

Gene therapy for oral squamous cell carcinoma with *IAI.3B* promoter-driven oncolytic adenovirus-infected carrier cells

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Abstract. Although replication-competent oncolytic viral vectors have been developed to improve antitumor activity, the generation of high titers of neutralizing antibodies inhibits repetitive viral infection. Many studies have reported that oncolytic virus-infected carrier cells can overcome this viral induced immunogenicity. However, the effects of oncolytic virus-infected carrier cells in human oral squamous cell carcinoma (OSCC) have not yet been examined. In the present study, simulating the clinical trial, we examined the antitumor activity of carrier cells infected with oncolytic adenovirus AdE3-*IAI.3B* in human OSCC. *IAI.3B* was highly activated in OSCC cells but not in normal cells. AdE3-*IAI.3B* killed OSCC cells *in vitro* but not normal cells. AdE3-*IAI.3B*-infected A549 carrier cells eradicated OSCC GFP-SAS tumors in nude mice. Anti-adenovirus neutralizing antibodies completely blocked the antitumor effect of AdE3-*IAI.3B* but did not block that of carrier cells. After the induction of anti-adenoviral CTL responses by immunization of adenovirus, administration of carrier cells induced complete regression of murine squamous cell carcinoma SCC7 tumors. Adenovirus-*GM-CSF* augmented the antitumor effect of carrier cells. The *IAI.3B*-driven oncolytic adenovirus-infected carrier cell system might prove useful in the treatment of OSCC and clinical trials of it should be conducted in the near future.

Introduction

More than 700 clinical trials of cancer gene therapy have been conducted in the USA, but encouraging clinical results have yet to be obtained. Recently, replication-competent oncolytic viral vectors have been developed to improve antitumor activity. However, there remain two major concerns with the use of these oncolytic vectors: frequent relapse of tumors despite temporary inhibition of tumor progression (1), and the generation of high titers of neutralizing antibodies which subsequently inhibit repetitive viral infection (2). Repetitive infection is difficult to achieve, although anti-CD3 antibody (2), polyethylene glycol (3), liposome (4), etoposide (5), and cyclophosphamide (6) have been tested in attempts to overcome the humoral immune responses to viral vectors.

Many studies of oncolytic vector-infected carrier cells have been reported, including intraperitoneal injection of PA-1 ovarian carcinoma cells infected with an oncolytic HSV-1 to treat intraperitoneal ovarian carcinoma xenografts (7), intravenous injection of MDA-MG-231 breast carcinoma cells infected with wild-type adenovirus to treat lung metastatic foci of breast carcinoma xenografts (8), intravenous injection of mesenchymal stem cells infected with oncolytic adenovirus to treat lung and breast tumor xenografts (9), intravenous injection of myeloma cells infected with oncolytic measles, vaccinia, vesicular stomatitis virus, and coxsackievirus A21 to treat orthotopic myeloma xenografts (10), intravenous injection of cytokine-induced killer cells infected with modified vaccinia virus to treat intraperitoneal ovarian tumors and subcutaneous breast tumors in syngeneic mice (11), intravenous injection of rat hepatoma cells infected with oncolytic parvovirus to treat lung metastatic foci of syngeneic rat hepatoma tumors (12), intravenous injection of CT26 tumor cells infected with oncolytic vesicular stomatitis virus to treat lung metastatic foci of syngeneic mouse CT26 colon tumors (13), intravenous injection of autologous CD8⁺ lymphocytes infected with oncolytic vesicular stomatitis virus to treat lymphnode metastatic foci of syngeneic mouse melanoma tumors (14), and

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intratumoral injection of A549 tumor cells infected with oncolytic adenovirus to treat syngeneic mouse ovarian tumors (15). These carrier cell treatments have yielded significant antitumor effects in syngeneic and non-syngeneic mouse tumors and completely overcome the inhibition of virus infection by antiviral antibody production (13,15).

The *IAI.3B* gene was originally isolated using polyclonal antibodies to a high-molecular-weight fraction derived from ovarian carcinoma (16). The promoter activity of the *IAI.3B* gene is specific for ovarian carcinoma, and an oncolytic adenovirus termed AdE3-*IAI.3B*, in which the *E1A* gene is under the control of the human *IAI.3B* promoter, replicates as efficiently as the wild-type adenovirus in ovarian carcinoma cells but causes regrowth following temporary inhibition of growth of ovarian carcinoma cell tumors (17). However, the promoter activity of the *IAI.3B* and the effects of AdE3-*IAI.3B* in human oral squamous cell carcinoma (OSCC) have not yet been examined. In a previous study (15), human non-small cell lung cancer A549 carrier cells were infected with AdE3-*IAI.3B* and the adenoviral particle-containing cell fragments derived from the carrier cells were engulfed by target cancer cells. This non-receptor-mediated adenoviral transfection system circumvented neutralization by anti-adenovirus antibodies and enhanced antitumor activity after the induction of anti-adenoviral CTL responses by preimmunization with adenovirus in syngeneic mice, and an antitumoral immune response was then induced. Combination with GM-CSF augments the antitumor effects of carrier cells and induces complete tumor reduction (15). In the present study, we examined the promoter activity of *IAI.3B*, the antitumor activity of AdE3-*IAI.3B*, and the antitumor activity of carrier cells infected with AdE3-*IAI.3B* in OSCC.

