Fusogenic Membrane Glycoproteins As a Novel Class of Genes for the Local and Immune-mediated Control of Tumor Growth

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Abstract

We report here the use of viral fusogenic membrane glycoproteins (FMGs) as a new class of therapeutic genes for the control of tumor growth. FMGs kill cells by fusing them into large multinucleated syncytia, which die by sequestration of cell nuclei and subsequent nuclear fusion by a mechanism that is nonapoptotic, as assessed by multiple criteria. Direct and bystander killing of three different FMGs were at least one log more potent than that of herpes simplex virus thymidine kinase or cytosine deaminase suicide genes. Transduction of human tumor xenografts with plasmid DNA prevented tumor outgrowth in vivo, and cytotoxicity could be regulated through transcriptional targeting. Syncytial formation is accompanied by the induction of immunostimulatory heat shock proteins, and tumor-associated FMG expression in immunocompetent animals generated specific antitumor immunity.

Introduction

Ideally, the transfer of therapeutic genes into tumor cells should lead both to direct local cell destruction and to activation of antitumor immunity to clear tumor deposits to which the genes cannot be delivered. The genes most commonly used in clinical trials for the control of local tumor growth have been the suicide gene/prodrug systems such as HSVtk\(^3\)/GCV or cytosine deaminase/5-fluorocytosine system (1). Importantly, these systems have local bystander effects (1) that are crucial to compensate for the relatively poor efficiencies of gene transfer that are currently possible (2). Other classes of genes have also been effective via nonspecific immune activation and inflammatory killing (3). In addition, gene transfer has been successful in stimulating tumor cell-specific immune responses (4), often through cross priming of host professional APCs with released tumor antigens (5, 6). In this respect, we have shown that HSVtk-mediated tumor cell killing in vivo can generate potent antitumor immunity (7), in part via induction of heat shock protein expression, which both attracts APCs to the tumor site and promotes their cross-priming with tumor antigens (8, 9). However, both better delivery systems and more effective genes are clearly still required to improve the efficiency of both local tumor control and of antitumor immune responses. In this respect, several different viruses kill their target cells by inducing fusion between infected and noninfected cells via the interaction of the viral envelope with its cellular receptor (10). Here we report that this activity of viral FMG can be exploited therapeutically to kill tumor cells more efficiently than conventional suicide genes and that they do so by mechanisms that are also highly immunostimulatory.

Materials and Methods

FMG and Suicide Gene Vectors.

All genes were subcloned into the pCR3.1 vector (Invitrogen) to be expressed from the same CMV promoter. Transfections were carried out using the Profection (calcium phosphate coprecipitation) method (Promega, Madison, WI) or the Electroporation method (Qiagen).

Apoptosis Detection.

Cells were cultured in Labtek chamber slides (Nalge Nunc International), plated at a density of 1 \times 10^6/chamber, and transected on day 0. Cells were collected on days 1–4, washed with PBS, and fixed with 2% paraformaldehyde. Then cells were washed and permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate. Next, the cells were washed, incubated for 60 min with TUNEL reaction mixture (In situ Cell Death Detection kit, fluorescein; Boehringer Mannheim), washed again, air dried, and mounted with Vectashield (Vector Laboratories) containing 2 μg/ml 4’,6-diamidino-2’-phenylindole dihydrochloride (Boehringer Mannheim).

hsp Detection Using RT-PCR.

RNA was prepared from transfected cells with RNAzol (Biogenesis, Bournemouth, United Kingdom). RNA concentrations were measured, and 1 μg of total cellular RNA was reverse transcribed in a 20-μl volume using oligo-(dT) as a primer and Moloney murine leukemia virus reverse transcriptase (Pharmacia LKB Biotechnology, Milton Keynes, United Kingdom). A cDNA equivalent of 1 ng of RNA was amplified by the PCR using primers specific for inducible human hsp70, gpt96, or glyceraldehyde-3-phosphate dehydrogenase (details of primers available on request). In all experiments, a mock PCR (without added DNA) was performed to exclude contamination. To exclude carry-over of genomic DNA during the RNA preparation step, controls were also carried out in which the reverse transcriptase enzyme was omitted.

In Vivo Studies.

