A Replication-Selective Adenoviral Vector for Head and Neck Cancers

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Objective: To test the oncolytic activity of cyclooxygenase 2 (COX-2) promoter–based conditional replication-selective adenovirus vector for squamous cell carcinoma cells of the head and neck.

Design: In vitro study.

Subjects: None.

Interventions: A conditional replication-selective adenovirus vector in which the expression of E1a, required for viral replication, is controlled by the COX-2 promoter, Ad-COX2-E1a, was generated. Its oncolytic activity according to the levels of COX-2 and of Coxsackie and adenovirus receptor expression was tested in a series of human head and neck squamous cell carcinoma cell lines.

Results: The respective COX-2 messenger RNA expression ratios of KB, H891, T891, T892, and L871 were 1.5, 60.0, 1.0, 14.6, and 1.3. The corresponding Coxsackie and adenovirus receptor messenger RNA expression ratios were 1, 1, 5, 3, and 1. In vitro assays showed significant growth suppression of cancer cell lines with strong expressions of COX-2.

Conclusion: This study demonstrated the possibility of oncolytic therapy using the COX-2 promoter–based conditional replication-selective adenovirus for head and neck squamous cell carcinoma expressing COX-2.


Despite recent progress in radiotherapy, chemotherapy, and surgical techniques, the survival of patients with head and neck squamous cell carcinomas has not improved significantly during the past 3 decades. To conquer these malignant tumors, various new therapies have been under development, including gene therapies using adenoviral vectors. At present, there are basically 2 types of adenovirus-based vectors for gene therapies against cancers: replication-defective adenoviral vectors (RDAVs) and replication-selective adenoviral vectors (RSAVs).1,2

In RDAVs, genes such as E1a and E1b, which are mandatory to replicate the adenoviruses, are artificially removed to eliminate proliferation of the vectors. Since RDAVs carry no danger of uncontrolled proliferation, they have been widely used as convenient and safe vectors for transferring therapeutic genes into target cancer cells. However, these vectors have limited the efficacy of treatment by restricting the number of tumor cells to which the therapeutic genes can be delivered.

On the other hand, RSAVs are designed to have a limited ability to replicate themselves in the targeted tumor cells but not in other normal tissues. Tumor cell killing is achieved not by the genes delivered by the vectors but by the oncolysis induced by the replicated viruses through their original nature as adenoviruses.3 Amplified viral vectors also spread to the adjacent tumor cells and kill these cells in the same manner.1 In addition, adenoviral infection has the potential to generate an antitumoral immune response.4

Cyclooxygenase (COX) is the rate-limiting enzyme in the formation of prostaglandins from arachidonic acid after its release by the enzyme phospholipase. To date, 2 isoforms of COX, COX-1 and COX-2, have been identified. The COX-1 form is the constitutive isoform present in most tissues. It mediates the synthesis of prostaglandins required for normal physiologic functions, including production of protective mucus by gastrointestinal mucosa and platelet aggregation. The COX-2 form, on the other hand, is an inducible gene and is overexpressed at sites of inflammation. It has also been reported that...
COX-2 is up-regulated in various malignancies, such as colon, lung, breast, pancreatic, and cervical cancers, as well as head and neck squamous cell carcinomas, but not in normal tissues.

Taking advantage of this specific COX-2 expression in tumor cells, we recently generated the COX-2 promoter–based RSAV Ad-COX2-E1a, in which expression of E1a is controlled by the COX-2 promoter. Since Ad-COX2-E1a requires COX-2 to produce messenger RNA (mRNA) of E1a, which is required for viral replication, replication of Ad-COX2-E1a is limited in COX-2–negative cells, whereas cotransfection of pX1 and pBHGE3 creates an infectious virus by homologous recombination. Ad5 indicates adenovirus type 5; Ap’, ampicillin resistance; COX, cyclooxygenase; ITR, inverted terminal repeat; mu, map units; ori, replication origin; ψ, package signal; and Δψ, deleted package signal.

In this study, to examine the possibility of clinical application of this new therapeutic concept for the treatment of head and neck cancers, we tested the specificity of replication and oncolytic activity of Ad-COX2-E1a in head and neck squamous cell carcinomas.

**METHODS**

**CELLS AND CELL CULTURE**

Human floor-of-mouth cancer cell line KB, human hypopharyngeal cancer cell line H891, human tongue cancer cell lines T891 and T892, human larynx cancer cell line L871, and transformed human embryonic kidney cell line 293 were used in this study. All cancer cell lines were kindly provided by Mamoru Tsukuda, MD, PhD (Department of Otolaryngology, Yokohama City University School of Medicine, Yokohama, Japan), and 293 cells were provided by the American Type Culture Collection (Rockville, Md).