Materials and methods

Cell lines and culture conditions. Human non-small cell lung cancer A549, human cervical squamous cell carcinoma SKGIIIa, human OSCC HSC-2, HSC-3 and HSC-4, human skin squamous cell carcinoma HSC-5, and human breast carcinoma MCF-7 cells were obtained from the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan). The human ovarian clear cell carcinoma KK and human ovarian adenocarcinoma KF cells were gifts from Dr Y. Kikuchi, National Defense Medical College, Tokorozawa, Japan. Human ovarian adenocarcinomas OCC1, 420, OVCAR3, 429, and HEY cells were obtained from Dr G. Mills and murine squamous cell carcinoma SCC7 from Dr L. Milas (The University of Texas, M.D. Anderson Cancer Center, TX, USA). Normal human keratinocyte K42 and normal human fibroblast F27 cells were established by Dr K. Hashimoto, Ehime University, Japan. Normal human ovarian fibroblast NOE-1 cells were established in our laboratory. Human umbilical vein endothelial HUVECs were obtained from Cambrex Bio Science Walkersville Inc. (Walkersville, MD). Human OSCC GFP-SAS was established by S. Shintani (Showa University, Tokyo, Japan). Human OSCC Ca9-22 was obtained from the Cell Resource Center for Biomedical Research (Institute of Development, Aging and Cancer, Tohoku University). Human cervical squamous cell carcinoma HT-III and CaSki cells, human cervical adenocarcinoma

HeLa, human colorectal adenocarcinoma HT-29, and human pancreas carcinoma PANC-1 cells were obtained from the American Type Culture Collection (Rockville, MD).

Cells were maintained in a humidified 5% CO₂/95% air incubator at 37°C. All cell lines except K42 and HUVECs were grown in RPMI medium supplemented with 10% heat-inactivated fetal bovine serum. K42 cells were grown in MCDB153 (Nissui Co., Tokyo, Japan) with bovine hypothalamus extract. HUVEC cells were grown in EBM-2 (Cambrex, MD).

Construction of the AdE3-*IAI.3B* vector. The pXC1 plasmid has adenovirus 5 sequences from nt 22 to nt 5790 containing the *E1* gene (Microbix Biosystems Inc., Toronto, Canada). A unique AgeI site was introduced to generate the plasmid pXC1-AgeI. The *IAI.3B* promoter was ligated to pXC1-AgeI plasmid to obtain pXC1-*IAI.3B*. To construct the AdE3-*IAI.3B* virus, homologous recombination was performed between pXC1-*IAI.3B* plasmid and the right-hand side of pBHGE3 adenovirus DNA containing the *E3* region in 293 cells by a standard technique (17). To construct the wild-type adenovirus AdE3, homologous recombination was performed between pXC1 and pBHGE3 in 293 cells. The replication-defective *E1*-deleted Ad- β -gal virus was used as control adenovirus. All viruses were purified with double Cesium Chloride gradients using standard methods, and titered with standard spectrophotometry and plaque assay (17). AxCAmGM-CSF was obtained from RIKEN Bioresource Center (Tsukuba, Japan).

Real-time quantitative RT-PCR. RNA samples (100 ng) were used in RT and real-time PCR for RNA expression studies. Reverse transcriptase and real-time PCR reactions were carried out with the ABI prism 7700 sequence detection system (Applied Biosystems, Foster City, CA) in a total volume of 50 μ l that contained TaqMan one step RT-PCR master mix (Applied Biosystems), 0.3 μ M of each forward and reverse primer, and 0.21 μ M of MGB probe. The forward and reverse primer and MGB probe for *IAI.3B* were respectively 5'-CCACTTGTTCCATGTGACACAGA-3', 5'-CCGTTTCGTTAACCCTTGTCTC-3' and 5'-AAGACAAGCCCCAGACTGGTTCACAAG-3', respectively. The reaction was performed with the following thermal cycling method: 30 min at 48°C for reverse transcription, 5 min at 95°C for AmpliTaq Gold activation, 15 sec at 95°C and 60 sec at 60°C for 40 cycles. GAPDH was chosen as a house-keeping gene to be tested as an endogenous control.

