Viral Fusogenic Membrane Glycoprotein Expression Causes Syncytia Formation with Bioenergetic Cell Death: Implications for Gene Therapy

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ABSTRACT

Viral fusogenic membrane glycoproteins (FMGs) are candidates for gene therapy of solid tumors because they cause cell fusion, leading to formation of lethal multinucleated syncytia. However, the cellular mechanisms mediating cell death after FMG-induced cell fusion remain unclear. The present study was designed to examine the mechanisms by which FMG expression in hepatocellular carcinoma cells lead to cell death. Transfection of Hep3B cells with the Gibbon Ape leukemia virus hyperfusogenic envelope protein (GALV-FMG) resulted in the formation of multinucleated syncytia that reached a maximum 5 days after transfection (100 nuclei/syncytium). The syncytia were viable for a period of 2 days and then rapidly lost viability by day 5. Mitochondrial dysfunction occurred in GALV-FMG-induced syncydia prior to loss of viability with loss of the mitochondrial membrane potential, cellular ATP depletion, and release of mitochondrial cytochrome c-GFP into the cytosol. The pancaspase inhibitor, Z-VAD-fmk, did not prevent cell death. However, glycolytic generation of ATP with fructose effectively increased cellular ATP and preserved syncytial viability. These data suggest that expression of FMG in hepatoma cells results in the formation of multinucleated syncytia, causing mitochondrial failure with ATP depletion, a bioenergetic form of cell death with necrosis. This form of cell death should be effective in vivo and enhance the bystander effect, suggesting that FMG-based gene therapy deserves further study for the treatment of hepatocellular and other cancers.

INTRODUCTION

Hepatocellular carcinoma is a significant worldwide health problem with limited treatment options and a high mortality rate. Gene therapy is emerging as an important potential approach for the treatment of hepatocellular carcinoma (1–3). Hepatocellular carcinoma lends itself to gene therapy approaches because of both anatomical and genetic features permitting gene targeting. Because these cancers are located within the livers as solid mass lesions, targeted constructs can be directly injected into these tumors using percutaneous approaches. The dependence of these tumors upon the arterial blood supply also permits targeting by intra-arterial infusion. Furthermore, expression of the constructs can be rendered tumor specific by using the α-fetoprotein promoter, a gene that is expressed by many hepatocellular cancers (4).

Transduction of tumor cells using suicide genes is a common strategy used in cancer gene therapy, in which tumor cells are transduced with genes for enzymes that convert otherwise nontoxic drugs into cytotoxic compounds (5). A key observation originating from these studies is the so-called “bystander effect” (6, 7). The bystander effect consists of loss of viability in nontransduced cells after death of transduced neighboring cells. The bystander effect is not well understood but has been observed in vitro and is thought to be even more pronounced in vivo, where the immune system and inflammatory responses enhance this phenomenon (8). The bystander effect is crucial for effective anticancer therapy because it compensates for the limited efficiency of gene transduction.

A unique gene therapy approach for human cancers is transduction with viral FMGs (9). Several different viruses kill their target cells by causing fusion between infected and noninfected cells via the interaction between the viral envelope with its receptor; the fusion of uninfected cells with infected cells represents a type of bystander effect. The fusogenic portions of membrane glycoproteins have been identified in varicella-zoster virus, measles virus, and human immunodeficiency virus (10–13). Transduction of the FMG alone in human cells has been shown to induce cell-cell fusion and formation of lethal multinucleated syncytia (10, 13). The ability of viral FMGs to fuse target cells with neighboring cells with a lethal outcome could be exploited as a treatment approach for hepatocellular carcinoma. The cytototoxic activity of the cDNAs of envelope genes from three different classes of viruses including the rhadoboviral VSV-G envelope gene (14), the combination of the F and H genes from measles virus (13), and mutated version of the retroviral Gibbon Ape Leukemia virus (GALV) has been evaluated previously (9).

