Antitumor Properties of Influenza Virus Vectors

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ABSTRACT

We are investigating the potential use of influenza virus vectors expressing selected tumor-associated antigens (TAAs) as therapeutic agents in anticancer strategies. Previously, we have shown that recombinant influenza viruses expressing a model TAA mediated the regression of established pulmonary metastases in mice through the induction of cytotoxic T-cell responses (N. P. Restifo et al., Virology, 249: 89–97, 1998). We have now expanded these observations in the mouse model using survival as the end point of the assay. Animals with a high tumor burden showed extended survival times when treated with a recombinant influenza virus expressing a TAA, but they finally succumbed to death. Death was associated with the presence of a small number of large tumors in lungs. Interestingly, these tumors were found to express undetectable levels of the TAAs because of a down-regulation in the TAA-specific mRNA levels. On the other hand, mice with five times lower tumor burden showed complete tumor regression and survival for >6 months when treated with the recombinant virus. These animals showed protection against a tumor challenge 6 months after treatment. Our results suggest that recombinant influenza viruses may be useful as therapeutic agents for the prevention and treatment of cancers with known TAAs.

INTRODUCTION

An active line of investigation in the area of cancer therapy concerns the design of clinical strategies resulting in the induction of robust cytotoxic immune responses against cancer cells. Ideally, this immune response should be able to eliminate most if not all cancer cells. TAAs are attractive targets for the induction of such immune responses. A number of different immunogenic delivery strategies for TAAs are currently being investigated, including peptide delivery (1, 2), the use of viral vectors (3–10), plasmid DNA-based vectors (11, 12), and ex vivo stimulation of dendritic cells (13–16).

Influenza virus vectors have several properties that make them attractive candidates as delivery vectors for TAAs: (a) influenza viruses are potent inducers of antigen-specific humoral and cellular responses (17). The presence of preexisting neutralizing antibody reactions in the host can be avoided by selecting appropriate antigenic strains of the virus. Specifically, there are many different antigenic strains of influenza A viruses for which little or no neutralizing immunity is currently present in humans (18); (b) the development of improved genetic engineering techniques to generate recombinant influenza viruses has greatly simplified the construction of influenza virus vectors (19, 20); and (c) influenza virus vectors have been successfully used in preclinical models to induce protective humoral and/or cellular immune responses against different viruses (21–26), bacteria (27, 28), and parasites (29–31).

We have demonstrated recently that a recombinant influenza virus expressing a model TAA was able to reduce the tumor number in an experimental tumor model system (32). In the present study, we extend this work to investigate the efficacy of these recombinant viruses in mediating survival of mice with established lung metastases. The duration of the antitumor protective immune responses was also investigated. In addition, our results support a general mechanism of escape of cancer cells under an immune selective pressure based on down-regulation of antigen expression.

MATERIALS AND METHODS

Animals, Viruses, and Cells. Female BALB/c mice, 6–8 weeks of age, were purchased from Taconic Farms (Germantown, NY). Transfectant influenza viruses MINIGAL, BHAGAL, and NAGAL were described previously (32). These transfected influenza A viruses were engineered to express the L1-restricted epitope TPHPARIGL contained in the amino acid sequence of β-galactosidase. The MINIGAL virus encodes a polypeptide containing the β-galactosidase epitope downstream of a leader sequence. BHAGAL and NAGAL viruses encode the β-galactosidase epitope inserted in the amino acid sequence of the viral glycoproteins hemagglutinin and neuraminidase, respectively. Transfectant viruses, as well as wild-type WSN virus, were grown and titrated in Madin-Darby bovine kidney cells as described previously (33). The CT26.CL25 tumor cell line is a cloned colon carcinoma cell line derived from BALB/c mice that has been transduced to express β-galactosidase (5). CT26.CL25 cells were maintained in RPMI 1640 (Life Technologies, Inc., Grand Island, NY) containing 10% heat-inactivated FBS (HyClone, Logan, Utah), 0.03% L-glutamine, 100 μg/ml streptomycin, 100 μg/ml penicillin, 50 μg/ml gentamicin sulfate, and 400 μg/ml G418. Madin-Darby bovine kidney cells were maintained in reinforced minimal essential medium (BioWhittaker, Walkersville, MD) containing 10% heat-inactivated FBS.

