Gene Therapy with Secretory Leukoprotease Inhibitor Promoter-Controlled Replication-Competent Adenovirus for Non-Small Cell Lung Cancer

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ABSTRACT

Secretory leukoprotease inhibitor (SLPI) is highly expressed in almost all non-small cell lung cancers (NSCLCs), but not in the majority of other tumor types. In an attempt to create a specific gene therapy for NSCLC, we constructed AdSLPI.E1AdB, an adenovirus vector with a double expression cassette consisting of E1A driven by the SLPI promoter gene followed by EIB-19K under the control of the cytomegalovirus (CMV) promoter that can selectively replicate only in NSCLC cells. Infection with AdSLPI.E1AdB yielded E1A protein expression and adenovirus replication resulting in a >100-fold increase of the virus titers only in SLPI-producing NSCLC cells (A549, H358, and HS24 cells). In contrast, neither E1A protein nor replication was detected in non-SLPI-producing HepG2 cells. Treatment with AdSLPI.E1AdB significantly inhibited the proliferation of NSCLC cells in vitro in a dose-dependent manner, whereas the cell growth of HepG2 or normal human bronchial epithelial cells was not affected by AdSLPI.E1AdB infection. Direct injection of AdSLPI.E1AdB into A549 and H358 tumors in nude mice resulted in a marked reduction in tumor growth compared with controls (A549, 57%, H358, 67%, P < 0.03). Histological examination revealed the replication of AdSLPI.E1AdB and strong induction of necrosis and apoptosis. In addition, we evaluated the combination of AdSLPI.E1AdB and AdCMV.NK4 encoding NK4 protein, which has strong antiangiogenic activity. E1A expressed by AdSLPI.E1AdB trans-acts on the replication of AdCMV.NK4 and thus increases the expression of NK4. Injection of these two vectors into H358 tumors resulted in a more striking reduction of tumor growth compared with single injection of each vector. These results suggest that AdSLPI.E1AdB could provide a selective therapeutic modality for NSCLC and that the combination of AdSLPI.E1AdB and AdCMV.NK4 may be a more effective gene therapy for NSCLC.

INTRODUCTION

Lung cancer has been a leading cause of cancer-related death in Japan, and its incidence is still increasing. Because patients with non-small cell lung cancer (NSCLC) tend to be diagnosed at advanced stages and are therefore resistant to treatment modalities including chemotherapy and radiotherapy, the prognosis for NSCLC is quite poor. In this context, as one of the new treatment modalities, gene therapy for NSCLC has been studied using different genes and different vectors. Gene therapy for solid tumors requires complete eradication of all tumor cells in the body. Adenovirus-mediated gene therapy has been most frequently used for cancer gene therapy because of the possibility of the large-sized DNA insertion and high expression level of the transgene. However, even with intratumoral injection of the adenoviral vectors, few clinical trials of gene therapies for NSCLC have demonstrated tumor responses because of the limited infection of cancer cells and therefore low expression of the transgene in the tumor tissues (1, 2).

The need to transduce all tumor cells might be partially accomplished by biological amplification of local and systemic antitumor effects by the transfer of immunostimulatory genes or by the bystander effects of certain suicide gene/prodrug combinations (3–5). An alternative method to enhance the transduction efficiency is to use a tumor-selective replication-competent adenovirus (6, 7).

The tumor-selective replication-competent adenovirus dl1520 conserves E1A, which is necessary for replication of the virus in the infected cells, but lacks EIB-55K, which prevents apoptosis of the infected cells and allows adenovirus replication by blocking the p53 function (6). A mutated adenovirus that lacks EIB-55K cannot inactivate the p53 function. Therefore, at least theoretically, dl1520 selectively replicates in cancer cells, lyses them, and infects neighboring tumor cells with a defective p53 pathway but does not infect normal cells. The specificity of dl1520 for p53-deficient cells has been debated (8–10). Dl1520 can replicate in some tumor cells that retain wild-type p53 because these tumor cells carry wild-type p53 but have defects in the p53 pathway (10). In addition, the adenoviral protein E4ORF6 also binds and inhibits p53 (11, 12). Therefore, the dl1520 adenovirus should improve replication selectivity in tumor cells.

Secretory leukoprotease inhibitor (SLPI) is a 12 kDa serine protease inhibitor distributed not only in the respiratory tract but also in the salivary, lacrimal, and genital glands (13–15). We confirmed that tissue-specific expression of SLPI is tightly regulated at the transcriptional level (16) and that SLPI protein was highly expressed in all tumors of NSCLC, but not in other cell types of tumors (17). These data suggest that the SLPI promoter might give rise to tumor-specific expression in addition to tissue-specific expression. Therefore, we used the SLPI promoter as a tissue-specific promoter for a tumor-selective replication-competent adenovirus vector.