The mechanism by which the FMG-induced multinucleated syncytia die is unknown. Two mechanisms of cell death are now recognized: (a) necrosis; and (b) apoptosis. Necrosis is characterized by cellular swelling and plasma membrane lysis. On a biochemical level, necrosis results from ATP depletion (15, 16). The morphological hallmarks of apoptosis include cell shrinkage, nuclear chromatin condensation, and separation of the cell into membrane-defined fragments (17, 18). Apoptosis is mediated by caspase protease activation and requires ATP (16, 19–21). Because necrosis involves release of cellular constituents into the interstitial space and apoptosis does not, necrosis elicits a stronger inflammatory response. Necrosis would, therefore, be expected to enhance the bystander effect because of the resulting inflammatory and immunological response (22).

Both necrosis and apoptosis involve mitochondrial dysfunction. In necrosis, mitochondrial dysfunction is so severe that oxidative phosphorylation fails, and cellular ATP depletion occurs (16, 19). In contrast, in apoptosis the mitochondrial dysfunction is associated with release of intermembrane constituents such as Cyt-c, which activate apoptotic death cascades. In both forms of cell death, mitochondria may lose their membrane potential and release Cyt-c from the intermembrane space. In this context, it may be the magnitude of ATP depletion that determines whether a cell dies by necrosis or apoptosis.

Our overall objective is to develop gene therapy for human hepatocellular carcinoma using FMG. As an initial step toward this goal, we used an in vitro approach to determine whether FMG transduction is cytotoxic for this cell type. We chose GALV-FMG for this study because of its documented efficacy in other cell types (9). Our specific aims were to answer the following questions: (a) Does GALV-FMG transfection induce multinucleated syncytia in human hepatocellular carcinoma cells? (b) Does the formation of the syncytia result in cell death?
death by a mitochondrial involved pathway? and (c) Does GALV-FMG-induced cell death occur by necrosis or apoptosis? Our results demonstrate that GALV-FMG transduction in a hepatocellular carcinoma cell line causes syncytia formation with mitochondrial dysfunction, ATP depletion, and necrosis. These promising results suggest that this gene therapy approach deserves further study for the treatment of this cancer.

MATERIALS AND METHODS

Cell Culture. Hep3B cells (10^4 cells/ml) were cultured in 35 × 10-mm Petri dishes (Becton Dickinson Labware; Becton Dickinson, Lincoln Park, NJ) on 22-mm square glass coverslips coated with type 1 collagen from rat tail tendon (23). The culture medium was EMEM supplemented with 10% fetal bovine serum, 100 mIU insulin, 100,000 units/l penicillin, 100 mg/l streptomycin, and 100 mg/l gentamicin. Cells were grown until ~70% subconfluent prior to each experiment.

GALV-FMG Transfection. A cDNA encoding for the fusogenic portion of the envelope glycoprotein of the GALV was subcloned into the pCR3.1 vector (Invitrogen). Hep3B cells were transfected with Lipofectamine (Life Technologies, Inc., Gaithersburg, MD). Briefly, the cells were transfected by adding 1 ml of a mixture of 6 μg of Lipofectamine and 1 μg of the DNA plasmid to each culture dish. The cells were incubated in the above mixture for 5 h at 37°C in a 5% CO_2/95% air incubator. After the incubation, 1 ml of EMEM containing 20% fetal bovine serum was added to the transfection medium in each culture dish. Twenty-four h later, the medium was aspirated and replaced with 2 ml of EMEM containing 10% fetal bovine serum.

Quantitation of Syncytium Formation. Syncytia formation was quantitated by a propidium iodide exclusion assay (26). Analogue to trypan blue, propidium iodide is excluded from viable cells but rapidly labels the nuclei of dead cells. Propidium iodide (5 μM) was added to the culture medium for 30 min to label dead cells, which were visualized by fluorescence microscopy using excitation and emission wavelengths of 520 and 605 nm, respectively. For the detection and quantitation of floating syncytia in the culture media, culture medium was collected, and the floating syncytia were collected by a cytospin technique. Two hundred μl of culture medium were collected and centrifuged using a cytospin (Cytospin 2; Shandon, Inc., Cheshire, United Kingdom) at 1000 rpm for 10 min. The total number of centrifuged multinucleated syncytia was counted, and data were expressed as the number of syncytia/200 μl of medium.