Treatment of Mice. Mice were inoculated with 200 μl of HBSS (Life Technologies, Inc., Grand Island, NY) containing 105 or 5 × 105 CT26.CL25 cells through the tail vein. Three days later, mice were treated i.p. with 100 μl of PBS containing 106 pfu transfected or wild-type influenza viruses. When indicated, mice received a second treatment at day 14 after tumor inoculation. Animals were maintained in observation and sacrificed when in extremis. All animal protocols were in accord with NIH guidelines on the care and use of laboratory animals.

Isolation and Culture of Cells from Lung Tumors. The lungs of sacrificed animals were analyzed for the presence of tumors. If present, tumors were disected, cut into little pieces, and washed with HBSS. These samples were incubated 3 h at 37°C with DMEM (Life Technologies, Inc.), 10% FCS containing antibiotics, and 200 units/ml of collagenase (Sigma Chemical Co., Saint Louis, MO). Detached cells were then collected, washed with HBSS, transferred to a tissue culture flask, and incubated overnight in the presence of RPMI 1640 containing 10% heat-inactivated FBS, 0.03% L-glutamine, 100 μg/ml streptomycin, 100 μg/ml penicillin, and 50 μg/ml gentamicin sulfate.

Analysis of β-Galactosidase Expression by Enzymatic Staining. Cells in 35-mm dishes were washed twice with PBS and fixed in 0.5% glutaraldehyde for 10 min at room temperature. Fixed cells were subsequently washed three times with PBS and stained for 3 h with a solution containing 0.67 mg/ml X-gal, 5 mM K4Fe(CN)6, 5 mM K3Fe(CN)6·3H2O, 1 mM MgCl2, and 0.05% Triton X-100. Cells were then washed with PBS and observed under a microscope.

Detection of β-Galactosidase-specific Genomic DNA. Genomic DNA was extracted from ~106 cells using DNAzol reagent according to the manufacturer’s instructions (Life Technologies, Inc.). One μg of genomic DNA was used as template in a PCR reaction using Expand High Fidelity polymerase (Roche Diagnostics Corp., Indianapolis, IN) and primers LacZf1948(+) and 5′-GGCGGAGCTCTCCGACTTGGATG-3′, annealing to nucleotide positions 1948 to 1966 of the LacZ gene, and LacZr3′(−) and 5′-CCGGGCGTATTATTTTGGACAGGAAAAGG-3′, annealing to nucleotide positions 3078–3084 of the LacZ gene (GenBank accession no. V00296). PCR reactions were analyzed by 1% agarose gel electrophoresis.

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3 The abbreviations used are: TAA, tumor-associated antigen; pfu, plaque forming unit(s); WSN, influenza A/WSN/33 virus; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; PBS, fetal bovine serum.
Detection of β-Galactosidase-specific mRNA. Cellular RNA was extracted from $10^6$ cells using RNAzol B reagent according to the manufacturer’s instructions (Tel-Test, Friendswood, TX). Five μg of extracted RNA were treated with DNase and used as template in a reverse transcription reaction using Superscript reverse transcriptase (Life Technologies, Inc.) and LacZ3′(−) primer. One tenth of this reaction was used in a PCR reaction using LacZ1948(+) and LacZ3′(−) primers. PCR products were analyzed by 1% agarose gel electrophoresis.

RESULTS

Treatment with Transfectant Influenza A Viruses Results in Increased Survival Times of Mice Inoculated Previously with $5 \times 10^5$ Tumor Cells. We have previously generated three transfectant influenza A viruses expressing the amino acid sequence TPHEARIGL in different polypeptide contexts. This sequence represents the naturally processed H-2 Ld-restricted epitope of β-galactosidase, which is recognized by specific CD8$^+$ T cells. We determined the therapeutic properties of these transfectant viruses for the treatment of mice bearing β-galactosidase-expressing tumors. We have already shown that this treatment results in a cytotoxic T-cell response against the β-galactosidase-expressing tumor cells (CT26.CL25 cells) and in a reduction in the number of tumors at day 12 after tumor inoculation (32). We next investigated the survival times of mice with established CT26.CL25 tumors when treated with the transfectant influenza viruses. Mice were inoculated with $5 \times 10^5$ CT26.CL25 cells through the tail vein, and 3 days later they were treated by i.p. injection with $10^6$ pfu of transfectant influenza A virus. As shown in Fig. 1, survival was prolonged by treatment with all three transfectant viruses (BHA-GAL, NAGAL, and MINIGAL viruses), whereas control treatment with wild-type influenza A/WSN/33 virus did not have any therapeutic effect.

