High Efficiency Gene Transfer into Primary Human Tumor Explants without Cell Selection


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Abstract

Preclinical studies with murine tumor models have demonstrated that autologous tumor cell vaccines engineered to secrete certain cytokines in a paracrine fashion elicit systemic immune responses capable of eliminating small amounts of established tumor. These results have engendered much interest in developing this strategy for gene therapy of human cancer. The major limitation to creating genetically modified autologous human tumor vaccines is efficient gene transfer into primary tumor explants, since the majority of human tumors fail to proliferate in long-term culture. Using the retroviral vector MFG in conjunction with short-term culture techniques, we have achieved, in the absence of selection, a mean transduction efficiency of 60% in primary renal, ovarian, and pancreatic tumor explants, and we have developed an autologous granulocyte-macrophage colony-stimulating factor secreting tumor vaccine for clinical trials.

Introduction

Recently, a new tumor vaccine approach using genetically altered autologous tumor cells to secrete local concentrations of cytokines has been developed in murine models (1–9). In some of these models, lymphokine gene transduced tumor cells have been shown to generate a local, tumor-specific immune response when administered as a s.c. vaccination (1–3, 5–9). In addition to rejecting the genetically modified tumor cells, vaccinated animals may develop a T-cell-dependent systemic immunity which in some cases can cure micrometastases established prior to treatment with the genetically altered tumor cells (1–3, 7, 8). Recently, a direct comparison of multiple cytokine genes transferred into a poorly immunological murine melanoma model identified GM-CSF as the most potent in generating a protective response (7). Two critical features of this approach include: (a) the ability to generate a T-cell dependent tumor-specific systemic immunity; and (b) the production of lymphokine only at the tumor site, thereby producing a strong antitumor immune response without systemic toxicity.

The extension of this strategy to human cancer therapy will require two technical advances. First, the gene transfer systems used must be able to routinely introduce lymphokine genes into human tumors efficiently and must be able to produce consistent levels of gene expression. Other investigators report an efficiency of transduction of no better than 1 in 100 tumor cells so that the vector carrying the cytokine gene must also transfer a selection marker. Second, the tumor cells that are transduced must be from primary human tumor cultures established at the time of surgery. All previous reports of gene transfer into human tumors have used vectors containing selectable markers and stable long-term cell lines rather than primary tumor cell cultures. Because stable long-term cultures cannot be established for the vast majority of human tumor explants (melanoma being the exception), these gene transfer approaches will fail to generate sufficient numbers of genetically modified cells (10, 11). Even for the rare circumstances in which long-term cell lines can be established, transduction of cell lines and posttransduction selection might result in selective loss of expression of critical tumor-specific antigens expressed by the parent tumor in vivo. Boon et al. (12, 13) have provided evidence to support this contention by showing that it is possible to isolate several tumor-specific T-cell clones from a patient with malignant melanoma. Evaluation of these T-cell clones for lysis of melanoma tumor cell clones obtained from the same patient revealed three melanoma-specific antigens. The first antigen was present on all melanoma clones tested, the second antigen was lost during long-term culture, and the third antigen was expressed on a minority of tumor clones (12, 13).

In this article we report the use of a retroviral vector system to achieve high efficiency transduction of primary human tumor explants without requiring long-term culture or selection. These results provide the basis for the routine production of genetically modified autologous tumor vaccines.

Materials and Methods

Patients. All surgical specimens were obtained from patients with a histological diagnosis of either renal cell carcinoma; ovarian carcinoma; adenocarcinoma of the lung, colon, and pancreas; squamous cell carcinoma of the hypopharynx; or carcinoma of the breast. All of the tumors were primary resections except for the five ovarian tumors which were obtained from ascites and the two breast carcinomas which were obtained from pleural fluid. Informed consent to use these surgical specimens was obtained from all patients prior to the surgical procedure.