Measurement of LDH Activity. LDH activity in the culture medium was measured as described previously (26). LDH activity after lysis of the cells with 375 μM digitonin was taken as maximal LDH release (26).

Multiparameter Digitized Fluorescent Microscopy. Experiments measuring mitochondrial membrane potential, oxidative stress, and cytosolic free calcium were performed using a multiparameter digitized fluorescent microscopy system (23). The microscope is an inverted phase/fluorescence microscope (Axiovert 35M; Carl Zeiss, Inc., Thornwood, NY) equipped with a thermostat-controlled, heated stage. The software for the system was the Metafluor quantitative fluorescence software package from Universal Imaging Corp. (West Chester, PA). Excitation light was provided by a 100-W mercury vapor lamp or a 75-W xenon lamp and passed through an interference and neutral density filter wheel assembly (Eastern Microscope, Research Triangle Park, NC) to select wavelength and intensity under computer control. A cooled charged coupling device camera (cooled-CCD camera, model KAF-1400; Photometrics, Tucson, AZ) collected fluorescent images that were fed to a computer (Data Store 486/50E). A 1316 × 1316 pixel area was digitized with 16-bit resolution using video acquisition for frame averaging, background subtraction and storage on a hard disk.

Measurement of Mitochondrial Membrane Potential. The mitochondrial membrane potential was monitored using the membrane potential-sensitive dye TMRM and digitized fluorescent microscopy (23). TMRM is a fluorescent lipophilic cation that electrotopically redistributes across the mitochondrial membrane according to the mitochondrial membrane potential (27). Cells were loaded with TMRM by incubation in Krebs’-Ringer-HEPES buffer (115 mM NaCl, 1 mM KH PO₄, 2 mM CaCl₂, 5 mM KCl, 1.2 mM MgSO₄, and 25 mM sodium-HEPES buffer, pH 7.4; Ref. 23) containing 50 nM TMRM for 15 min at 37°C. TMRM fluorescence was observed using a 546-nm
excitation filter, a 580-nm dichroic reflector, and a 590-nm long-pass filter. Cellular fluorescence was quantitated by multiplying the average fluorescence intensity in the cells or syncytia by the number of pixels above background using a threshold of zero.

Detection of Cyt-c Release from Mitochondria. Release of Cyt-c from cells was detected using both morphological and cell fractionation approaches. The expression vector pE-GFP-Cyt-c (a gift of Dr. Anna-Liisa Nieminen, Case Western Reserve University) encodes a polypeptide that contains GFP fused to the COOH terminus of Cyt-c (28). Hep3B cells (70% subconfluent) were transiently transfected using Lipofectamine (Life Technologies, Inc.) as described above. One ml of a mixture containing 10 μg of Lipofectamine and 1 μg of pE-GFP-Cyt-c DNA plasmid, or 1 ml of a mixture containing 10 μg of Lipofectamine and 1 μg of pE-GFP-Cyt-c plus 1 μg of pCR3.1-GALV-FMG, was used for the transfection. Confocal microscopy of transfected cells was performed using a laser scanning confocal microscope (LSM 510; Carl Zeiss, Inc., Thornwood, NY). GFP fluorescence was imaged using excitation and emission wavelengths of 488 and 505 nm, respectively.