Because E1A protein is essential for viral replication, E1A protein expressed by a tumor-selective replication-competent adenovirus can trans-act on commonly used replication-incompetent E1-deleted adenoviruses, resulting in the amplification of the transgene expression of the replication-incompetent adenovirus (18). Because cytotoxic genes induce the cell death of adenovirus-infected cells, noncytotoxic genes would be suitable candidates for use in combination with a tumor-selective replication-competent adenovirus. As one of the candidates for a transgene to combine with a replication-competent adenovirus, we have chosen NK4, an α chain fragment of human hepatocyte growth factor [HGF (19, 20)]. NK4, originally identified as a HGF antagonist, has a strong antiangiogenic activity involving both HGF-dependent and -independent pathways (21). In our previous study, we demonstrated strong antitumor activity against xenografts of human NSCLC in nude mice using an adenoviral vector expressing NK4 [AdCMV.NK4 (22)].

In this study, we demonstrate the strong and NSCLC-selective
antitumor activity of a tumor-selective replication-competent adenovirus controlled by the SLPI promoter (AdSLPI.E1AdB). In addition, the combination of AdSLPI.E1AdB and AdCMV.NK4 showed stronger antitumor effects than single-gene therapy with AdSLPI.E1AdB or AdCMV.NK4.

MATERIALS AND METHODS

Cell Cultures. Human NSCLC cell lines H358 (adenocarcinoma) and HS24 (squamous cell carcinoma) and other human cell lines [HepG2 (hepatocellular carcinoma), SBC-3 (small cell lung cancer), HT1080 (fibrosarcoma), SAOS-2 (osteosarcoma), and INR-90 (normal fibroblast)] were cultured in RPMI 1640 supplemented with 10% FCS. Normal human bronchial epithelium (NHBE) cells purchased from Takara Biomedical Co. (Shiga, Japan) were maintained in BEGM BulletKit (Takara Biomedical Co.). The A549 cell line was grown in DMEM with 10% FCS.

Construction of Conditionally Replication-Competent Adenovirus. A conditionally replication-competent adenovirus (cRCAd), AdSLPI.E1AdB expressing E1A-1.5S under the control of the SLPI promoter, was generated as described previously (23). Briefly, the adenovirus shuttle vector for AdSLPI.E1AdB was generated by manipulating plasmid pAdBIII (24, 25) as follows. The pRcCMV/BglII (26) was digested with HindIII to produce pRc/BglII. On the other hand, the SalII/HindIII fragment containing the SLPI promoter sequence excised from plasmid pAK6 (16) was cloned into the blunted BglII/HindIII site of pRc/BglII (pRc/SLPI/BglII). The pRc/SLPI/BglII was digested with HindIII and religated with the HindIII/HindIII fragment of pAdBglII-AFP.E1A13S/CMV.E1B19K (25) containing E1A without the promoter sequence and E1B-19K under the control of the cytomegalovirus (CMV) promoter to construct pRcSLPI.E1A/CMV.E1B19K. The fragment containing double expression cassette sequences of the pRcSLPI.E1A/CMV.E1B19K was blunted and cloned into the blunted BglII site of pAdBIII. Finally, the resulting shuttle vector, pAdBIII/SLPI.E1A/CMV.E1B19K, was cotransfected with pMM17 (Microbius Biosystems, Toronto, Canada) into 293 cells to generate the conditionally replication-competent adenovirus AdSLPI.E1AdB expressing E1A under the control of the SLPI promoter. As a control vector, we constructed a replication-deficient adenovirus vector, AdSLPI.LacZ, which expresses β-galactosidase protein by the SLPI promoter but lacks E1A and E1B-19K gene. Wild-type adenovirus and AxE1AdB, an E1B-55K-deleted replication-competent adenovirus without a specific promoter, were kindly provided by Dr. G.G. Crystal (Cornell University Medical College, New York, NY) and Dr. H. Hamada (Sapporo Medical University School of Medicine), respectively (18). The replication-deficient adenovirus vectors, AdCMV.LacZ and AdCMV.NK4, were generated as described previously (22). The total numbers of viral particles in the viral sample were measured by Abs405 nm, which was defined as 10^13 particles. The titer expressed as plaque-forming units (pfu)/milliliter of viral stocks were quantified by a plaque-forming assay using the 293 cells (27). Particle titers of 5 × 10^12 pfu/ml and infectious titers of 2 × 10^11 pfu/ml were obtained routinely in all recombinant adenoviral vectors.

ELISA for SLPI Protein and NK4 Protein. To evaluate the secretion of SLPI protein from the various cell lines, concentrations of SLPI protein in the culture medium after 72 h of incubation (1 × 10^6 cells/cm2) were determined by an ELISA kit for human SLPI (Quantikine; R&D Systems, Inc.). The minimal detectable amount was 25 pg/ml and calibrated with the total cell numbers. The secretion of NK4 in the culture medium from cells infected with AdSLPI.E1AdB was measured with an ELISA kit for human HGF (Quantikine; R&D Systems, Inc.). The minimal detectable amount of 40 pg/ml.