Assay for promoter activity. *IAI.3B* promoter fragment was inserted into the luciferase reporter vector PicaGene Basic, a promoterless and enhancerless vector (Toyo INK MFG Co., Tokyo, Japan). The sequence of the insert was confirmed with an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). Construct containing *IAI.3B* promoter sequence was fused to the *Luciferase* gene which was transfected into cells in the presence of Lipofectamine transfection reagent (Invitrogen, Carlsbad, CA) according to the protocol recommended by the manufacturer. Briefly, 1x10⁵ cells seeded in a 12-well culture dish were exposed to transfection mixtures containing 1 μ g of luciferase reporter plasmids and 0.2 μ g



SPANDIDOS PUBLICATIONS *luciferase-herpes simplex virus thymidine kinase* control vector (Promega, Madison, WI) at 37°C for 48 h. Dual luciferase assays were performed according to the manufacturer's protocol (Promega).

Crystal violet assay. Cells were plated at a density of 2000 cells/well in 96-well plates. Cells were infected with AdE3-*IAI.3B*, AdE3, and co-cultured with AdE3-*IAI.3B*- and AdE3-infected carrier cells with or without high titer ($\times 6,000$) of anti-adenovirus antibodies (Takeda Pharmaceutical, Tokyo, Japan). After 5 days, cells were fixed and stained with 0.5% crystal violet in 50% methanol for 1 h at room temperature, washed with water, and air-dried, and each well was scanned with a spectrophotometric microplate reader (Immuno Mini, NJ-2300, Nalge Nunc International K.K., Tokyo, Japan) at the wavelength of 540 nm. The 50% growth inhibitory concentration (IC_{50}) was determined.

Inhibition of subcutaneous tumor growth in vivo. To determine the inhibition of xenograft subcutaneous tumor growth, AdE3-*IAI.3B* was injected into subcutaneous tumors in female nude (*nu/nu*) mice (CLEA Japan Inc., Tokyo). In brief, 1×10^7 GFP-SAS cells in 100 μ l of RPMI were injected into the left posterior flank of each mouse through an insulin syringe with a 27 1/2-gauge needle. In each group 5-10 animals were used. After 20-30 days, small (4-5 mm) or large (8-10 mm) tumors were established. Then 100 μ l of AdE3-*IAI.3B* (1×10^{10} PFU, plaque forming units), AdE3 (1×10^{10} PFU), Ad- β -gal (1×10^{10} PFU), medium alone, or A549 carrier cells infected with AdE3-*IAI.3B* at 200 MOI (multiplicity of infection) were injected intratumorally on days 0, 1, 2, 3, 4 and 5. The tumors were measured every 2 days with calipers to determine two perpendicular diameters. Tumor volume was calculated by assuming a spherical shape, with average tumor diameter calculated as the square root of the product of cross-sectional diameters.

To determine inhibition of syngeneic subcutaneous tumor growth, murine SCC7 cells (1×10^6) were injected into the left thigh of female C3H mice (CLEA) and AdE3-*IAI.3B*-infected A549 carrier cells were injected into subcutaneous tumors. Five to ten animals were used for each group. Medium alone, AdE3-*IAI.3B* (1×10^{10} PFU), or A549 carrier cells (5×10^6) infected with AdE3-*IAI.3B* at 200 MOI with or without AxCamGM-CSF at 10 MOI were injected into the tumors on days 0, 1, and 2. Mice were preimmunized with Ad- β -gal (1×10^{10} PFU) 21 days before the inoculation of SCCA7 cells. The animal studies were approved by the Ehime University Review Board.

Statistical analyses. Values are the mean \pm SD, and were examined with the unpaired t-test, Cochran-Cox test, Welch test, and regression analysis. Survival data were plotted on Kaplan-Meier curves, and examined with the log-rank test using the LIFETEST procedure. Findings of $P < 0.05$ were considered significant.

Results

mRNA levels of *IAI.3B* in OSCC cells. To examine the mRNA levels of *IAI.3B* in OSCC cells, one-step real-time RT-PCR

was performed using MGB probe. The mRNA levels of *IAI.3B* in OSCC cells were not significantly different from those in ovarian, cervical and other cancer cells but 6 times higher than those in normal cells (Fig. 1A).

Transcriptional activities of *IAI.3B* in OSCC cells. To examine the transcriptional activities of *IAI.3B* in OSCC cells, transient expression assays were performed. Luciferase reporter plasmid containing the 5'-flanking regions of 1875 bp upstream from the transcriptional start site of the *IAI.3B* gene was constructed and transfected into each cell, and cell lysates were tested in luciferase assays. Fig. 1B demonstrates the transcriptional activities in each cell. The promoter activity of *IAI.3B* in OSCC cells was not significantly different from those in ovarian, cervical and other cancer cells but 36 times higher than those in normal cells.