Dissociation of Primary Human Tumor Explants. All tumors were transported from the operating room on ice and were mechanically dissociated into 1–5-mm fragments within 1 h. These tumor fragments were then enzymatically digested, initially by exposure to collagenase (GIBCO; 1 mg/ml; 173 units/ml) and DNase I (776785; 0.1 mg/ml; Boehringer Mannheim Biochemical) until all of the fragments were fully digested. This process yields approximately 5 × 10^6 viable malignant cells

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3 The abbreviations used are: GM-CSF, granulocyte-macrophage colony-stimulating factor; FBS, fetal bovine serum; PBS, phosphate-buffered saline.

from a 1.0-g tumor mass. Cells from all digested fractions were pooled and incubated in selected growth medium. Cells were passaged when each flask reached 80–100% confluence.

**In Vitro Growth of Primary Human Tumor Explants.** Conditions necessary for the short-term growth of primary human tumor cultures were evaluated in the following way. Freshly digested tumor cells were plated in duplicate at \(1 \times 10^5\) cells/75 cm\(^2\) flask. Each growth condition was evaluated both separately and in combination with other growth supplements (Tables 1 and 2). Different media including RPMI, Dulbecco's modified Eagle's medium, Ham's and AIM-V preparations, and lots of FBS were the initial components of growth medium screened (Tables 1 and 2). Following identification of the optimal medium and serum, additional additives were systematically evaluated (Table 2). Each supplement was evaluated for at least 2 in vitro passages/patient tumor and for enhancing the growth of at least 2 different patients' tumors of before routinely including it as a supplement for tumor growth. When tumor cells in each flask reached 100% confluence, they were trypsinized and counted before being replated.

**Transduction of Primary Human Tumor Cultures.** Transduction is performed with the MFG retroviral vector system. The structure of MFG has recently been described (7, 17). Briefly, in this vector, Moloney murine leukemia virus long terminal repeat sequences are used to generate both a full length viral RNA (for encapsidation into virus particles) and a subgenomic mRNA (analogous to the Moloney murine leukemia virus env mRNA) which is responsible for the expression of inserted sequences. The vector retains both sequences in the viral gag region shown to improve the encapsidation of viral RNA (16) and the normal 5' and 3' splice sites necessary for the generation of the env mRNA. Protein coding sequences are inserted between the NcoI and BamHI sites in such a way that the initiation codon of the inserted sequences is placed precisely at the position of the viral env initiation codon, and minimal 3' nontranslated sequences are included in the insert. No selectable marker exists in the vector. Complementary DNA sequences encoding the cytokine were inserted into MFG and the resulting vector constructs were introduced into CRIP cells as previously described (17) in order to generate recombinant virus with amphotropic host range.

The retroviral producer cell line, CRIP, is grown in culture to confluency in Dulbecco's modified Eagle's medium + 10% calf serum. Two days prior to transduction, the cells are trypsinized and replated at a density of \(2 \times 10^6\) cells/100-mm culture dish. One day prior to transduction, 10 cc of fresh medium are added to the cells. On the day of transduction, a 24-h supernatant is collected and filtered through a 0.45-um filter to remove contaminating cells and plated (100 ul/well) in 96-well flat plates at 1:3 dilutions. Medium and serum, additional additives were systematically evaluated (Table 2). Each supplement was evaluated for at least 2 in vitro passages/patient tumor and for enhancing the growth of at least 2 different patients' tumors of before routinely including it as a supplement for tumor growth. When tumor cells in each flask reached 100% confluence, they were trypsinized and counted before being replated.

**Establishing Primary Human Tumor Cultures.** Our method of digesting freshly excised tumor specimens routinely yields \(5 \times 10^8\) viable tumor cells/g of excised tumor. It should be pointed out that

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a Cultures were scored using the following scoring system: NC, no change in growth rate; --, decreased growth; ++, 2-fold increase in growth; ++++, 3-fold increase in growth; ++++, 4-fold increase in growth; NT = not tested (a 2-fold increase in growth = 2 times the number of cells obtained from the control flask during a 96-h incubation period). When the cells in each flask reached 100% confluence (total cell number = approximately \(2 \times 10^7\) cells/flask), they were trypsinized, counted, and replaced for further growth evaluation. The number of passages reached/given time period was also recorded. For some tumors the doubling time was estimated using a \(^{1}H\)thymidine uptake proliferation assay.

b Adenocarcinoma (Adeno) of the lung and squamous cell carcinoma (SCCA) of the tonsil.

c Only one tumor of each tested.

d DEMEM, Dulbecco's modified Eagle's medium.