Cytosolic extracts from FMG-transfected or untransfected control cells were obtained as described by Leist et al. (29). At the desired time points, the culture medium was exchanged with premeabilization buffer (210 mM β-mannitol, 70 mM sucrose, 10 mM HEPES, 5 mM succinate, 0.2 mM EGTA, 0.15% BSA, and 80 μg/ml digitonin, pH 7.2). The permeabilization buffer was removed and centrifuged for 10 min at 13,000 × g. Protein from the supernatant was separated on 14% polyacrylamide gels and electroblotted to nitrocellulose. The membrane was blocked with 1% w/v skim milk in 20 mM Tris, 0.5 M NaCl, 0.05% Tween 20 (pH 7.0) for 1 h and then incubated for 1 h with a 1:1000 dilution of mouse anti-Cyt-c (PharMingen, San Diego, CA). After washing, membranes were incubated for 1 h with peroxidase-conjugated goat antiserum IgG (Biosource, Camarillo, CA) and washed again. Bound antibody was visualized using chemiluminescent substrate (ECL; Amersham, Arlington Heights, IL) and exposed to Kodak X-OMAT film.

Measurement of Cellular ATP Level. Cellular ATP levels were quantitated by the luciferin/luciferase assay as described previously (30). Cells in the culture dish were rapidly quenched by the addition of 3% HClO4 at 4°C. The supernatant was neutralized with 1.0 mol/l KOH, and the precipitate was removed by centrifugation (7500 × g). ATP was measured following the directions of the manufacturer of the commercial assay kit. Bioluminescence was quantitated in a luminometer (TD-20/20; Turner Designs, Sunnyvale, CA).

Materials and Reagents. DAPI, propidium iodide, TMRM, fura-2, dihydroethidium, bis-(O-aminophenoxy)-ethane-N,N,N',N'-tetraacetic acid tetra-(acetoxymethyl)-ester were from Molecular Probes, Inc. (Eugene, OR). Z-VAD-fmk was obtained from Enzyme Systems Products (Livermore, CA). Fructose, NaCl, KH2PO4, CaCl2, KCl, MgSO4, and sodium-HEPES were from Sigma Chemical Co. (St. Louis, MO).

RESULTS

FMG Transfection Induces Cell-Cell Fusion and the Formation of Multinucleated Syncytia of Hep3B Cells. After transfection of Hep3B cells with GALV-FMG, the formation of multinucleated syncytia was observed within 24 h. At this time point, the maximum number of nuclei/syncytia was ~20 (Fig. 1A). These syncytia increased in size over time, reaching a maximum of >100 nuclei/syncytium at 72 h after the transfection (Fig. 1B). To quantitate the formation of syncytia, we calculated the “fusion index” (the percentage of the nuclei in syncytia versus total nuclei in the microscopic field). The fusion index was maximal at 72 h after transfection with a
value of 18.2 ± 3.2% (Fig. 1C). Syncytia were never observed in cells transfected with the empty plasmid. Thus, GALV-FMG transfection in a hepatocellular carcinoma cell line does result in significant syncytia formation.

The Formation of Syncytia Results in Cell Death. The viability of the syncytia was assessed by propidium iodide exclusion. Syncytia became propidium iodide positive over time, indicating loss of viability (Fig. 2A). The number of propidium iodide-
FMG-induced Cell Death Is Not Caspase Dependent. Mitochondrial dysfunction with loss of oxidative phosphorylation can cause a bioenergetic form of cell death that is ATP dependent. Therefore, total cellular ATP in FMG-transfected or untransfected cells were evaluated in this study (Fig. 3A). GALV-FMG transfection reduced the total cellular ATP level to 20% of untransfected cells. High concentrations of fructose have been shown to prevent ATP depletion in hepatocytes during mitochondrial dysfunction by enhancing glycolytic generation of ATP (32). Therefore, we determined the effects of fructose on FMG-induced ATP depletion and cell death. Fructose (20 mM) effectively attenuated the decrease in cellular ATP level (Fig. 5A) and reduced the number of propidium iodide-positive syncytia (Fig. 5B), or LDH release (Fig. 4C) induced by FMG transfection. Thus, as assessed by complementary approaches, loss of syncytial viability occurred by a caspase-independent mechanism.