Transcriptionally targeted AdE3-*IAI.3B* has potent anti-proliferative effects in OSCC, ovarian, cervical, and other cancer cells but not in normal cells. To determine the most appropriate infection condition, AdE3-*IAI.3B* was infected in A549 cells at various MOIs and infection times. A549 carrier cells infected with AdE3-*IAI.3B* at 200 MOI for 33-48 h exhibited the most potent anti-proliferative effect on HEY cells in the presence of antiadenovirus antibodies (Fig. 2A and B). Furthermore, 12-36 h of infection induced the least injury of carrier cells infected with AdE3-*IAI.3B* at 200 MOI (Fig. 2C). Therefore, 200 MOI for 33-36 h was considered the best set of conditions for infection of carrier cells. More than 48 h of infection induced severe injury in carrier cells infected with AdE3-*IAI.3B* at 200 MOI (Fig. 2C). Furthermore, 33 h of infection was adopted as the infection time, since it is more convenient than 36 h.

To estimate the potential of *IAI.3B* promoter for use in gene therapy of OSCC, AdE3-*IAI.3B* was transfected into each cell line. Fig. 3A shows the growth-inhibitory effects (IC_{50}) of AdE3-*IAI.3B* in various types of cell lines. The IC_{50} of AdE3 did not differ significantly among OSCC, ovarian, cervical, other cancer cells, and normal cells. AdE3-*IAI.3B* did not kill normal cells but significantly suppressed the growth of OSCC, ovarian, cervical and other cancer cells. Fig. 3B shows the growth-inhibitory effects (IC_{50}) of AdE3-*IAI.3B*-infected carrier cells in various types of cell lines. The IC_{50} of AdE3-infected carrier cells did not differ significantly among OSCC, ovarian, cervical, other cancer cells, and normal cells. However, AdE3-*IAI.3B*-infected carrier cells did not kill normal cells but significantly suppressed the growth of OSCC, ovarian, cervical, and other cancer cells.

AdE3-*IAI.3B*- and AdE3-*IAI.3B*-infected A549 carrier cells suppress subcutaneous tumor growth of OSCC, respectively, in nude and syngeneic mice. To evaluate the antitumor effect of AdE3-*IAI.3B* in small tumors, subcutaneous xenograft tumors, 4-5 mm diameter were established in the left flanks of nude mice using GFP-SAS OSCC cells. By 12 days, we observed significant reduction in tumor size in the AdE3-*IAI.3B*- and AdE3-treated groups compared with the medium-alone- and Ad- β -gal-treated groups in the GFP-SAS tumor models. By 30 days, complete tumor regression was transiently