Loss of TAA Expression in Tumor-bearing Animals Treated with Transfectant Influenza A Viruses. Despite the increased survival times of CT26.CL25 tumor-bearing mice when treated with a transfectant influenza virus expressing a CD8$^+$ T-cell epitope from β-galactosidase, most of the animals finally succumbed to death. Lungs from these animals were extracted at the moment of death and analyzed for the presence of tumors. Interestingly, all treated animals showed a small number (<10) of large-size tumors. This is in contrast with lungs from untreated animals, which showed a large number (>500) of small-size tumors. We then isolated cells from the tumors of treated and untreated animals, and after overnight culture, we stained the cells with X-gal. Cells expressing β-galactosidase stain blue using this technique. As shown in Fig. 2, no differences were detected in the number of cells (~100%) expressing β-galactosidase between tumor cells isolated from untreated animals and CT26.CL25 cultured cells prior to inoculation into mice. In contrast, <0.1% of cells isolated from tumors of treated animals showed detectable levels of β-galactosidase staining (Fig. 2, C–E).
Reduced Levels of TAA-specific mRNA in Tumor-bearing Animals Treated with Transfectant Influenza A Viruses. We next determined whether the loss of detectable β-galactosidase staining in tumor cells derived from tumor-bearing animals treated with transfectant influenza viruses was attributable to a loss of the LacZ gene or to reduced β-galactosidase-specific mRNA levels. For this purpose, total RNA and genomic DNA were extracted from tumor cells isolated from treated and untreated animals. Isolated RNA was subjected to reverse transcription using a primer specific for β-galactosidase RNA. Reverse-transcribed RNA and isolated genomic DNA were used as PCR templates using β-galactosidase-specific primers (Fig. 3). A product of the expected length (1145 bp) was obtained when genomic DNA was used as template, indicating that there were no major rearrangements, insertions, or deletions in the LacZ gene. In contrast to this finding, β-galactosidase-specific mRNA levels were drastically reduced and not detectable by the PCR-based assay in tumor cells isolated from treated animals (Fig. 3B).

Treatment with Transfectant Influenza Virus Results in Protection against Death of Mice Inoculated Previously with 10⁵ Tumor Cells. We next examined the therapeutic effect of transfectant influenza A viruses expressing the β-galactosidase epitope in mice that received a five times lower dose of tumor cells. Because no major differences were detected in our previous experiments among the three transfectant viruses, we chose the NAGAL virus for this experiment. Mice receiving an i.v. dose of 10⁵ CT26.CL25 tumor cells and then vaccinated i.p. 3 and 14 days later with 10⁶ pfu of the transfectant influenza A virus NAGAL, or they were mock treated. Animals were monitored daily for survival for >6 months.

Treatment with Transfectant Influenza Virus Induces a Long-lasting Protection against Tumors in Mice. To determine the duration of the protective immune response induced by transfectant NAGAL virus in mice, survivors from the previous experiment (Fig. 4) were challenged i.v. with a second dose of 10⁵ CT26.CL25 cells 6 months after treatment and followed for survival. Fig. 5 shows the results from this experiment. Although all naïve control animals died, most of the previously immunized animals (80%) survived the challenge. These results demonstrate that the protective immune response induced in mice by NAGAL virus against tumor cells expressing β-galactosidase was effective even 6 months after treatment.