Southern Blot and Western Blot Analysis of Adenovirus. DNA isolated from cells infected with either AdSLPI.E1AdB, AxE1AdB, AdSLPI.LacZ, or AdCMV.NK4 at a multiplicity of infection (m.o.i.) of 10 was digested with HindIII, subjected to 2.2 M formaldehyde-1% agarose gel electrophoresis, transferred to a nylon membrane, and hybridized with a 32P-labeled probe of the adenovirus E4 regions or NK4 region. The probes of E4 and CMV/NK4 coding region were prepared by amplifying them with the PCR primer pair E4-S (GTAGAGTCATAATCGTGCATCAGG) and E4-AS (TTTATATGGTACCGGAGGGTTGTG) or the PCR primer pair CMV-S (TAGGGTAGTCTTTGCTGATT) and NK4-AS (TAGGGTAGTCTTTGCTGATT). As an internal control, the same membrane was subsequently rehybridized with a human glyceraldehyde-3-phosphate dehydrogenase cDNA probe.

To confirm the expression of AdSLPI.E1AdB, 72 h after infection with AdSLPI.E1AdB at a m.o.i. of 100, the cells were scraped, centrifuged for 5 min at 1,400 rpm, washed twice with ice-cold PBS, and then resuspended in 200 μl of cell lysis buffer (1% Triton X-100, 0.5% NaDOC, 0.1% SDS, 20 μM EDTA, 1 μg/ml aprotinin, 0.5 mM phenylmethylsulfonyl fluoride). The cell lysates were centrifuged for 10 min at 15,000 rpm and stored at −80°C. The protein concentration of the cell lysates was determined using Bio-Rad protein assay reagent (Bio-Rad, Fremont, CA). Samples (20 μg each) were electrophoresed on an 8% SDS-PAGE gel and transferred to polyvinylidene difluoride membranes. After being blocked in 10% dry milk blocking solution overnight, membranes were incubated with a 1:1,000 dilution of primary mouse anti-adenovirus E1A antibodies (Clone M73; NeoMarkers, Fremont, CA). Immunocomplexes were detected with the secondary alkaline phosphatase-conjugated goat antirabbit antibodies (Promega, Madison, WI) using 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium kit (Promega).

PCR Analysis. To evaluate replication of viral DNA in vivo, DNA isolated from tumors infected with AdSLPI.E1AdB (1 × 10^6 pfu) was subjected to the semiquantitative PCR analysis with primer pair E1A-S (GTATGATTTAGACGGTCACGCCG) and E1A-AS (GATAAGCAGGCGCCCATTTT) using the same primer pair for E4 used for Southern blotting, or the primer pair β-actin-S (ATGGTGCAGAAGATTCCT) and β-actin-AS (ACTGAATCAACG-CAATTG). Twenty to 30 cycles of PCR were carried out in a thermal cycle. Each cycle included 30 s of denaturation at 94°C, 30 s of primer annealing at 55°C, and 30 s of extension at 72°C. Amplification of the β-actin gene was used to control the amount of DNA in each sample.

Adenovirus Replication Assay. The cells cultured in 12-well dishes (1 × 10^5 cells/well) were infected with AdSLPLacZ or AdSLPI.E1AdB at a m.o.i. of 20. The medium was exchanged with fresh medium after 2 h, and each cell line was subsequently incubated for 24 or 72 h. Cell lysates were prepared with three cycles of freezing and thawing. Serial dilutions of the lysates were titrated on the 293 cells.

Cell Survival Assay in Vitro. The cells plated into 96-well plates (1 × 10^4 cells/well) were infected with AdSLPLacZ or AdSLPI.E1AdB at a m.o.i. of 20. The medium was exchanged with fresh medium after 2 h, and each cell line was subsequently incubated for 24 or 72 h. Cell lysates were removed with three cycles of freezing and thawing. Serial dilutions of the lysates were titrated on the 293 cells.

In Vivo Animal Models. All animal experiments were carried out in accordance with the institutional guidelines of the Institute of Development, Aging and Cancer, Tohoku University. A549 or H9262 cells (1 × 10^7 cells/injection) were injected into the flanks of female BALB/c nu/nu mice. HepG2 or A549 cells (1 × 10^7 cells/mouse) were injected into female C.B-17 severe combined immunodeficient (SCID) mice. A single vector administration of AdSLPI.E1AdB was performed in A549 and HepG2 tumors, and combined administration of AdSLPI.E1AdB and AdCMV.NK4 was performed in H358 tumors. AdSLPI.E1AdB (1 × 10^6 pfu), AdSLPI.LacZ (1 × 10^6 pfu), or PBS (50 μl) was administered intratumorally to the A549 and HepG2 tumors twice on days 5 and 7 after tumor inoculation, when the tumor had grown to about 50 mm^3, and the combination of AdSLPI.E1AdB (5 × 10^5 pfu) and AdSLPI.LacZ (5 × 10^5 pfu) or the single vector of AdSLPI.E1AdB (1 × 10^6 pfu), AdSLPI.LacZ (1 × 10^6 pfu), or PBS (50 μl) was injected into the H358 tumor twice, on days 10 and 12 after tumor inoculation, when the tumor had grown to 50–100 mm^3. Each treatment group had at least five mice. In an additional experiment, when the H358 tumors were grown to 500 mm^3 in the combination of AdSLPI.E1AdB (5 × 10^5 pfu) and AdCMV.NK4 (5 × 10^6 pfu) was injected intratumorally. To quantify tumor growth, two perpendicular diameters of the tumors were measured with calipers every 3–5 days. The tumor volume was calculated using the following formula: tumor volume (mm^3) = 0.52 × (width^2) × (length) (28).