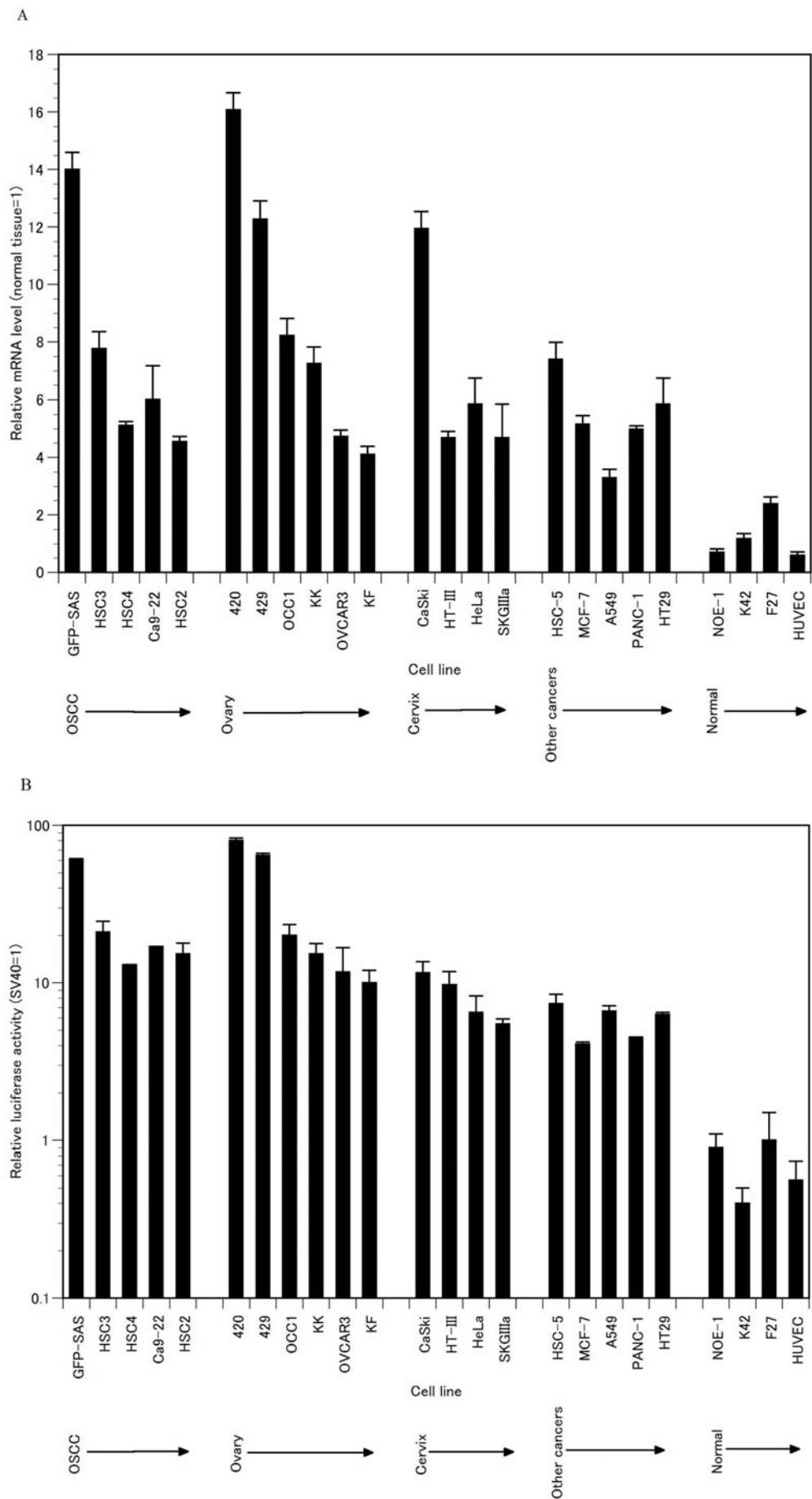


Figure 1. (A) *IAI.3B* mRNA levels determined by real-time RT-PCR and (B) transcriptional activity of *IAI.3B* promoter in head and neck squamous cell, ovarian, cervical, other cancer and normal cells. mRNA and luciferase activities were plotted as ratios to those in the normal tissue and the control plasmid (pGV-SV40) driven by the SV40 enhancer/promoter, respectively. Bars, SD.

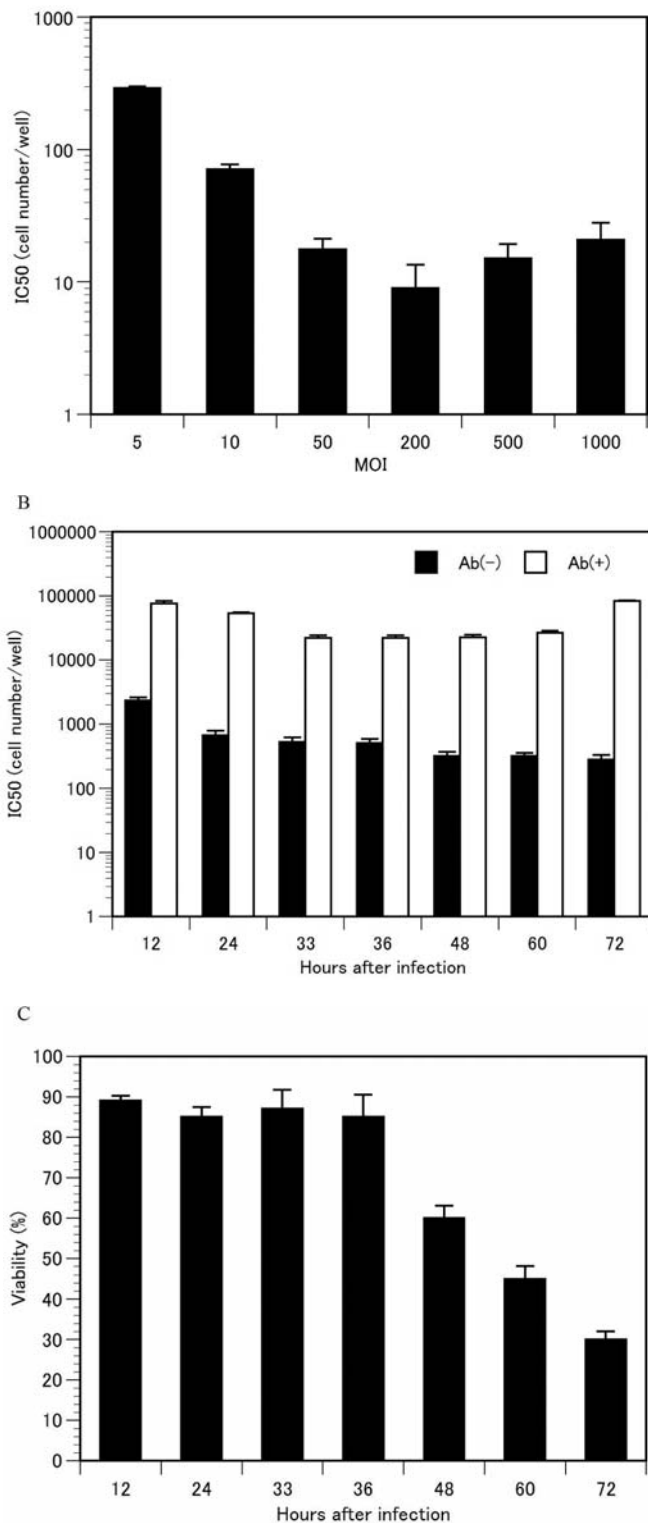


Figure 2. (A) The growth-inhibitory effects (IC₅₀) of AdE3-*IAI.3B* at various MOIs with anti-adenovirus neutralizing antibodies. (B) The growth-inhibitory effects (IC₅₀) of AdE3-*IAI.3B*-infected carrier cells at various infection times with or without anti-adenovirus neutralizing antibodies (Ab). (C) The viability of A549 carrier cells infected with AdE3-*IAI.3B* at various infection times. Bars, SD.