All procedures were approved by the Institutional Animal Care and Use Committee at the Mayo Foundation and by the Imperial Cancer Research Fund Animal Research Committee. To assess the efficacy of FMG expression in human tumors, athymic nude mice were injected with 10^5 human tumor cells to establish s.c. tumors. C57BL/6 mice were obtained from colonies bred at the Imperial Cancer Research Fund. Murine colorectal CMT93 cells or melanoma B16 cells were transfected with the PCR3-GALV or empty PCR3 plasmid. After 48 h, the cells were placed in selection, Geneticin (Life Technologies, Inc.), 1 mg/ml CMT93, and 5 mg/ml B16. A pooled population from each tumor line was obtained after 2 weeks. Two \times 10^5 CMT93-GALV or CMT93-neo or \times 10^6 B16-GALV or B16-neo cells were inoculated s.c. into the flank region. Animals were examined daily, and the tumor was excised once it reached a diameter of 1.0 × 1.0 cm. At a time >14 days after primary excision, the mice were then rechallenged with parental tumor cells (s.c. injection on the opposite flank of 2 \times 10^5 CMT93 or 10^6 B16). Animals were examined daily until tumor became palpable and killed if the tumor size reached 1.0 × 1.0 cm. An individual mouse was considered tumor free if the tumor was less than 0.3 × 0.3 cm.
Fig. 1. Expression of FMG cDNAs is highly cytotoxic in susceptible cell lines in a density-dependent fashion and has a bystander killing effect at least one log greater than HSVtk. A, 293 cells plated at a high seeding density (50,000 cells/well) were transfected with 5 μg of plasmid DNA (HSVtk, CD, F alone, or H alone) or cotransfected with 2.5 μg of F with 2.5 μg of H. Cells transfected with HSVtk or CD were incubated 24 h later in ganciclovir (5 μg/ml) or 5-fluorocytosine (3 μM). Surviving cell counts were determined using trypan blue exclusion cell counting or by lactate dehydrogenase release assays 5 days after transfection. Transfections with 5 μg of a control CMV-β-gal plasmid showed a mean transfection efficiency of about 7%. Bars, SD. B, comparison of cytotoxicity of three FMGs with HSVtk at high plating density. 293 cells were transfected as in A with HSVtk, F and H together (F+H), GALV, or VSV-G. The results reported in A and B are representative of at least three separate experiments in each case. Bars, SD. C, arresting cells in S phase does not affect the efficiency of cell killing by FMG. Cells were preincubated in normal medium or medium containing 5 μg/ml of aphidicolin (Aphid) to block DNA synthesis. Twenty-four h later, the cells were transfected, in triplicates, with 5 μg of plasmid encoding GALV, no DNA, or GALV-EGF. GALV-EGF contains the EGF ligand NH₂-terminally displayed on the fusogenic GALV protein, leading to >90% inhibition of its fusogenic capacity. Bars, SD. D, 293-β-Gal cells were plated in triplicates in 96-well plates at a density of 10⁵ cells/well. Twenty-four h later, increasing numbers of GALV-transfected (upper row of triplicates) or HSVtk-transfected (lower row) 293 cells were added to the wells. The number of transfected cells was estimated using transfection of parental 293 cells with CMV-β-Gal 24 h previously. From left to right, the number of cells added per triplicate set of wells was 0, 1, 10, 100, 10², 10³, and 10⁴. Both FMG- and HSVtk-transfected wells were treated with GCV. Five days later, wells were washed and stained for β-galactosidase as a measure of surviving cells. The data shown are representative of three similar experiments.
Fig. 2. Syncytial formation proceeds through nuclear accumulation and fusion. Cells transfected with 5 μg of GALV plasmid DNA were followed by confocal microscopy for up to 120 h. Cells were stained with 4',6-diamidine-2'-phenylindole dihydrochloride to visualize the nuclei (blue/violet, A–C). A, a typical syncytium formed 24 h after transfection with GALV cDNA. The cellular membrane bounding the syncytium is shown as red staining (DII). B, transfected cells were stained with the DM1A antibody (Sigma), which recognizes α-tubulin as part of the cellular cytoskeleton. A single syncytium is shown 24 h after transfection. Incoming nuclei from newly fused cells can be seen being recruited at the periphery and being shuttled toward the accumulated nuclear concentration from previous fusions (top right). C, a syncytium at 48 h after transfection stained for apoptosis by the TUNEL assay (Boehringer Mannheim). Apoptotic nuclei appear as green staining at the periphery of the syncytium, but no positive staining was observed in the nuclei within syncytia. D and E, at late stages of syncytial formation, individual nuclei break down releasing chromosomes (D) until finally all nuclear structure is lost, leaving only chromosomes and interphase DNA (E).
Results