For histological analysis, the tumors were excised and embedded both with paraffin and with OCT compound as frozen sections. To detect apoptotic cells, paraffin-embedded sections were processed using the in situ Apoptosis Detection Kit (Takara Biomedical Co., Tokyo, Japan). The apoptotic cells were estimated by the percentage of positively stained cells visualized in five randomly chosen high-power fields for each section (×400). To assess the
complete viruses and vascularity in the tumor injected with AdSLPI.E1AdB or the combination of AdSLPI.E1AdB and AdCMV.NK4, OCT compound-embedded frozen sections fixed in 1% paraformaldehyde were incubated with mouse antibody for the hexon of the adenovirus (1:200 dilution; clone 2/6; Chemicon International Inc., Temecula, CA) or rat antimouse CD31 antibodies (Ref. 29; 1:200 dilution; PharMingen, San Diego, CA) and visualized with a streptavidin-peroxidase complex (Histofine; Nichirei Corp., Tokyo, Japan). More than five areas (0.25 mm $^3 \times 5$) of microvessels invading into tumor were evaluated with NIH Image software for quantification (30).

To evaluate the transgene expression of the adenovirus controlled by the CMV promoter or that controlled by the SLPI promoter, we measured the expression of the reporter gene LacZ in liver after an i.v. injection or intra-tracheal injection of AdCMV.LacZ or AdSLPI.LacZ (1 × 10$^9$ pfu). Mice were sacrificed, and the liver and lung were removed at 3 days after infection. Half of the liver and the entire lung were embedded with OCT compound and stained with 5-bromo-4-chloro-3-indolyl-$\beta$-D-galactopyranoside. The other half of the liver was homogenized and centrifuged for 10 min at 15,000 rpm, and the supernatant was measured for $\beta$-galactosidase activity using the $\beta$-Galactosidase Enzyme Assay System with Reporter Lysis Buffer (Promega) according to the manual.

**Statistical Evaluation.** Statistical values are presented as the mean ± SD; all comparisons were made using the unpaired two-tailed Student’s $t$ test. Statistical significance was defined as $P < 0.05$.

**RESULTS**

**Construction and Expression of SLPI-Promoting Replication-Competent Adenovirus.** The three NSCLC cells (A549, H358, and HS24) and NHBE cells specifically secreted high levels of SLPI protein, whereas the other cell lines (HepG2, SBC-3, SAOS-2, and HS24) and NHBE cells specifically secreted high levels of SLPI (Fig. 1). AINR-90 (normal fibroblast) did not secrete detectable amounts of SLPI (Fig. 1A). The three NSCLC cells (A549, H358, and H9252) and NHBE cells specifically secreted high levels of SLPI whereas the other cell lines (HepG2, SBC-3, SAOS-2, and HS24) and NHBE cells specifically secreted high levels of SLPI (Fig. 1). Infection with AINR-90 (normal fibroblast) did not secrete detectable amounts of SLPI (Fig. 1A). We constructed AdSLPI.E1AdB, an adenovirus vector with a double expression cassette consisting of E1A driven by the SLPI promoter followed by E1B-19K under the control of the CMV promoter that can selectively replicate in SLPI-producing cells such as NSCLC cells (Fig. 1B).

Western blot analysis of SLPI-producing A549 cells infected with AdSLPI.E1AdB vector demonstrated E1A protein expression in vitro (Fig. 1C, Lane 2). In contrast, non-SLPI-producing HepG2 cells did not express the E1A protein. The expression of E1A was also detected in both H358 and HS24 cells (data not shown). Infection with AxE1AdB, a restricted replication-competent adenovirus that lacks the SLPI promoter, demonstrated expression of E1A protein in both A549 and HepG2 cells (Fig. 1C, Lanes 3). To determine whether the virus vector actually replicates in the infected cells, we evaluated adenoviral replication in the two cell lines HepG2 and A549 by Southern blotting of the E4 region of the adenoviral genome. As expected, AdSLPI.E1AdB was replicated only in the A549 cells (Fig. 1D, Lanes 2), whereas AxE1AdB replicated in both cells (Fig. 1D, Lanes 3). Neither E1A protein nor E4 gene was detected in the cells infected with AdSLPI.LacZ (Fig. 1C and D, Lanes 1).

