Induction of Immunological Antitumor Effects by Adenovirus-Mediated Gene Transfer of B7-1 in a Murine Squamous Cell Carcinoma Cell Line

Yasuhiro Hoshitani, MD; Haruhiko Ishida, MD, PhD; Naoki Otsuki, MD, PhD; Toshiro Shirakawa, MD, PhD; Akinobu Gotoh, MD, PhD; Ken-ichi Nibu, MD, PhD

Objective: To evaluate the antitumor immune effects of B7-1 gene expression mediated by adenoviral vectors against squamous cell carcinoma. Transfection of the costimulatory molecule B7-1 gene into certain murine tumors increases antitumor immunity and suppresses tumor growth.

Design: In vitro and in vivo study.

Interventions: A murine squamous cell carcinoma cell line, KLN205, was infected with adenoviral vectors carrying either B7-1 (AdB7) or LacZ (AdCL) genes. Infected cells were injected subcutaneously into the flanks of DBA/2 mice.

Main Outcome Measures: The growth of tumors infected with adenoviral vectors was measured.

Results: AdB7-infected cells grew significantly slower than AdCL-infected cells in vivo, while there was no significant difference in the growth rates between the 2 groups in vitro. Moreover, significant growth suppression of rechallenged noninfected parental cells was observed in the mice immunized with AdB7-infected cells but not in those immunized with AdCL-infected cells.

Conclusion: These results suggest that the B7-1 gene has therapeutic potential for immunotherapy against head and neck squamous cell carcinoma.

Arch Otolaryngol Head Neck Surg. 2007;133:270-275

Among the known costimulatory molecules, the B7 family is the most potent, and B7-1 (CD80) is the first identified member of this family. B7-1 binds to CD28 and CTLA-4, counterreceptors on T lymphocytes, and is regarded to be mandatory in their activation. B7-1 is expressed on most antigen-presenting cells, such as dendritic cells, activated macrophages, and B cells, but is not expressed in most solid tumor cells. Low or negative expression of B7-1 in tumor cells is thought to provide an opportunity for escape from the antitumor immune system. Indeed, several studies have reported promising antitumor effects of B7-1 gene transfection in tumor cells. However, the antitumor effect of B7-1 on SCC is still controversial.

In the present study, we evaluated the antitumor effect of B7-1 gene expression mediated by an adenoviral vector in a murine SCC model to further explore the potential of the B7-1 gene as a candidate for immunotherapy against head and neck cancers. To our knowledge, this is the first study to report identification of the antitumor effect of B7-1 mediated by an adenoviral vector against murine SCC in immunocompetent mice.
ANIMALS AND CELL LINE

Male DBA/2 mice purchased from Japan Clea Co Ltd (Tokyo, Japan) were maintained in a specific pathogen-free environment. Sterilized food and water were provided ad libitum. Mice were 6 weeks of age at the time of the experiment, which was approved by the Committee for Animal Experiments of Kobe University School of Medicine, Kobe, Japan, and carried out under the Guidelines for Animal Experiments at Kobe University School of Medicine.

KLN205, which was obtained from Dainippon Pharmaceuti
cal Company (Osaka, Japan), is an established cell line of SCC generated from a rat tumor. The cells were maintained as monolayer cultures in minimum essential medium containing 10% fetal bovine serum, L-glutamine, and penicillin G potassium at 37°C in 5% carbon dioxide.

ADENOVIRAL VECTORS

The adenoviral vector carrying the B7-1 gene (AdexCAmB7: AdB7), which was obtained from RIKEN BioResource Center (Tsukuba, Japan), is a recombinant adenovirus expressing the mouse B7-1 (CD80) gene and is regulated by the cytomegalovirus enhancer/chicken β-actin (CAG) promoter. An E1-deleted human type-5 adenovirus, which contains the lacZ gene that encodes β-galactosidase (AdCL), was used as a negative control. The viruses were grown to high titers in the human embryonic kidney 293 cell line containing E1. They were then released from the cells by sonication and were purified twice by means of cesium chloride density ultracentrifugation. Finally, the viruses were dialysed by passage through a dialysis filter and frozen until use. The titers were determined by plaque-forming assay on the kidney 293 cell line containing E1.

