008131, Dr C.-O. Yun), and KOSEF through National Core Research Center for Nanomedical Technology (R15-2004-024-02001-0, Dr C.-O. Yun and Dr J.-H. Kim). The sponsors had no role in the study design, data collection, analysis, interpretation of the data, or preparation of the manuscript.

We thank Dr Chung Mo Nam, Department of Preventive Medicine, and Byoung Chul Cho, Department of Internal Medicine, Yonsei University College of Medicine, Seoul, South Korea, for statistical evaluation.

Funding to pay the Open Access publication charges for this article was provided by Korea Biotech R & D Group of MoST (M10416130002-04N1613-00210 to Dr C.-O. Yun).

Manuscript received February 2, 2006; revised July 28, 2006; accepted August 30, 2006.