All cancer cell lines were maintained at 37°C in complete RPMI 1640 medium (Sigma Chemical Co, St Louis, Mo) supplemented with 10% fetal bovine serum, penicillin, and streptomycin. The cells were supplied twice a week with fresh growth media, and the 293 cells were maintained in minimum essential medium (Gibco BRL, Rockville, Md) containing 10% fetal bovine serum supplemented with penicillin and streptomycin. The cells were supplied 3 times per week with fresh medium and maintained at 37°C in 5% carbon dioxide.

**CONSTRUCTION AND PRODUCTION OF REPLICATION-COMPETENT ADENOVIRAL VECTOR, Ad-COX2-E1a**

The details of the production of COX-2–based replication-selective adenoviral vector were described in our previous report. Briefly, a unique AgeI site was introduced at nucleotide position 552 in the pXC1 plasmid, which possesses the adenovirus 3 sequences from base pairs 22 to 3790 containing the E1 gene (Microbix Biosystems Inc, Toronto, Ontario) to generate the plasmid pXC1-AgeI. Next, the COX2-E1a promoter was ligated to the pXC1-AgeI plasmid to obtain pXC1-COX2-E1a. The recombinant Ad-COX2-E1a virus was prepared by means of homologous recombination using 10 µg of pXC1-COX2-E1a mixed with 20 µg of pBHGE3 plasmid containing Ad5 sequences with a wild-type E3 region and E1 deletion of base pairs 188 to 1339 (Figure 1). The resulting Ad-COX2-E1a virus was precipitated with cesium chloride and then used to transfect 293 cells, as described elsewhere.

**REAL-TIME QUANTITATIVE REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION ASSAY OF COX-2 AND COX-2 Promoter E1a mRNA EXPRESSION**

The cancer cells were trypsinized and collected by centrifugation at 1000g for 5 minutes. Acid guanidinium thiocyanatephenol-chloroform (Isogen; Nippon Gene, Tokyo, Japan) was used to extract total RNA from the cells. Quantitative reverse transcriptase polymerase chain reaction using a fluorogenic detection system (TaqMan; Applied Biosystems, Foster City, Calif) was performed according to previously described methods. Briefly, primers and the detection system probe for COX-2 and for Coxackie and adenovirus receptor (CAR) were designed by means of primer design software (Primer Express; Applied Biosystems). The RNA for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an endogenous control. The primers and the detection system probe for GAPDH were purchased (GAPDH and TaqMan GAPDH control reagent kit; Applied Biosystems) (Table 1). A sequence detector (ABI Prism 7700; Applied Biosystems) was used to measure fluorescence in real-time polymerase chain reaction. Obtained data were analyzed with software (Sequence Detector; Applied Biosystems).

**IN VITRO CYTOTOXICITY ASSAY**

Cancer cells were seeded at a density of 150 per well in 96-well tissue-culture plates. The 293 cells were seeded at a density of 500 per well. After 24 hours of incubation, the cells were infected with Ad-COX2-E1a or Ad-CMV-βgal, an adenovirus containing the β-galactosidase reporter gene under the control of the CMV promoter, as a viral control at concentrations from 5 to 75 multiplicities of infection for 4 hours. After a 5-day incubation, the numbers of viable cells of triplicate cultures of
relative quantification of CAR mRNA expression. The relative quantification was calculated by the value of KB cells. The expression ratios for KB, H891, T891, T892, and L871 were 1.0, 1.0, 1.0, 1.0, and 1.0, respectively.

In summary, H891 cell lines showed the highest levels of COX-2 mRNA expression of all the cell lines but the lowest levels of CAR mRNA expression. The T892 cell lines showed relatively high levels of both COX-2 and CAR mRNA expression, and T891 cell lines had the highest levels of CAR mRNA expression but relatively low levels of COX-2 mRNA expression.

IN VITRO CYTOTOXICITY ASSAY OF Ad-COX2-E1a AND Ad-CMV-βgal

We used Ad-CMV-βgal as a negative control vector and 293 cells, which expressed the transforming E1 gene of adenovirus type 5, as positive control cells. The 293 cells showed significant growth inhibition of cells infected with both Ad-COX2-E1a and Ad-CMV-βgal (Figure 3). The Ad-COX2-E1a significantly inhibited cell growth of the H891 and T892 cell lines, which expressed high levels of COX-2 mRNA, at 25 to 75 multiplicities of infection in a viral concentration–dependent manner, in comparison with the other 3 cell lines (Figure 3A and Table 2). The L871 cell lines with low levels of COX-2 and CAR did not show any sign of growth inhibition. Statistical analysis using a mixed-effect model showed the significant interaction of Ad-COX2-E1a with expression of COX-2 (P = .002). A significant interaction of Ad-COX2-E1a with CAR or a synergistic effect of COX-2 and CAR was not observed.