DISCUSSION

In this report, we have studied the efficacy of transfectant influenza A viruses in mediating tumor clearance and survival of mice bearing tumors expressing a model TAA. The recombinant viruses expressed a single epitope (9 amino acids) that was derived from the model TAA and that was recognized by murine CD8+ T cells. The observed effects were dependent on the expression of the tumor epitope by the
recombinant virus, because treatment with wild-type influenza A virus did not mediate extended survival times in tumor-bearing animals. Our experiments were performed in animals that were inoculated with two different doses of tumor cells. All animals receiving a tumor dose of $10^5$ tumor cells survived for >6 months when treated with a recombinant influenza virus expressing the tumor epitope. Treatment started at day 3 after tumor inoculation, when metastatic tumors have already been established in lungs (5). It should be noted that the treatment of established tumors by vaccination strategies is more challenging than the prevention of tumor development by vaccination prior to tumor inoculation. Moreover, most of the animals (80%) that were cured by the treatment showed protection against a secondary challenge with the same tumor cells 6 months later, demonstrating a long-lasting antitumor immunity induced by the recombinant influenza A virus. When animals from the same group were challenged with non-β-galactosidase-expressing tumor cells, only one of five animals survived. These results indicate that most of the long-term tumor immunity is mediated by memory CTLs specific for the tumor epitope.

Animals receiving a higher dose ($5 \times 10^5$) of tumor cells showed extended survival times when treated with the recombinant influenza A viruses. However, in this case the treatment was not able to completely clear all tumors in the mice. The low number of tumors in the treated mice suggested that the treatment resulted in the selection of a few tumor cells that were able to escape the induced antitumor response. This hypothesis is supported by our results showing that treatment with the recombinant viruses selected for tumor cells in which the TAA (β-galactosidase) expression was down-regulated. Tumor cells not expressing β-galactosidase are not recognized and not killed by CTLs specific for the TAA antigen expressed by the recombinant influenza virus (32). Consistent with antigen down-regulation, we could not detect TAA-specific mRNA in the tumor cells derived from treated animals. However, the LacZ gene was readily detected by PCR techniques, suggesting that the most likely explanation for the lack of antigen expression in the tumor cells was a down-regulation of the LacZ promoter. In fact, promoter down-regulation of transgenes after in vivo delivery is not uncommon (34, 35). Moreover, loss of antigen expression in tumor cells as a result of immuno-selection has been reported for other tumors (36–38). However, the precise molecular mechanism of the down-regulation remains unknown, and we cannot exclude other possibilities, such as deletions in the promoter region of the LacZ gene.

Therapeutic regimes based on the induction of cellular responses against TAAs are promising strategies against cancer, especially if used in combination with other techniques. For this purpose, vaccination strategies inducing efficient CTL responses against the cancer cells are being explored. Our results demonstrate that recombinant influenza viruses expressing TAA epitopes are good inducers of antitumor responses with therapeutic properties in mice. Our results also suggest that strategies based on vaccination against multiple TAA determinants might be more effective in the treatment of cancer, because tumor escape by down-regulation of expression of multiple TAAs would be more difficult. The inclusion of both CD8 and CD4 tumor-specific epitopes is also likely to improve the antitumor efficacy of such vaccination approaches. However, other potential mechanisms for antigenic escape, such as defects in MHC class I presentation are possible (39, 40), and they might be difficult to prevent. In these studies, we have used a TAA model system based on the expression of a foreign protein (bacterial β-galactosidase) by the tumor cells. Expression of foreign antigens by tumor cells is specially relevant in the case of human papillomavirus-induced carcinomas, where the viral E6 and E7 oncoproteins are expressed by the tumor cells. These proteins might then be good targets for the induction of therapeutic immune responses against cervical carcinomas in humans (41). However, in several other tumors, only self-antigens have been defined as TAAs. For example, most of the described TAAs in melanoma cells recognized by T cells are also present in normal melanocytes (42). In this case, vaccination strategies based on expression of TAAs need to break the immunotolerance against the self-antigen to be effective. Moreover, a melanoma-induced immune response might also be responsible for clearance of normal melanocytes. Nevertheless, it seems that such responses can be induced in patients or in animal models resulting in melanoma regression, and the only side effect associated in some instances with tumor clearance seems to be a general depigmentation of the skin (vitiligo; Refs. 43–47). Our results then suggest that recombinant influenza viruses expressing TAAs might be effective inducers of protective antitumor responses against human cancers with known TAAs.

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