**Effect of AdSLPI.E1AdB on Cancer Cell Proliferation in Vitro.** To assess the cytotoxicity of AdSLPI.E1AdB, we evaluated the cell growth inhibition after infection with either AdSLPI.E1AdB or AdSLPI.LacZ at various doses (Fig. 2A). AdSLPI.E1AdB inhibited the cell growth of SLPI-producing cancer cell lines in accordance with the dose escalation (A549, H358, and HS24) but did not inhibit the growth of HepG2. Although the cytotoxicity of AdSLPI.E1AdB on H358 was slightly inferior to that of the wild-type adenovirus, cytotoxicities of AdSLPI.E1AdB on A549 and HS24 were equivalent to those of the wild-type adenovirus. In contrast with AdSLPI.E1AdB, the wild-type adenovirus showed nonspecific cytotoxicities in all cell lines including the normal bronchial cell line. Because the NHBE cells secreted SLPI protein as did the NSCLC cells (Fig. 1A), cytotoxic effects on the NHBE cells by AdSLPI.E1AdB were a concern. However, AdSLPI.E1AdB did not suppress the growth of NHBE cells with normal p53 function because E1B-55K was deleted from AdSLPI.E1AdB. These results demonstrate the tumor specificity together with the tissue selectivity of AdSLPI.E1AdB. Furthermore, we determined cell viability of A549 serially after infection with
AdSLPLE1A at a m.o.i. of 1. The apparent cytotoxicity appeared 10 days after infection (Fig. 2B). These data suggest that actual virus replication induces cytotoxicity.

**Cell-Specific Replication of AdSLPLE1AdB.** Although we had already revealed replication of AdSLPLE1AdB in A549 cells with Southern blotting, we performed a plaque-forming assay using cell lysates of AdSLPLE1AdB-infected cells to confirm whether these replicated viruses really maintain their infection ability. The titers of adenovirus from both A549 and H358 infected with AdSLPLE1AdB increased >100-fold from day 1 to day 3, whereas the virus titers from HepG2 did not increase at all. The wild-type adenovirus replicated in both A549 and HepG2 (Table 1). These results indicated that

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**Fig. 2.** Cytotoxic effect of AdSLPLE1AdB on human cell lines in vitro. The cells plated in 96-well plates (1 \( \times \) 10^3/well) were infected with either AdSLPLE1AdB (▲), AdSLPLE1LacZ (○), or wild-type adenovirus (□) at a multiplicity of infection of 12–200. The cell viabilities were evaluated by AlamarBlue 72 h after infection (A). A549 was infected with AdSLPLE1AdB (▲) or AdSLPLE1LacZ (○) at a multiplicity of infection of 1. The cell viabilities were analyzed serially after infection (B). Cell viabilities were presented as a percentage in comparison with cell viability without infection. Data are presented as the mean ± SD of triplicate samples.
AdSLPI.E1AdB replicates and increases infectivity only in SLPI-producing tumor cells.

**SLPI Promoter Has Tissue Selectivity.** To clarify the intrinsic effect of the SLPI promoter, we used LacZ gene controlled by the SLPI promoter (AdSLPI.LacZ) or CMV promoter (AdCMV.LacZ). We analyzed the expression of LacZ protein in the liver, which showed the highest distribution of adnoviral vectors after i.v. administration of AdSLPI.LacZ or AdCMV.LacZ to C57BL/6 mice. In the immunohistochemistry of LacZ, AdSLPI.LacZ did not express a detectable amount of LacZ protein, whereas AdCMV.LacZ highly expressed LacZ protein (Fig. 3A). Quantitative analysis of LacZ expression in the liver after i.v. injection revealed that AdSLPI.LacZ expressed a remarkably inferior amount of LacZ protein compared with AdCMV.LacZ (75 versus 4,752 ng/g, respectively; Fig. 3G). It was reported previously that an adenovirus administered i.v. was mostly distributed to the liver and often induced liver damage (31, 32). It was expected that SLPI promoter would reduce liver damage after systemic injection of adenovirus vector. The LacZ protein levels of both lung and kidney after i.v. injection were below the level of detection of the ELISA kit. To evaluate the expression of AdSLPI.LacZ in the lung, we administered AdSLPI.LacZ or AdCMV.LacZ to mice intratracheally. Although AdCMV.LacZ was expressed in the bronchial and alveolar cells, AdSLPI.LacZ was expressed only in a few cells of large-sized bronchi (Fig. 3, C–F), suggesting that SLPI-expressing normal cells in the lung are very limited. In this context, AdSLPI.E1AdB might be able to suppress the adverse effect on liver and lung after intravascular administration of AdSLPI.E1AdB.