Cytotoxic Effects of FMGs and Comparison to Suicide Genes. We tested the cytotoxic activity of the cDNAs of envelope genes from three different classes of viruses: the rhabdoviral VSV-G envelope gene (11), the combination of the F and H genes from measles virus (12), and a hyperfusogenic version of the retroviral GALV in which the COOH-terminal R peptide sequence of the cytoplasmic domain of the envelope had been deleted. In transient transfections, the cytotoxicity of all three FMGs was superior to that of either the HSVtk or CD suicide genes when cells were plated at either low or high plating density, although cytotoxicity was greatest at high plating densities (Fig. 1A). GALV or the F and H combination was consistently the most potent gene (Fig. 1B) when tested with HSVtk or CD in 10 human tumor lines/explants: Tel.CeB6 (human rhabdomyosarcoma) and HT1080 (human fibrosarcoma), 293, Mel624 (human melanoma), HeLa (cervical carcinoma), as well as in two freshly resected human colorectal and three freshly resected human melanoma explants (data not shown), confirming that the relative efficacies are not attributable to the selective choice of cell lines. The efficacy of VSV-G transfection approached that of either GALV or F+H when the ambient pH was reduced to <6.0 (data not shown). Moreover, transfections of GALV cDNA into cells blocked in S phase of the cell cycle using the drug aphidicolin showed that cytotoxicity is independent of the cell cycle (Fig. 1C).

To compare directly the relative efficacies of the bystander effects of the FMG and the HSVtk suicide gene, Fig. 1D shows that in excess of 10^4 HSVtk-transfected cells had to be added to wells containing 10^5 293-b-Gal cells to come close to complete killing of the target population. In contrast, at least 1 log fewer GALV-transfected 293 cells were sufficient to eradicate completely the target population. Over several different experiments, we calculated that a single HSVtk-transfected cell would kill approximately 8–10 bystander cells at most under these particular conditions. However, a single GALV-expressing cell would kill, on average, in excess of 150–200 bystander cells. Similar data were obtained using the HT1080 and Mel624 cell lines, confirming that these effects are not specific to individual cell lines.

FMG Cytotoxicity Occurs Through Nonapoptotic Mechanisms. Transfection with GALV induces the formation of multinucleated syncytia bounded by a single cellular membrane encompassing nuclei that individually still retain clearly defined nuclear membranes (Fig. 2A). Recruitment of bystander cells into the syncytium proceeds via a process that appears to involve streaming of the incoming nuclei along thick, organized microtubule bundles that feed into the perinuclear

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region such that large numbers of nuclei become localized in close juxtaposition (Fig. 2B). These structures can remain stable and metabolically and transcriptionally active for several days. At later time points, individual nuclei within the syncytia lose their nuclear membranes and release their chromosomes (Fig. 2D). The end result of the process, at least in vitro, is the disintegration of the syncytium with concomitant release of DNA (Fig. 2E).

At no stage of the sequence of syncytium development do nuclei stain positive for apoptosis by TUNEL analysis, although apoptotic cells or debris was occasionally observed at the periphery of well-developed syncytia (Fig. 2C). DNA ladders and electron microscopy also failed to indicate significant levels of apoptotic cell death in FMG-transfected as opposed to mock-transfected cultures (data not shown). Importantly, FMG-mediated tumor cell killing was not inhibited by the addition of caspase inhibitors, such as ZVAD, indicating that the mechanism of cell killing is not apoptotic (data not shown). We are currently investigating the dependence for killing on genes such as p53, p21, and other cell cycle controlling elements. This pattern of nonapoptotic cell killing has also been confirmed using the F+H combination and different cell lines (data not shown). The observation of interphase DNA and condensed chromosomes within syncytia (Fig. 2) suggest that a process similar to premature chromosome condensation may be occurring in which fusion of dividing cells occurs with nondividing cells and eventually precipitates a mitosis-like state in the syncytium but from which other mitotic events cannot occur (13), probably because the multiple nuclei can no longer organize an effective mitotic spindle.5

Established Tumors Can Be Eradicated by Transduction with Plasmid DNA Encoding FMG cDNA. Human tumor xenografts of HT1080 or Mel624 cells were injected s.c. into nude athymic mice at a dose of $10^6$ tumor cells/mouse. At this dose, 90–100% of mice develop small, palpable tumors by 72 h after tumor cell seeding. Tumors were transduced with $10 \mu$g of plasmid DNA complexed with Efectene lipid (Qiagen). The subsequent development of tumor growth was then measured with time as shown in Fig. 3. Both HT1080 fibrosarcoma and Mel624 melanoma tumors transduced with the CMV-β-Gal construct grew progressively in both tumor types (Fig. 3). In contrast, the CMV-GALV cDNA eradicated tumor growth in 100% of HT1080 and 90% of Mel624 tumor-bearing mice (Fig. 3, C and D), despite the initial progression of the tumors after DNA transduction (Fig. 3A). Tumor-free mice in Fig. 3, C and D, were scored as having no palpable tumor mass by day 90 after initial tumor cell seeding; in addition, no outgrowths of tumor were observed in any of these mice over this period of 90 days. The in vivo delivery of the HSVβ gene, followed by treatment with GCV, was never capable of achieving similar levels of tumor killing (data not shown), even in immunocompetent models where the immune system boosts the antitumor efficacy of this treatment (14, 15). In subsequent experiments, we observed extensive syncytia in tumors recovered from mice that had received injections 48 h previously with FMG vector. No syncytia were observed from tumors injected with β-Gal vector or PBS. In addition, RT-PCR studies from these tumors also showed the expression of hsp5, molecules that are also associated with the fusion process (see below and Fig. 4). These observations strongly suggest that the in vivo mechanism of cell killing is the same as that seen in vitro. When tumors greater than 0.2–0.3 cm in diameter were injected with plasmid DNA, we were unable to obtain complete regressions of tumors with the same efficiency as seen in Fig. 3. However, ongoing experiments with vectors of higher efficiency than plasmid DNA demon