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Table 1 Basal media and FBS screened for primary human tumor growth support in vitro

At least 2 histologically similar tumor cell types were grown in each of the basal media listed above and studied for enhanced in vitro proliferation. Varying amounts of FBS were also evaluated. Growth rate was initially evaluated by daily observation and scoring of duplicate flasks. *

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these results take into account our recent data which suggest that mechanical dissociation into 5-mm tumor fragments prior to digestion is superior to enzymatically digesting smaller tumor fragments, especially when only collagenase is used for the initial enzymatic digestion. In fact, when tumor cells that are mechanically dissociated are grown separately from collagenase-digested tumor cells but in the same growth medium, the initial growth rate of the mechanically dissociated tumor cell population is much slower, resulting in roughly one-half the number of expanded cells during the first two in vitro passages (data not shown).

We have evaluated 24 renal cell carcinomas, 26 ovarian carcinomas, 8 colon carcinomas, 5 pancreatic carcinomas, 3 breast carcinomas, an adenocarcinoma of the lung, and a squamous cell carcinoma of the tonsil for short-term in vitro growth. Growth conditions were studied using the procedure described in "Materials and Methods." Initially, the optimal base medium and FBS were determined for each histologic type (Table 1). Lots of characterized and defined FBS were screened. Once an adequate lot of serum was identified, the percentage of FBS was evaluated. A list of the base media and percentage of defined fetal bovine sera is shown in Table 1. Additional supplements were subsequently evaluated. A list of these growth supplements can be found in Table 2. Optimal short-term growth of fresh human tumor explants is dependent on several conditions. Common to all histological tumor types with the exception of breast carcinoma is the percentage of high grade FBS used in the buffered medium. All of the other tumors grown in our laboratory to date grow well in 20% characterized or defined FBS. Breast carcinomas require 5% FBS or less. Greater than 5% FBS can result in overgrowth of fibroblasts (14). Ovarian, colon, and pancreatic tumor explants also require the addition of human insulin (0.2 units/ml). The addition of transferrin and selenium will often enhance the growth of ovarian and breast carcinomas. Renal cell carcinoma explants require the addition of transferrin and selenium (10%; Difco; 0060-01-6) and occasionally, bovine pituitary extract (Sigma; P1167). Colon tumor explants grow well in a medium that is also supplemented with bovine pituitary extract. With these defined conditions an expansion of the tumor cell population of 10-fold or greater is routinely obtained during a period of 2–4 weeks (Table 3).

In addition, an attempt was made to identify characteristics of the initial tumor specimen that were associated with enhanced or inhibited in vitro growth. In particular, histological diagnosis, degree of malignant cell differentiation, and degree of necrosis were compared with the last in vitro passage achieved by the tumor. Interestingly, only the degree of necrosis adversely affected the success of short-term in vitro growth. In contrast, all malignant histologies could be grown equally well provided that the conditions for each histology were optimized. To illustrate this point, the results for 26 nephrectomy specimens evaluated for in vitro expansion are shown in Table 3.