**AdSLPI.E1AdB Treatment Suppressed Tumor Growth of A549 but not HepG2 in Vivo.** Direct injections of AdSLPI.E1AdB (1 × 10⁸ pfu × 2 times on day 5 and 7) into A549 tumors established in BALB/c nu/nu mice strikingly suppressed tumor growth by 57.0% on day 27 compared with AdSLPI.LacZ treatment (n = 6; P < 0.01; Fig. 4A). We also performed a similar experiment using SCID mice. A remarkable tumor suppression was observed in SCID mice by intratumoral injection of AdSLPI.E1AdB (69.4% reduction on day 29 compared with AdSLPI.LacZ; n = 6; P < 0.01; Fig. 4B). This antitumor effect by AdSLPI.E1AdB was not less effective compared with E1B-55K-deleted replication-competent adenovirus AxE1AdB (data not shown). Because HepG2 tumors were established only in the SCID mice, we injected AdSLPI.E1AdB into the HepG2 tumors of SCID mice. As we expected, AdSLPI.E1AdB did not affect the tumor growth of HepG2 at all (Fig. 4C). To assess the antitumor effect achieved by AdSLPI.E1AdB treatment of A549, tumors treated with AdSLPI.E1AdB or AdSLPI.LacZ were removed on day 27 and subjected to histological examination. In the H&E stain, massive cell death considered as necrosis was recognized in the extensive central area of the tumor treated with AdSLPI.E1AdB (Fig. 5, A and B). Terminal deoxynucleotidyl transferase-mediated nick end labeling assay demonstrated that the number of apoptotic cells in tumors treated with AdSLPI.E1AdB was 2.5× greater than that in tumors treated with control vectors (n = 4; P < 0.05; Fig. 5, C, D, and G). This result indicates that treatment with AdSLPI.E1AdB suppressed tumor growth by leading tumor cells to both necrosis and apoptosis.

To evaluate whether capsid protein of the adenovirus in addition to the virus genome was composed in SLPI-producing tumors, we performed immunohistochemical analysis using the antihexon virus antibody. Positive stains were recognized only in the tumor treated with...
AdSLPI.E1AdB and not in the tumor treated with control vector (Fig. 5, E and F). Furthermore, we evaluated viral replication in the tumor with semiquantitative PCR amplification using E1A and E4 primers. As shown in Fig. 5H, more adenovirus DNA existed in the A549 tumors resected on day 14 than on day 7. This result suggested that treatment with AdSLPI.E1AdB yields virus replication and virus particle with capsid protein in the SLPI-producing tumor.

**Combined Treatment with AdSLPI.E1AdB and AdCMV.NK4 in Vitro.** To increase antitumor activity, we infected SLPI-producing tumor cells with AdSLPI.E1AdB in combination with AdCMV.NK4. Although AdCMV.NK4 does not affect tumor cell growth in vitro, AdCMV.NK4 has a strong in vivo antitumor effect through both HGF-dependent and -independent antiangiogenic activities, as we demonstrated previously (21, 22). When both AdSLPI.E1AdB and AdCMV.NK4 are used to infect SLPI-producing tumor cells, it is expected that E1A protein expressed by AdSLPI.E1AdB will trans-act on AdCMV.NK4, which is originally replication incompetent, and thus induce replication of AdCMV.NK4 and lead to amplification of NK4 protein expression. The NK4 protein level in medium from H358 cells treated with AdSLPI.E1AdB and AdCMV.NK4 increased remarkably (m.o.i. of 25, 22×; m.o.i. of 50, 55×) compared with that in cells treated with AdSLPI.LacZ and AdCMV.NK4 3 days after

![Graphs showing tumor growth inhibition due to gene therapy with SLPI promoting adenovirus](image-url)
infection with AdSLPI.E1AdB or AdSLPL.lacZ (m.o.i. of 25 or 50) in addition to AdCMV.NK4 (m.o.i. of 25; Fig. 6A), whereas no significant increase in NK4 protein was obtained from HepG2 cells (data not shown). The increased expression of NK4 can be explained by either replication of AdCMV.NK4 by E1A of AdSLPI.E1AdB or up-regulation of CMV promoter by E1A of AdSLPI.E1AdB. To determine this mechanism, we analyzed the replication of AdCMV.NK4 with Southern blotting using CMV-NK4 probe. We could detect virus DNA that is the product of replication of AdCMV.NK4 after coinfection with AdCMV.NK4 and AdSLPI.E1A in the SLPI-producing A549 cell line, whereas we could not detect the virus DNA in non-SLPI-producing HepG2 cells (Fig. 6B). When AxE1AdB was coinfected with AdCMV.NK4, AxE1AdB promoted the replication of AdCMV.NK4 in both A549 and HepG2 cell