observed in the AdE3-*IAI.3B*- and AdE3-treated groups, though all tumors regrew by 60 days (Fig. 4A). To evaluate

the antitumor effect of AdE3-*IAI.3B* in large tumors, xenograftic subcutaneous tumors with 8-10-mm diameter were established in the left flanks of nude mice using GFP-SAS. By 20-50 days after initiation of intratumoral injections, AdE3-*IAI.3B* had significantly repressed the growth of GFP-SAS tumors, but AdE3-*IAI.3B*-injected tumors regrew 60 days after the initiation of treatment, to an extent not significantly different from the medium-treated control and Ad- β -gal (Fig. 4B). In contrast, carrier cells infected with AdE3-*IAI.3B* completely suppressed the growth of GFP-SAS tumors 40 days after initiation of treatment and induced complete tumor regression.

To evaluate the antitumor effects of AdE3-*IAI.3B* after immunization in an immunocompetent syngeneic mouse model, C3H mice were immunized with Ad- β -gal and subcutaneous tumors with 5-8-mm diameter were established in the left thigh of C3H female mice using SCC7 cells (Fig. 4C). Survival of control mice was not significantly different from that of the mice treated with AdE3-*IAI.3B* alone. The survival of mice treated with A549 carrier cells infected with AdE3-*IAI.3B* was significantly longer than those of the mice treated with medium control or AdE3-*IAI.3B*. Furthermore, simultaneous infection with AxCamGM-CSF augmented the antitumor effect of A549 carrier cells infected with AdE3-*IAI.3B* and induced complete elimination of tumor in all treated mice. Mice that exhibited complete tumor regression were resistant to subsequent inoculation of SCC7 cells.

Discussion

The *IAI.3B* promoter-introduced replication-competent oncolytic adenovirus AdE3-*IAI.3B* replicates as efficiently as wild-type adenovirus in human OSCC, human ovarian carcinoma, human cervical carcinoma, and other cancer cells expressing high levels of *IAI.3B*, but does not in normal cells expressing low levels of *IAI.3B* (17). However, intratumoral administration of AdE3-*IAI.3B* alone yielded no prominent therapeutic effects in nude mice bearing large xenografts; nevertheless, injection of AdE3-*IAI.3B*-infected A549 carrier cells into the tumors produced significant antitumor effects. The antitumor effect of carrier cells in nude mice could be due to increases in local retention time and local concentration of AdE3-*IAI.3B* by delivery from carrier cells. Direct intratumoral administration of oncolytic adenoviruses such as ONYX-015 did not prevent the recurrence of treated tumors, although ONYX-015 transiently eradicated tumors in nude mice (1). More than 90% of intratumorally injected adenoviruses in nude mice migrate into the systemic circulation and accumulate in the liver (15). Therefore, oncolytic adenovirus itself is unable to induce complete tumor regression in nude mice, though oncolytic adenovirus-infected carrier cells are able to.

Treatment with PEGylation (3), liposome-encapsulation (4), etoposide (5) and cyclophosphamide (6) decreased the titer of neutralizing antibodies against adenoviruses and yielded partial recovery of adenoviral infectivity in target cells on the second injection. However, such modifications were ineffective in the case of third or later injections, due to the increase in anti-adenovirus antibody titers, and therefore,