Transduction of Human Tumor Explants. We have identified three conditions that are critical for high efficiency gene transfer to primary cultures of human tumor cells. First, successful transduction requires a vector system that can transduce cells efficiently, resulting in consistent levels of gene expression. Although the quality of retroviral supernatants can vary, this problem is easily controlled by titrating of the retroviral supernatants using easily transducible cell lines prior to use in gene transfer to the fresh human tumor explants. Second, efficient retroviral gene transfer and expression depends on the percentage of tumor cells within the tumor population that are actively proliferating at the time of gene transfer. In general, transduction efficiency correlates with the percentage of tumor cells undergoing cell cycling since integration of the retroviral vector into the host genome is required for expression of the transferred gene. Third,

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transduction efficiency can be enhanced by the addition of polymers to the retroviral supernatant just prior to exposure of the target cells to the retroviral vector. Enhanced gene transfer is thought to occur via a charge-mediated mechanism that affects virus binding to or penetration of the target cell. The polycations protamine, polybrene, and DEAE-dextran are routinely used for this purpose (20). However, protamine, which has been commonly used for previous gene therapy trials, gives low efficiencies of gene transfer to human lymphocytes and tumor cell lines, and therefore requires the cotransfer of a selectable marker.

We therefore performed an extensive comparison of polybrene and DEAE-dextran, using a wide range of concentrations and exposure times of these agents to the target cell. Although both agents increased the transduction efficiency of human tumor cells in primary culture, DEAE-dextran resulted in less cell toxicity than polybrene at equivalent enhancement doses. Thus, we find that DEAE-dextran is a potent enhancer of gene transfer to primary human tumor cultures. It also results in less target cell toxicity.

An ovarian tumor cell line derived from a patient with malignant ascites was initially used to evaluate optimal transduction conditions. These conditions are described in "Materials and Methods."

These conditions were then used to evaluate 8 renal cell carcinomas, 5 ovarian carcinomas, 5 colorectal carcinomas, 2 pancreatic carcinomas, 2 breast tumors, 1 squamous cell carcinoma of the tonsil, and 1 adenocarcinoma of the lung for transduction efficiency using the MFG vector carrying the E. coli LacZ gene (Fig. 1A). Using the conditions described above, we were able to show expression of the LacZ marker gene in at least 20% of each tumor population, with a mean of 70% for renal cell carcinomas, 65% for ovarian carcinomas, and 43% for colon carcinomas. In 16 of 21 tumors, we were able to achieve at least a 40% transduction efficiency. We have also attempted to transduce several other histological human tumors, including a squamous cell carcinoma arising from a tonsil (40% transduction), an adenocarcinoma of the lung (82% transduction), and 2 breast carcinomas (mean transduction efficiency of 28%).

Recent studies evaluating the antitumor immune response generated by a variety of single lymphokines using the B16 melanoma murine model revealed that the cytokine GM-CSF can generate an enhanced antitumor immune response that is much greater than the response generated by any other cytokine tested (7). In vivo depletion studies revealed that this response is dependent on both CD4+ and CD8+ T-cells. Additional experiments indicated that maximal systemic immunity was achieved when the average level of GM-CSF production by the vaccine cells was equal to or greater than 36 ng/10⁶ cells/24 h. These preclinical studies provide the immunological data needed to begin to apply this approach to the treatment of cancer in patients.

As a prelude to initiating clinical vaccine trials, we used the same MFG retroviral vector to transfer the human GM-CSF gene to 3 renal cell carcinomas, 2 colon carcinomas, and 3 pancreatic carcinomas (Fig. 1B). In 6 of the 8 tumors, GM-CSF production was at least 50 ng/10⁶ cells/24 h. It was even possible to improve production of GM-CSF by the less efficiently transduced tumors, 1 to more than 50 ng/10⁶ cells/24 h, after a second retroviral transduction was performed. Southern blot analysis of 5 of the renal cell carcinoma cultures genetically altered to secrete GM-CSF revealed a range of integrated vector copy numbers between 0.5 and 2 copies/cell. This correlated with a range of GM-CSF secretion between 26 and 74 ng/10⁶ cells/24 h (Table 4). In addition, the transduced cells can freeze and thaw easily, with minimal loss of the number of viable, lymphokine-producing cells (Fig. 1B). This confirms that the MFG vector system has the ability to very efficiently transfer human cytokine genes to fresh human tumor explants.