Fig. 5. Histological examination and PCR of tumors after injection of AdSLPI.E1AdB. A549 tumors treated with AdSLPL.lacZ (A, C, and E) or AdSLPI.E1AdB (B, D, and F) were removed on day 27 and assessed by H&E staining (A and B; \( \times 40 \)), terminal deoxynucleotidyl transferase-mediated nick end labeling assay (C and D; \( \times 40 \)), and immunohistochemistry with antibody for adenovirus hexon protein (E and F; \( \times 100 \)). Note that virus capsid protein was detected only in tumors treated with AdSLPI.E1AdB. G, quantification of the number of terminal deoxynucleotidyl transferase-mediated nick end labeling-positive cells. Data are presented as the mean \( \pm SD \) of four tumor samples. H, PCR amplification of the E1A gene and E4 gene from A549 tumors 7 or 14 days after injection of PBS (Lane 1) or AdSLPI.E1AdB (Lanes 2–5). AdSLPI.E1AdB virus DNA was used as positive control (Lane 6).
These results demonstrated that AdSLPI.E1A increases expression of AdCMV.NK4 by inducing replication of AdCMV.NK4 after coinfection with these vectors.

**Combined Treatment with AdSLPI.E1AdB and AdCMV.NK4 in Vivo.** To evaluate the antitumor effect of the combined treatment of AdSLPI.E1AdB and AdCMV.NK4 in vivo, we injected AdSLPI.E1AdB plus AdCMV.NK4 into established H358 tumors at the same dose (a total of 1 × 10^9 pfu/mouse). As expected, the combined treatment with AdSLPI.E1AdB and AdCMV.NK4 demonstrated a striking reduction of tumor growth compared with each single treatment (80% reduction compared with control; n = 5; P < 0.01; Fig. 7A). Although it was usually difficult to show apparent antitumor effect on large tumors, we injected the combination of AdSLPI.E1AdB and AdCMV.NK4 into large-sized tumors (>500 mm³) and succeeded in shrinking the tumors to close to half-volume. Histological examination of tumors resected on day 40 also demonstrated that the combination of AdSLPI.E1AdB and AdCMV.NK4 not only provoked necrosis and apoptosis of the tumor cells but also remarkably suppressed tumor angiogenesis compared with AdCMV.NK4 alone (n = 4; combination, 85% suppression; AdCMV.NK4 alone, 65% suppression; Fig. 7, B–E) in expectation of amplification of NK4 protein by coinfection with AdSLPI.E1AdB.

**DISCUSSION**

In this report, we constructed a replication-competent adenoviral vector controlled by the SLPI promoter (AdSLPI.E1AdB). AdSLPI.E1AdB specifically replicates in and lyses human NSCLC cells overexpressing SLPI both in vitro and in vivo, but it does not do so in non-SLPI-expressing tumor cells. In addition, we assessed whether the combination of AdSLPI.E1AdB and AdCMV.NK4 can enhance the antitumor effect. This combined gene therapy successfully demonstrated a stronger antitumor effect than single-gene therapy against even a large tumor xenograft.

One of the main issues of replication-competent adenovirus is tumor specificity. Although initial reports suggested that the E1B-55K-deleted mutant, dl520, selectively destroyed tumor cells harboring adenovirus, the expression of NK4 protein by coinfection with AdSLPI.E1AdB.
ing a defective p53 gene (6, 33), several later reports demonstrated that d1520 replicates and induces cytosis of tumor cells retaining wild-type p53 (8, 9, 34). Ries et al. (10) identified loss of ARF as a key mechanism of p53 status-independent cytosis. The ARF protein is a major negative regulator of Mdm2, which binds and inactivates p53 (35–37). The A549 cell line used in this report is a representative cell line sensitive to E1B-55K-deleted mutant adenovirus because A549 cells express wild-type p53 but not ARF protein (10, 34). Furthermore, few reports have demonstrated that E1B-55K-deleted mutant can replicate in and lyse to normal human fibroblasts (8, 38). In this context, a tumor-specific promoter is an ideal strategy for improving tumor selectivity of replication-competent adenovirus.

SLPI is restrictedly expressed in both normal cells and tumor cells in specific organs (13–15). NSCLC expressed a high level of SLPI promoter (17). We constructed a replication-competent adenovirus controlled by a tissue-specific SLPI promoter. Our data showed that AdSLPI.E1AdB expressed E1A and replicated only in NSCLC tumors without any decrease in antitumor effect compared with E1B-55K-deleted adenovirus d1520 in vitro and in vivo. In this construct, the CMV promoter was inserted to express a sufficient level of E1B-19K near the SLPI promoter. Although we did not see any effect on the SLPI promoter by the strong CMV promoter/enhancer, the CMV promoter should be deleted and E1B-19K should be controlled by the SLPI promoter because strong enhancer may bidirectionally affect the other specific promoter, which can be much more than 1 kb. When an adenovirus is administered systemically to a mouse, liver damage is a critical adverse effect because the liver is the organ in which the transgene expression is the highest (22, 31). Because systemic administration of AdSLPI.LacZ resulted in expression of only a minimal amount of LacZ protein in the liver compared with administration of AdCMV.LacZ, the SLPI promoter may decrease the adverse effect on other organs as well as the liver after systemic administration of an adenovirus vector. After intratracheal injection of AdSLPI.LacZ, few cells in the bronchi were positive for LacZ expression, and the positive cells could be goblet cells because immunohistochemistry of SLPI protein demonstrated that goblet cells in human bronchial/bronchiolar epithelium expressed SLPI (39). These data suggested a possible adverse effect of AdSLPI.E1AdB when administered intratracheally. Nevertheless, further examinations in association with safety issues are needed for clinical application of AdSLPI.E1AdB.