DETECTION OF B7-1 EXPRESSION ON KLN205 CELLS INFECTED WITH AdB7

KLN205 cells planted in a cell culture dish (100 x 20 mm) were infected with AdB7 at various concentrations (multiplicity of infections [MOIs] of 0, 8, 64, and 512), and incubated for 72 hours at 37°C in 5% carbon dioxide. Real-time polymerase chain reaction was used for detection of B7-1 expression on these cells.

DETERMINATION OF OPTIMAL DOSE FOR AdB7 INFECTION

KLN205 cells planted at a density of 5.0 x 10⁵ cells per 10-mL medium in a cell culture dish (100 x 20 mm) were infected with AdB7 at various MOIs. After incubation at 37°C for 24, 48, and 72 hours, the living cells were counted, and the optimal dose for AdB7 infection was estimated from the cell proliferation curves.

INJECTION OF KLN205 CELLS IN DBA/2 MICE

After incubation with AdB7 or AdCL in a cell culture dish (100 x 20 mm) for 72 hours at 37°C, the KLN205 cells were trypsinized and centrifuged. After the mice were anesthetized with diethyl ether, a single-cell suspension of AdB7-infected KLN205 cells was injected subcutaneously into the flanks of DBA/2 mice with a 27-gauge needle at a dose of 5 x 10⁵ cells in a volume of 200 μL (AdB7-infected group). Two control groups were prepared in the following manner: the noninfected group was injected with noninfected KLN205 cells (parental cells) and the AdCL-infected group was injected with AdCL-transfected KLN205 cells in the same manner as for the AdB7-infected group. The largest diameters of the injected tumors (width x length) were measured in millimeters with a caliper. Tumor-bearing mice were killed when the diameter had become greater than 30 mm. At least 6 mice were used for each group.

IMMUNOCYTOCHEMICAL AND STATISTICAL ANALYSIS

Two mice in each of the 3 groups were killed 14 days after inoculation. Tumors were resected and were stored in TissueTek OCT compound (Sakura Finetechnical Co Ltd, Tokyo, Japan) at –80°C until immunohistochemical analysis.

Sections were cut to a thickness of 4 μm with a freezing microtome and fixed with acetone for 10 minutes at 4°C, followed by rinsing with phosphate-buffered saline. Endogenous peroxidase was blocked with 3% hydrogen peroxide, and non-specific binding was blocked with normal serum. The primary antibodies against mouse CD80 (RMM-P1-1; Serotec, Oxford, England), MHC class I (ER-HR 52; BMA Biomedicals, Augst, Switzerland), CD8 (33-6-7; GT Urological, Minneapolis, Minn), and CD4 (sc-13573; Santa Cruz Biotechnology, Santa Cruz, Calif) were used at a dilution of 1:500, 1:100, 1:500, and 1:500, respectively. Slides were incubated with the respective antibodies for 30 minutes at room temperature. Antibody binding was visualized with Vectastain ABC kit Elite (Vector Laboratories, Burlingame, Calif). Diaminobenzidine was used for coloration, and nuclei were counterstained with hematoxylin. A negative control was included using phosphate-buffered saline as a replacement for the primary antibody.

The paired t-test was used to identify statistical differences. P<.05 was considered statistically significant.

DETECTION OF B7-1 EXPRESSION IN KLN205 CELLS INFECTED WITH AdB7

First, the expression of the B7-1 gene mediated by AdB7 was verified by means of real-time polymerase chain reaction. As shown in Figure 1, B7-1 expression in KLN205 cells infected with AdB7 increased in proportion to the concentration of the adenoviral vector.

OPTIMAL DOSE FOR VECTOR INFECTION

Next, we assessed the cytotoxic effects of AdB7 for KLN205 cells. As shown in Figure 2, proliferation of the AdB7-infected KLN205 cells was clearly inhibited at an MOI of 343, while there was no significant difference in proliferation between noninfected and AdB7-infected KLN205 cells at an MOI of 216 or less. Thus, we determined that an MOI of 216 was the optimal dose of vector to attain maximum expression of the B7-1 gene without cytotoxic effects caused by the adenoviral vector itself.