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Mitochondrial Dysfunction Occurs in the Syncytia. Because mitochondrial dysfunction is a common mechanism of cell death, mitochondrial function in the syncytia was assessed. Fig. 3A is a representative photomicrograph of Hep3B cells loaded with TMRM. The TMRM fluorescence intensity in syncytia demonstrated that the mitochondrial membrane potential was ~15% of control cells at all observation periods (Fig. 3B). Thus, syncytia formation appears to result in early mitochondrial dysfunction.

Loss of the membrane potential has been associated with release of Cyt-c from mitochondria into the cytoplasm (28). Therefore, we monitored the cellular compartmentation of Cyt-c-GFP after cotransfection with GALV-FMG (Fig. 3C). Cyt-c-GFP displayed a punctate pattern of fluorescence that was indistinguishable from that of TMRM in the control cells and single cells in the transfected cultures. Overlay images of Cyt-c-GFP with TMRM demonstrated that the transfected protein was in the mitochondria. In the syncytia induced by cotransfection of Cyt-c-GFP with GALV-FMG, Cyt-c-GFP displayed a diffuse pattern of fluorescence, indicating a redistribution of Cyt-c-GFP from mitochondria to the cytoplasm. Biochemical cell fractionation studies also demonstrated Cyt-c release into the cytosol, as assessed by immunoblot analysis (see Fig. 5D). These results suggest that Cyt-c-GFP was released from mitochondria during multinucleated syncytia formation.

Positive syncytia in the FMG-transfected groups began to increase 3 days after the transfection and increased to 50% of total syncytia at 5 days (Fig. 2B). In contrast, propidium iodide-positive nuclei were not observed in the two control groups (untransfected and empty plasmid-transfected cells). We noted that syncytia frequently detached from the substratum, resulting in an underestimation of syncytia formation and cell death. Therefore, we also quantitated the number of detached syncytia in the culture media. The appearance of floating syncytia also increased 3 days after transfection and reached a maximum at 5 days (Fig. 2C). To more fully quantitate cell death, LDH activity in the culture medium was measured. LDH activity increased in the same time course as the increase in the number of propidium iodide-positive syncytia and reached a maximum of ~70% at 5 days after the transfection (Fig. 2D). These data demonstrate that GALV-FMG-induced syncytia result in cell death in the majority of Hep3B cells. However, the onset of cell death is delayed as compared with syncytia formation. These data suggest that the act of cell fusion alone is not lethal but requires additional cellular mechanisms.
DISCUSSION

In the present study, we have demonstrated that transfection of GALV-FMG causes cell-cell fusion and induces the formation of lethal multinucleated syncytia in Hep3B cells. These syncytia were still viable within 2 days, and they further fuse with surrounding cells until they finally form large syncytia containing up to 100 nuclei. Thereafter, these large syncytia lose viability. The early fusion of syncytia with adjacent cells before loss of viability results in a significant bystander effect. Indeed, transfection of GALV-FMG was associated with cell killing of 70% of the cells as assessed by LDH release, whereas the initial transfection efficiency was only 7%, as assessed by expression of a GFP construct. This significant bystander effect of GALV-FMG has been observed in other transfected cells (9). Comparative data suggest that the bystander killing of untransfected cells after GALV-FMG is at least 1 log greater than the herpes simplex virus thymidine kinase/ganciclovir model (9). Collectively, these data suggest that GALV-FMG would appear to be a promising construct for gene therapy of hepatocellular carcinoma cells.

Information regarding the mechanisms of cell death in the syncytia is important because cancers are often resistant to many forms of cell death (e.g., overexpression of antiapoptotic proteins, loss of cell cycle checkpoint functions, and others). Several observations suggest that the multinucleated syncytia die by a necrotic process: (a) the syncytia developed lysis of the plasma membrane, a cardinal feature of necrosis, as manifest by uptake of propidium iodide and leakage of LDH into the media; (b) the nuclear morphology was unchanged during loss of viability, an observation consistent with cell death by necrosis but not apoptosis, where chromatin condensation and nuclear fragmentation are morphological hallmarks; (c) caspase inhibitors, which block cell death by apoptosis, did not prevent cell killing after GALV-FMG transduction; and (d) we have shown in other studies that apoptotic DNA cleavage into multiples of oligonucleosomes does not occur in these syncytia (9). Cell death by necrosis may be advantageous for gene therapy because necrosis elicits an inflammatory response, further enhancing the bystander effect by inflammatory/immune-mediated mechanisms (22).