Recently, SLPI promoter was applied for another adenovirus vector expressing suicide gene (thymidine kinase) for ovarian cancer (40). This adenovirus highly expressed the transgene in both ovarian cancer cell lines and primary ovarian cancer cells, but not in normal cells. The therapeutic efficacy was similar to the ubiquitous CMV promoter in vitro and in a murine model of peritoneally disseminated ovarian cancer, with higher activity than controls. Our results suggest that the SLPI promoter is a suitable promoter for gene therapy for tumors expressing SLPI protein without any decrease in antitumor effect compared with other commonly used promoters.

Other tumor- or tissue-specific promoters for replication-competent adenovirus have been reported. Among the replication-competent adenoviruses driven by a tissue-specific promoter, Calydon CV706, in which the prostate-specific antigen promoter drives E1A, is already being clinically developed (41). Trans-Rectal administration of CV706 to locally recurrent prostate tumor was well tolerated, and tumor responses were observed in the high-dose group in a Phase I clinical trial. Doromin et al. (42) demonstrated a NSCLC-specific antitumor effect by a replication-competent adenovirus driven by surfactant protein B promoter. Surfactant protein B promoter activity is restricted to adult type II alveolar epithelial cells and bronchial epithelial cells. These tumor- or tissue-specific promoters could reduce adverse effects to the organs without decreasing any antitumor effects.

An E1B-55K-deleted replication-competent adenovirus d1520, ONX-015, has been most extensively studied in clinical trials. Although ONX-015 has been applied on different types of solid tumors including lung cancer, pancreatic cancer, liver tumor, colon cancer, and head and neck cancers, durable clinical tumor response with ONX-015 as a single agent has not been very encouraging (43–46). A higher tumor response was observed only when ONX-015 was combined with a chemotherapy regimen using cisplatin and 5-fluorouracil, compared with ONX-015 or chemotherapy alone, in head and neck cancers in a clinical trial and a preclinical study (47).

The oncolytic adenovirus efficacy can be improved other than by combining with chemotherapy or radiotherapy by adding cytotoxic genes to the viral genome to couple gene-based therapy with virus oncolysis. Replication-defective adenovirus vector distributes poorly within a tumor mass and therefore fails to distribute such a cytotoxic gene efficiently. Transgene delivery by an oncolytic virus reaches an anatomically larger area of tumor compared with that reached by a replication-defective adenoviral vector. The strategy of using replication-competent adenovirus as a helper virus is another candidate for improvement of antitumor effect by gene therapy. Oncolytic adenovirus can amplify the transgene of replication-incompetent adenovirus by E1A protein of replication-competent adenovirus. The scaffolds in live cells are necessary for propagation of replication-incompetent virus. In this respect, transgene related with immune response or antiangiogenesis without direct cytotoxicity is suitable for combination with replication-competent adenovirus (18).

We used the NK4 gene in combination with replication-competent adenovirus. NK4 was found as a specific antigen for HGF; NK4 contains an NH2-terminal hairpin domain and four subsequent kringle domains of the HGF α chain, binds to c-Met, and suppresses HGF-induced tumor growth and invasion (20). Recently, Kuba et al. (21) demonstrated that NK4 not only blocks HGF-induced angiogenesis but also inhibits angiogenesis induced by basic fibroblast growth factor and vascular endothelial growth factor through a mechanism that has not yet been elucidated. We also determined that NK4 had a strong antiangiogenic effect using AdCMV.NK4 in vivo (22). As we expected, AdSLPI.E1AdB amplified NK4 expression, and therefore the combination of AdSLPI.E1AdB and AdCMV.NK4 showed an improved antitumor effect compared with other single-gene therapy. In conclusion, AdSLPI.E1AdB, a tumor-restricted competent adenovirus driven by the SLPI promoter, demonstrated a specific and strong antitumor effect on NSCLC tumor xenografts. In addition, the combination of AdSLPI.E1AdB and AdCMV.NK4 demonstrated a greater effect than single-gene therapy and significant shrinkage of large tumors, which are usually resistant to regular gene therapy, AdSLPI.E1AdB, alone and in combination with AdCMV.NK4, can be a suitable candidate for a clinical gene therapy trial of NSCLC.

ACKNOWLEDGMENTS

We thank Dr. H. Hamada for kindly providing us with replication-competent adenovirus AxE1AdB.

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Gene Therapy with Secretory Leukoprotease Inhibitor Promoter-Controlled Replication-Competent Adenovirus for Non-Small Cell Lung Cancer

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