B7-1 ANITUMOR EFFECTS IN VIVO

We next studied the primary antitumor effect of AdB7 infection. After incubation with AdCL or AdB7 at an MOI...
of 216 in a cell culture dish (100 x 20 mm) for 72 hours at 37°C, cells were collected and injected into the mice. Figure 3 shows representative tumors of mice from the 3 groups 5 weeks after tumor cell injection. Over time, the tumors of the noninfected and AdCL-infected groups became evidently larger than those of the AdB7-infected group. Two weeks after injection, the size of tumors in the AdB7-infected group was significantly smaller than those of tumors in the noninfected or AdCL-infected group (Figure 4; P = .007), while there was no statistical difference between the size of tumors in the noninfected and in the AdCL-infected group (P = .17).

VACCINATION EFFECT OF ADB7

To study the antitumor effect of AdB7 in greater depth, we examined the vaccination effect of AdB7-infected cells. Two weeks after the tumor cell injection, 5 x 10⁵ of the noninfected KN205 cells (parental cells) were injected into the contralateral flank of the mice in all 3 groups. Fourteen days after the rechallenge of the parental cells, significant growth suppression was observed in the rechallenged tumors of AdB7-infected tumor-bearing mice in comparison with those in AdCL-infected or noninfected tumor-bearing mice (Figure 5).

IMMUNOHISTOCHEMICAL ANALYSIS

Finally, we performed immunohistochemical analyses to verify the production of CD80 encoded by B7-1 gene expression and of the MHC class I antigen of KN206 cells, as well as to determine the type of infiltrating T lymphocytes in the tumor tissues.

Positive staining for CD80 was detected in most tumor cells in the AdB7-infected group but only in occasional cells in the AdCL-infected or noninfected groups (Figure 6D-F). In contrast, staining for the MHC class I antigen was observed in most of the tumor cells in all 3 groups (Figure 6G-I). A large number of CD8-positive cells was seen in AdB7-infected tumors, while there were few CD8-positive cells in the AdCL-infected or noninfected group (Figure 6J-L). CD4-positive cells were rare in all 3 groups (Figure 6M-O).

COMMENT

The importance of B7-1 as a costimulator for generating antitumor immune response has been suggested by results obtained with a number of in vivo experimental systems including melanomas, adenocarcinomas, and gliomas. These studies found evidence of the direct antitumor effect of the B7-1 gene by using murine models of the tumors. Additional studies have demonstrated that immunizing mice with B7-1-transduced tumor cells elicits protective and sometimes curative immunity against wild-type tumors. These studies imply the potential of adenoviral gene transfer for the treatment of cancer. However, the antitumor effect of B7-1 remains controversial. In a murine model of oral SCC cell lines established by chemical stimulation, progressive growth was observed when cells lost their B7-1 expression. Lymphocytes stimulated with B7-1-transfected SCC cells proved to be cytotoxic against parental cells in vitro, and liposomal transfection of B7-1 plasmids enhanced the immunological response in vitro. On the other hand, transgenic mice constitutively expressing B7-1 on keratinocytes could not inhibit the development of carcinomas following chemical carcinogenesis in skin, and SCC cell lines transfected with B7-1 via a plasmid vector did not alter tumor growing capability in vivo.

Adenovirus has proven to be an effective vehicle for transferring exogenes to target cells. Replication-deficient adenoviral vectors provide a number of advantages for the transfer and expression of therapeutic genes. Recently, it has been reported that adenovirus-mediated B7-1 gene transfer has an antitumor effect on several tumors. Encouraged by these findings, we performed this study to evaluate the antitumor effect of the
B7-1 gene by using an adenoviral vector harboring the B7-1 gene and to determine the potential of the B7-1 gene for tumor immunotherapy against SCC.