Discussion

Preclinical murine studies have shown that tumor cells, genetically altered to secrete lymphokines, will increase the immunogenicity of a tumor when given s.c. in the form of a vaccine. The gene transfer of GM-CSF, in particular, stands out as the cytokine that generates the greatest antitumor immune response in murine models (7). Furthermore, the local secretion of GM-CSF at the site of the tumor leads to the production of both helper and cytolytic cells that can circulate and eradicate existing tumor at distant sites. In addition, our previous studies have also shown that tumor cells genetically altered to secrete local concentrations of GM-CSF will cure mice of micrometastatic melanoma. These studies therefore provide the theoretical basis for using this approach to treat human cancers.

We now report that it is technically possible to produce a genetically altered autologous human tumor vaccine for patient trials. In addition, we provide evidence for successful gene transfer to short-term, primary tumor cultures, which is a critical advantage over previous reports of genetically altered long-term human tumor cell lines. Since the goal of a genetically altered tumor vaccine is to activate the immune system of a patient to recognize and eradicate existing tumor at other sites, therapeutic efficacy will depend on reinjecting a population of vaccine cells that represent the antigenic diversity of the parent population. There is now evidence that suggests that long-term in vitro culture of human tumor cell lines results in the loss of expression of relevant tumor antigens. For example, Boon et al. (12, 13) found that the immunodominant T-cell recognized antigen in a human melanoma was spontaneously lost upon long-term culture and subcloning. To our knowledge, this is the first report of high efficiency gene transfer to primary human tumor cultures without requiring simultaneous transfer of a selection marker for in vitro selection of the transduced cells.
GM-CSF production by 1 million transduced tumor cells over a 24-h period at least 3 days following transduction was determined using the bioassay described in "Materials and Methods." Two transduced renal tumor cell cultures were evaluated for GM-CSF production before and after freezing and thawing the tumor cells. One pancreatic tumor culture at the corresponding in vitro passage was also determined. GM-CSF production was detected by 2 renal cell carcinomas (12 versus 83 and 3.2 versus 78.6 ng/10^6 cells/24 h, untransduced versus transduced, respectively), and 1 colon tumor culture long enough to successfully undergo gene transfer.

A proliferating population of primary human tumor cells is critical for retroviral mediated transduction since proliferation of the majority of tumor cells within the culture is necessary to facilitate integration. For this reason, growth conditions necessary for in vitro expansion of several histologically different fresh human tumor explants were optimized. It is also worth noting that even the short-term culture of the primary tumor cells yielded a significant increase (greater than 10-fold) in the total number of tumor cells. In fact, 26 fresh human renal cell explants received after nephrectomy, 21 of the 24 specimens (88%) with malignant histological subtypes were propagated in culture long enough to successfully undergo gene transfer. Murine tumor vaccine studies have revealed that for GM-CSF a full antitumor immunization potential is obtained over a greater than 10-fold range of cytokine concentrations (7). However, immunization potential was extremely dependent on vaccinating cell dose, with increasing doses providing increased systemic protection against tumor challenge. It is therefore likely that at least 1 x 10^7 cytokine-secreting tumor cells will be needed to generate an optimal antitumor immune response in patients. Thus, to produce tumor vaccines from the majority of patient specimens, either 10 g of viable tumor must be available or the primary culture must be expanded at least 10-fold. Since the average weight of excised tumor specimens received by our laboratory is 2-3 g, in most cases vaccine development will depend on the success of in vitro expansion. Given that the majority of tumor cells proliferate for 2-3 passages under the growth conditions we have developed, it is unlikely that major populations of antigen-bearing cells will be selected out during the short-term culture period.

Our system has two further advantages over past approaches. First, we have found that it is possible to freeze and thaw previously transduced tumor cells without loss of cell viability and gene expression. This should allow for flexibility in therapy administration. Second, these cells can be irradiated following transduction, resulting in the inhibition of cell proliferation without loss of in vitro GM-CSF production. Our preclinical animal studies confirm the in vivo efficacy of these irradiated tumor vaccines (7). Thus, this vaccine should be as safe as it is effective.