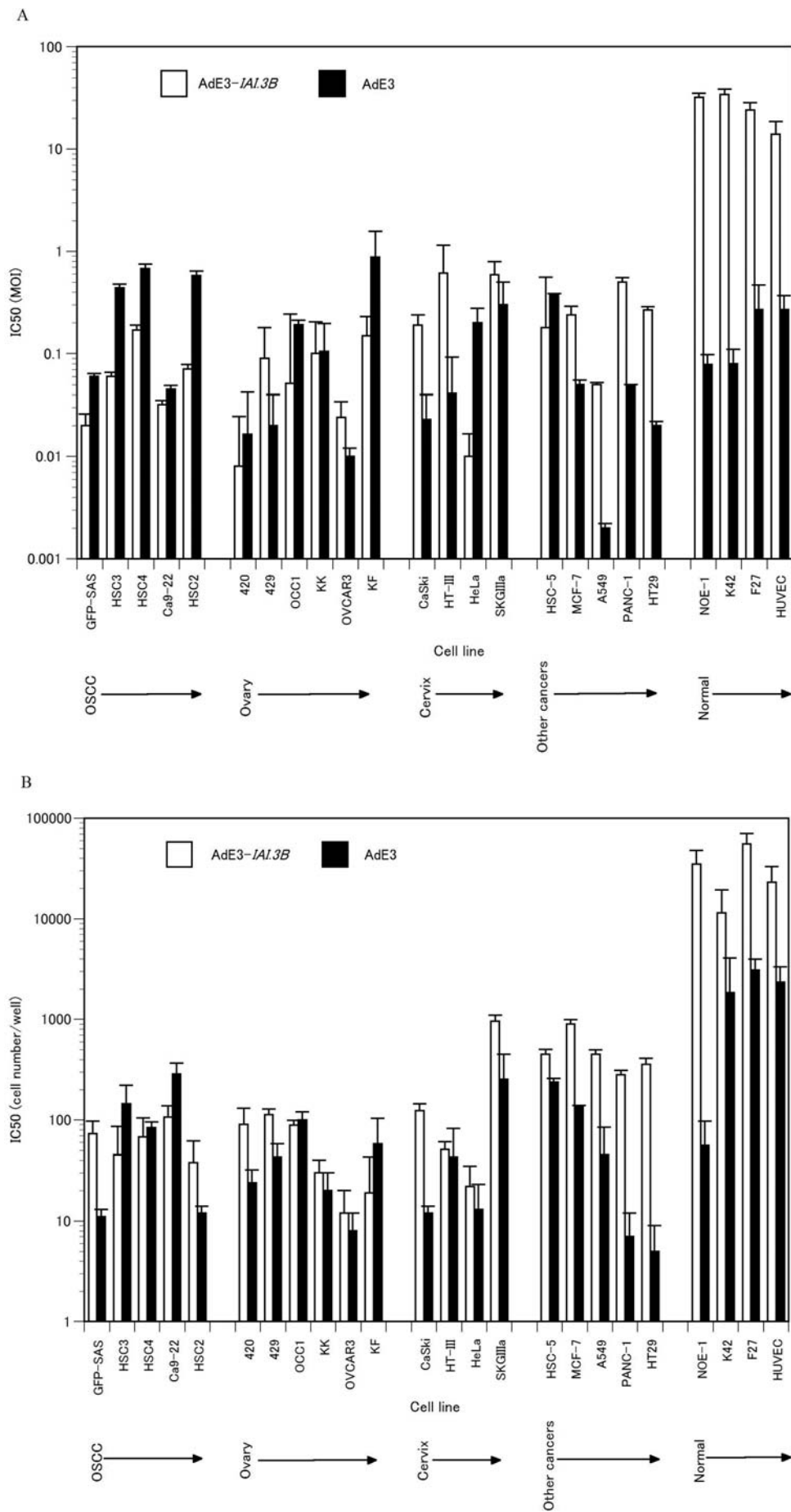


Figure 3. (A) The growth-inhibitory effects (IC₅₀) of AdE3-*IAI.3B* and AdE3 and (B) the IC₅₀ of AdE3-*IAI.3B*- and AdE3-infected A549 carrier cells in OSCC, ovarian, cervical, other cancer and normal cells without anti-adenovirus neutralizing antibodies. Bars, SD.