Mitochondrial dysfunction with loss of oxidative phosphorylation resulted in a bioenergetic form of syncytial cell death. This conclusion is based on our observation of early and significant mitochondrial depolarization in the syncytia, loss of cellular ATP, and protection against cell death by increasing cellular ATP with the glycolytic substrate fructose. The mitochondrial membrane potential is a critical, if not the predominant mechanism, driving the proton motive force responsible for oxidative phosphorylation. Loss of the mitochondrial membrane potential would therefore be predictive of impairment in oxidative phosphorylation (16, 20). In support of this concept, we demonstrated loss of cellular ATP after transfection of GALV-FMG. Moreover, high concentrations of fructose used to generate ATP glycolytically increased ATP to 60% of control values, an observation consistent with previous studies in anoxic liver (32). This increase in ATP by fructose appeared to be sufficient to inhibit cell death.

The mitochondrial dysfunction in the syncytia was associated with release of Cyt-c from the intermembrane space of mitochondria into the cytosol, indicating structural alterations in the outer mitochondrial membrane. This structural dysfunction of mitochondria is a feature of cell death either by necrosis or apoptosis (15). Interestingly, fructose

![Fig. 5. Fructose-attenuated death of multinucleated syncytia. Hep3B cells transfected with GALV-FMG plasmid or empty plasmid were incubated for 5 days in the presence of 20 mM fructose. Cellular ATP levels were quantitated 1 day after the transfection (A). The number of propidium iodide-positive syncytia (B) and LDH activity in the culture media (C) were evaluated at 0, 1, 2, 3, 4, and 5 days after the transfection. Mitochondrial membrane potential (D) and Cyt-c release (E) were evaluated at 0, 1 (1d), 2 (2d), and 3 (3d) days as described in “Materials and Methods.” A–D: bars, SD.]
attenuated the release of Cyt-c from mitochondria into the cytosol. This observation suggests that the initial cellular insult after GALV-FMG syncytia formation is failure to synthesize ATP. Loss of cellular ATP then appears to further potentiate mitochondrial dysfunction, causing Cyt-c release. These data suggest that Cyt-c release can be a secondary process in cell death and need not be implicated as a primary event. GALV-FMG may cause mitochondrial dysfunction by being mistargeted to mitochondria in the syncytia, causing failure of oxidative phosphorylation. Unfortunately, in the absence of anti-GALV-FMG antiserum, we were unable to test this possibility. Nonetheless, because mammalian cells cannot survive long-term without ATP, cells would not be expected to develop resistance to this form of cell death, further supporting the potential utility of GALV-FMG gene therapy for cancer.

Because hepatocellular carcinomas frequently have mutations in p53 (33–35), we used the p53 null Hep3B cells for these studies (36). GALV-FMG-induced syncytia formation and cell death were efficient in this cell type. These data demonstrate that GALV-FMG cell killing is p53 independent, and therefore, a viable gene therapy approach for cancers, such as hepatocellular carcinoma with p53 mutations.

In conclusion, transduction of GALV-FMG results in efficient formation of giant syncytia, causing death of hepatocellular carcinoma cells. The incorporation of adjacent, nontransduced cells into the syncytia is responsible for a significant bystander effect. It would be anticipated that because cell death occurs by necrosis and would elicit an inflammatory response in vivo, the bystander effect would be accentuated in vivo. Gene therapy with GALV-FMG is, therefore, a promising approach for the treatment of hepatocellular carcinoma. Its efficiency in inducing tumor regression in xenografts is currently being evaluated using plasmid and viral vectors for gene delivery. Cancer-specific expression constructs using the α-fetoprotein promoter also should be engineered and tested before its therapeutic promise can be further assessed in humans.

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