As our results show, high-level expression of B7-1 in mouse SCC cells via an adenoviral vector was obtained at a vector concentration without cytotoxic effects caused by the adenoviral vector itself. The growth of the subcutaneous tumors was markedly retarded in the AdB7-infected group, whereas tumors progressively increased in size in the 2 control groups. Immunohistochemical examination confirmed strong CD80 expression (product of the B7-1 gene) on tumor cells and infiltration of a large amount of CD8-positive cells in the AdB7-infected group. Expression of MHC class I was also confirmed in most parental tumor cells. These results indicate that AdB7-infected SCC cells obtained a function as antigen-presenting cells. They then activated CD8-positive T lymphocytes by means of CD80 costimulation with MHC class I, resulting in the antitumor effect. Moreover, in the “vaccine experiment,” an antitumor effect was attained in the mice immunized with AdB7-infected cells, suggesting that vaccination augments not only local but also systemic immune response.

In our experiments, we used an immunogenic cell line distinctly expressing MHC class I as a wild-type tumor as shown in Figure 6. On the other hand, in nonimmunogenic or low-immunogenic tumors expressing a low level of MHC class I, transduction of B7-1 alone is not sufficient to induce an antitumor effect against tumor cells. In an effort to compensate for the low expression of MHC class I, the synergistic effect of B7-1 gene transfer combined with other costimulatory molecules or cytokines including intercellular adhesion molecule-1, interleukin (IL)-2, IL-4, IL-12, and interferon gamma has been studied with promising results. Thus, we are currently preparing the further experiments with various cytokines to conquer not only the immunogenic but also the low- or nonimmunogenic type of SCC.

In conclusion, the findings of this study show that adenovirus-mediated B7-1 gene expression in mouse SCC cells leads to a direct antitumor effect as well as a
Figure 6. Photographs of immunohistochemical studies using antibodies against CD80, major histocompatibility complex (MHC) class I, CD8, and CD4 (hematoxylin; scale, 50 µm [A-O]). Noninfected group (A, D, G, J, and M); AdCL-infected group (B, E, H, K, and N); AdB7-infected group (C, F, I, L, and O); negative control (A-C). The majority of tumor cells in the AdB7-infected group strongly expressed CD80, while there were few CD80-positive tumor cells in the other 2 groups (D-F). There were no differences among the 3 groups for staining for MHC class I (G-I). A large number of CD8-positive cells infiltrated into tumors in the AdB7-infected group but not in the other 2 groups (J-L). Only a few CD4-positive cells were observed in the groups (M-O).
vaccination effect against SCC cell lines. Further studies to achieve high-level and long-acting expression of B7-1 and to examine the synergistic effect of various cytokines may lead to the clinical application of B7-1 gene transfer as a new therapeutic alternative for head and neck cancers.

Submitted for Publication: September 21, 2006; accepted November 7, 2006.

Correspondence: Ken-ichi Nibu, MD, PhD, Department of Otolaryngology—Head and Neck Surgery, Kobe University Graduate School of Medicine, 7-5-1 Kusunoki-Cho, Chuo-ku, Kobe 650-0017, Japan (nibu@med.kobe-u.ac.jp).

Author Contributions: Dr Nibu had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: Hoshitani, Ishida, Shirakawa, Gotoh, and Nibu. Acquisition of data: Hoshitani. Analysis and interpretation of data: Hoshitani, Ishida, Otsuki, Gotoh, and Nibu. Drafting of the manuscript: Hoshitani. Critical revision of the manuscript for important intellectual content: Ishida, Otsuki, Shirakawa, Gotoh, and Nibu. Obtained funding: Ishida, Gotoh, and Nibu. Study supervision: Ishida, Otsuki, Shirakawa, Gotoh, and Nibu.

Financial Disclosure: None reported.

Funding/Support: This work was supported by Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science for Dr Ishida (grant 16591707) and for Dr Nibu (grant 18659500) and a Health and Labour Sciences Research Grant for Clinical Cancer Research from the Japan Ministry of Health, Labour, and Welfare for Dr Nibu (grant H17-Gannrinshou-001).

REFERENCES