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(17), HT1080 and Mel624 tumors were also transduced in vivo with the TYR-GALV plasmid (10 μg/tumor). HT1080 tumors continued to grow progressively after transduction with the TYR-GALV DNA (Fig. 3, A–C). In contrast, Mel624 tumors were eradicated in 100% of the mice that received injections (Fig. 3D), indicating that transcriptional control of expression is an effective means of targeting FMG-mediated gene therapy to specific tumor types.

**FMG Gene Expression Is Associated with Increased Expression of Immunostimulatory Signals and Acts as a Potent Immunogen.** The mechanisms by which tumor cells are killed influence the potency of the subsequent immune response to nontransduced tumors elsewhere in the animal (7, 8, 18). Fig. 4 shows that FMG transfection is associated with induction of mRNA of two different heat shock proteins, hsp70 and gp96, both of which are known to play important roles in enhancing tumor immunogenicity (8, 19). Transfection of a nonfusogenic GALV-EGF cDNA did not induce either hsp over levels seen in resting cells (Fig. 4). These RT-PCR results were confirmed in three different cell lines (Tel.CeB6, HT1080, and Mel624) and by immunofluorescence (data not shown). In addition, the expression of viral FMGs would be expected to be highly immunogenic per se (that is separate from any effects of syncytial induction and hsp expression), which may itself promote generation of tumor-specific immunity, through cross-priming of APCs with tumor antigens. Murine cell lines are not fused by GALV as the murine homologue of the Pit-1 receptor is not recognized by GALV. Therefore, murine colorectal CMT93 (20) and melanoma B16 tumor cell lines were transfected with the GALV gene and transected cells were used as live vaccine cells in syngeneic C57/B1 mice. Both CMT93-GALV and B16-GALV tumors grew as primary tumors but at a reduced rate compared with the parental G418r CMT93-neo and B16-neo controls (data not shown). However, after surgery, animals vaccinated with GALV-expressing cells were significantly protected against rechallenge with parental, unmodified cells compared with animals vaccinated with parental cells transfected with neo alone (Fig. 4). Similar results were obtained with the B16 murine melanoma line (data not shown). These data confirm that expression of FMG in tumor cells can serve as a potent immunostimulatory signal that generates in vivo protection against unmodified parental tumor.

**Discussion**

Our data show that FMG transfection is a much more effective treatment for killing human tumor lines in vitro than commonly used suicide genes. Killing is cell density dependent, independent of cell division, and does not require administration of a prodrug, thereby alleviating the problems of drug delivery and bioavailability. In addition, the local bystander effect of FMG killing is generally at least a log higher than that of HSVtk. Direct delivery of plasmid DNA encoding the GALV FMG to actively growing tumors in vivo is sufficient to eradicate tumor progression and growth, despite the relatively low efficiency of gene transfer.

Syncytial-mediated cell killing occurs by nonapoptotic mechanisms, through a process of nuclear recruitment, fusion, and disintegration and is associated with induction of hsps that should prove to be highly immune stimulatory in vivo (8, 18). In addition, expression of FMG is highly immunogenic per se (in the absence of syncytial induction), adding to the potency of the FMG as a mechanism for inducing antitumor immunization. Because neither GALV nor F+H fuse murine cells, we cannot yet assess fully the added contribution of syncytial induction to immune stimulation in an immunocompetent model. However, we are currently generating murine tumor lines and mice transgenic for the GALV receptor Pit-1 to address both these issues, as well as that of FMG-mediated toxicity to tissues surrounding targeted tumors. We have shown here that transcriptional targeting of FMG expression is possible to reduce toxicity, and we are currently developing molecular switches to ensure that FMG expression is extinguished when normal cells are recruited into the syncytia.

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**References**


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Details of the expression vector are available on request.
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