No cancer cell lines were affected by infection with the control virus Ad-CMV-βgal (Figure 3B).

COMMENT

Oncolytic viral therapy was established by Huebner et al in 1956. They detected responses in 26 of 40 patients with cervical cancer injected with wild-type adenovirus. After a half-century, recent advances in genetic engineering techniques have produced tumor-selective replication adenoviruses. At present, 2 basic approaches are used for tumor-selective adenoviral replication. The first approach is to delete any gene functions that are critical for efficient viral replication in normal cells but not in tumor cells. In the case of ONYX-015, for example, the E1b 55-kDa gene is deleted so that the virus replicates in p53-deficient tumor cells but not in normal cells that contain wild-type p53. This virus is reportedly molecularly identical to one that is being evaluated in clinical trials for head and neck squamous cell carcinomas.

The second approach is to use tumor-selective promoters to control the expression of E1a, an adenoviral early gene required for viral replication, as shown in this study. In the case of CN706, for example, the prostate-specific antigen gene promoter-enhancer element is in-
Relative messenger RNA (mRNA) levels of cyclooxygenase 2 (COX-2) (A) and Coxsackie and adenovirus receptor (CAR) (B) determined with quantitative reverse transcriptase polymerase chain reaction of cell lines. Values represent relative levels of mRNA normalized to the T891 cell line (A) and KB cell line (B) and established with triplicate determinations of each sample. Glyceraldehyde-3-phosphate dehydrogenase was selected as an endogenous RNA control to normalize for differences in the amounts of total RNA.

Table 2. Results of Linear Regression Analysis

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<th>Estimate at 75 MOIs</th>
<th>SE</th>
<th>Comparison</th>
<th>Difference</th>
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<td>T892 vs T891</td>
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Abbreviations: MOIs, multiplicities of infection; NA, not applicable.
serted upstream of the E1a gene for prostate cancer. The functions of several other promoters, including osteocalcin, α-fetoprotein, Muc-1, L-plastin, and midkine, are being evaluated by other investigators. These viruses should only replicate in tumor cells expressing the promoters. Thus, the choice of promoters is most important for selectivity and accuracy of regulation.

Cyclo-oxygenase 2, an enzyme that catalyzes the synthesis of prostaglandins, is overexpressed in a variety of premalignant and malignant conditions, including leukoplakia and squamous cell carcinoma of the head and neck. Increased levels of COX-2 are thought to contribute to carcinogenesis by modulating apoptosis, immune surveillance, and angiogenesis. Recent experiments and clinical studies have shown promising results indicating that the newly developed selective COX-2 inhibitors may prevent or treat human head and neck cancers.

To further take advantage of this specific COX-2 expression in head and neck cancers, we tested the oncolytic activity of COX-2 promoter–based RSAd in head and neck squamous cell carcinoma cell lines. As we expected, significant cytotoxicity was observed in the cells strongly expressing COX-2 mRNA. The strongest inhibition was observed in H891 cells, which showed the highest COX-2 but the lowest CAR levels. The second strongest cytotoxicity was observed in T892 cells, which expressed relatively high levels of COX-2 and CAR. Statistical analysis also confirmed that COX-2 expression is a significant important factor for oncolysis by Ad-COX2-E1a.

The initial binding of the adenovirus to the cell surface has been shown to be a receptor-mediated process. Several studies have reported that the effect of adeno viral gene transfer on tumors correlates with the expression of CAR. However, the present study failed to show the significant effect of CAR expression on the oncolysis by Ad-COX2-E1a. Further studies should be performed to determine the role of CAR in head and neck squamous cell carcinoma cell lines in the cytotoxicity of Ad-COX2-E1a.

CONCLUSIONS

In the present study, we demonstrated the specificity and effectiveness of Ad-COX-2-E1a, a COX-2 promoter–specific RSAd, for suppressing COX-2–expressing head and neck cancer cells. Currently, we are conducting further in vitro studies using other promoters and experiments with animal models. These studies will lead to the clinical application of the RSAd for the oncolytic therapy of human head and neck cancers.

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