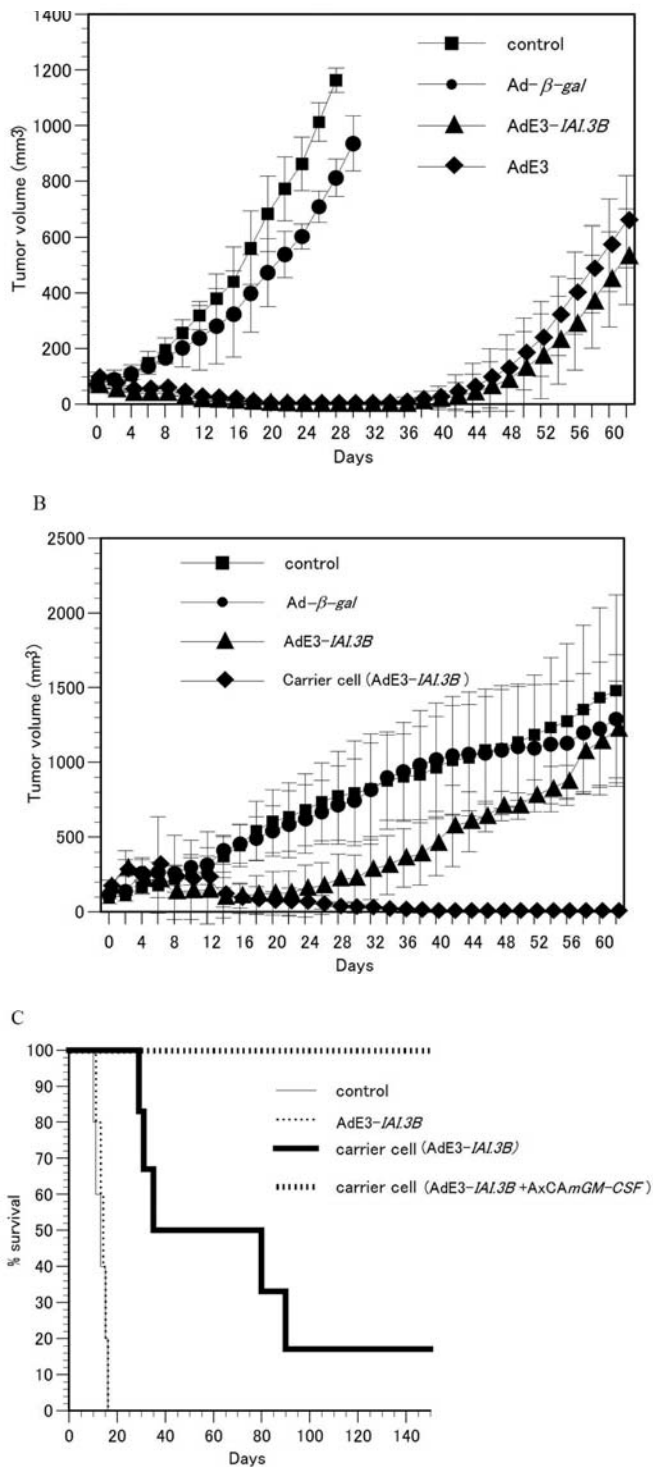


Figure 4. (A) The antitumor effects of AdE3-IAI.3B in subcutaneous OSCC GFP-SAS small (4-5 mm) tumors in nude mice. (B) The antitumor effects of A549 carrier cells infected with AdE3-IAI.3B in large GFP-SAS subcutaneous OSCC (8-10 mm) tumors in nude mice. (C) The antitumor effects of A549 carrier cells infected with AdE3-IAI.3B against subcutaneous syngeneic SCC7 tumors established in C3H mice after immunization. RPMI medium, Ad-β-gal, AdE3-IAI.3B, and A549 carrier cells infected with AdE3-IAI.3B with or without AxCaGM-CSF were injected into each tumor. Bars, ±SD.

could not produce significant antitumor effects in immuno-competent animals. Moreover, murine cancer cells are

resistant to replication-competent viral gene therapy because of their lower expression of adenoviral receptors (18). However, our carrier cell system completely eradicated murine tumors in syngeneic mice and produced significant antitumor effects despite high titers of anti-adenovirus antibodies.

Our previous study demonstrated that A549 carrier cells bearing oncolytic adenoviruses delivered adenoviral particles through their cell fragments to target cells (15). Engulfment of cell fragments was observed only in proliferative malignant cells and not in non-proliferative normal cells. Carrier cell-mediated adenoviral infection consequently produced CTL responses against adenoviruses. Furthermore, mice with complete tumor regression obtained protective immunity. It thus appears that the cell fragment-mediated transfer of adenovirus is independent of adenovirus receptors and is not blocked by anti-adenovirus antibodies. Many studies of oncolytic virus-infected carrier cell systems have been reported in oncolytic HSV-1 mutant-infected PA-1 cells (7), in modified vaccinia virus-infected cytokine-induced killer (CIK) cells (11), in oncolytic adenovirus-infected A549 cells (15), in vesicular stomatitis virus (VSV)-infected colon carcinoma cells (13), in nerve growth factor-transduced autologous fibroblasts (19), and in human interferon β-transduced umbilical cord matrix stem cell (20). In this study, AdE3-IAI.3B selectively killed OSCC cells *in vitro* and *in vivo*. IAI.3B promoter-driven oncolytic virotherapy has not yet been reported in OSCC cells. The present study is the first report of IAI.3B-specific gene therapy for OSCC. Although clinical trials for adenovirus-p53 (21) and oncolytic adenovirus (22) for OSCC have been reported, the results of such clinical trials were clinically insufficient, since adenoviral infection was completely blocked by the production of anti-adenovirus neutralizing antibodies. This finding is identical to those for gene therapy with other viruses.

It has been reported that oncolytic virus-infected carrier cells overcome the viral-induced humoral immune response, the viral-induced cellular immune response kills virus-infected target cancer cells, and GM-CSF augments the anti-tumor effect of carrier cells (15). The present study also demonstrated that oncolytic adenovirus-infected A549 carrier cells induced elimination of tumor and adenovirus-GM-CSF augmented the anti-tumor effect of carrier cells, resulting in complete regression of tumor in mice. Furthermore, second challenge with syngeneic mouse squamous cell carcinoma was completely rejected by a specific antitumor response, also suggesting that the systemic tumor immune response induced by carrier cell treatment may cure not only the carrier cells-injected local tumors but also non-injected metastatic tumors. In conclusion, IAI.3B promoter-driven oncolytic adenovirus-infected carrier cells may cure OSCC and human clinical trials with AdE3-IAI.3B-infected carrier cells should be possible in the near future.

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