The transduction efficiency ranged from 39 to 100%. This was particularly true when the vector was used to transfer the LacZ marker gene. β-galactosidase activity was assayed using the substrate staining procedure described in "Materials and Methods." Percentage transduction was calculated as the total gene, β-galactosidase activity was assayed using the substrate staining procedure described in "Materials and Methods." Percentage transduction was calculated as the total gene copy number in primary tumor cells yielded a significant increase (greater than 10-fold) in the total number of tumor cells. In fact, 26 fresh human renal cell explants received after nephrectomy, 21 of the 24 specimens (88%) with malignant histological subtypes were propagated in culture long enough to successfully undergo gene transfer. Murine tumor vaccine studies have revealed that for GM-CSF a full antitumor immunization potential is obtained over a greater than 10-fold range of cytokine concentrations (7). However, immunization potential was extremely dependent on vaccinating cell dose, with increasing doses providing increased systemic protection against tumor challenge. It is therefore likely that at least 1 x 10^7 cytokine-secreting tumor cells will be needed to generate an optimal antitumor immune response in patients. Thus, to produce tumor vaccines from the majority of patient specimens, either 10 g of viable tumor must be available or the primary culture must be expanded at least 10-fold. Since the average weight of excised tumor specimens received by our laboratory is 2-3 g, in most cases vaccine development will depend on the success of in vitro expansion. Given that the majority of tumor cells proliferate for 2-3 passages under the growth conditions we have developed, it is unlikely that major populations of antigen-bearing cells will be selected out during the short-term culture period.

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gene. There are at least three possible explanations for variability. First, successful gene transfer may be dependent on the histological tumor type or the degree of cellular differentiation of the tumor cells that are being transduced. Our data do not demonstrate a correlation of transduction efficiency with the histological cell type or degree of differentiation of the renal cell, pancreatic, and colon carcinomas that have been evaluated so far. In contrast, all of the ovarian carcinomas were from ascites, which may explain the less variable range of transduction efficiency among these more advanced populations of tumor cells. A comparison could not be made for the breast, lung, and squamous cell carcinomas because too few tumors of these histological types were evaluated. Second, integration of the transferred gene is dependent on proliferation of the tumor cell population. Therefore, efficient gene transfer requires proliferation of the majority of tumor cells within the explanted population. It may be that suboptimal tumor cell proliferation of some primary tumor cultures explains the wide range of transduction efficiencies among the initial gene transfer experiments performed using the LacZ marker gene. However, it does not account for the entire problem since we see less variation in transduction efficiency with improvement of our technique of retroviral supernatant collection. Third, transduction efficiency is dependent on the virus titer of retroviral supernatant, which will vary with different titer collections. This should no longer represent a significant practical problem as recent advances in long-term freezing of amphotropic retroviral supernatants will allow lots to be tested for titer prior to their use in vaccine preparation.

In addition, when GM-CSF secretion was compared with the vector copy number for 5 genetically altered renal cell cultures, the number of integrated copies/cell did not perfectly correlate with the concentration of GM-CSF produced. A possible explanation for this is that expression of retrovirally transferred genes may be dependent on host cell-derived transcription factors that vary among different histologically similar tumor cultures. We also noted that with increasing passage of the human renal tumors cells, endogenous GM-CSF production was also induced. However, levels of endogenous GM-CSF (which ranged from 0 to 20 ng/10^6 cells/24 h, with an average of 4.1 ng/10^6 cells/24 h) were far below the threshold for maximal vaccine potency (36 ng/10^6 cells/24 h) as determined in our animal studies.

In conclusion, we have shown that it is possible to establish and efficiently transduce short-term, primary human tumor cultures. The MFG retroviral vector system has made it technically feasible to provide safe, efficient gene therapy to patients with cancer. In addition, this vector appears flexible enough to transduce a wide variety of histological tumor types. We are planning to use this vector system in a Phase I study to evaluate the antitumor immune response generated by autologous GM-CSF-secreting renal tumor cells in patients in the near future.

References

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