

# Gene Therapy for Oral Cancer: Efficient Delivery of a 'Suicide Gene' to Murine Oral Cancer Cells in Physiological Milieu

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## ABSTRACT

Gene therapy is a new therapeutic modality in which defective genes are replaced with functional ones, or genes are delivered that can specifically kill cancer cells. Efficient gene delivery is an important component of gene therapy approaches. Potential safety problems with viral vectors necessitate the development of efficient nonviral vectors.

DNA complexes with synthetic cationic liposomes or polymers constitute a simple means of transferring DNA into target cells. Gene delivery mediated by many nonviral vectors, however, is inhibited by serum components, and this is expected to limit the efficiency of gene delivery in vivo. In this study, the authors examined two novel gene transfection reagents, Metafectene and GeneJammer, for their ability to deliver a reporter gene to SCCVII murine squamous cell carcinoma cells in the presence of high concentrations of mouse serum. After establishing conditions that achieved significant gene delivery, the authors introduced the Herpes Simplex Virus Thymidine kinase (HSV-tk) gene into the cells using the cationic liposome reagent, Metafectene, followed by the administration of ganciclovir. After seven days of incubation, 90 percent and 82 percent cytotoxicity was observed in 0 percent and 60 percent mouse serum, respectively. The authors' observations suggest that Metafectene may be useful for the gene therapy of oral squamous cell carcinoma in a murine model involving the induction of oral tumors by SCCVII cells.

**H**ead and neck cancers constitute about 3 percent to 5 percent of all cancers in the United States and are more common in persons over age 50.<sup>1</sup> About 39,000 individuals are predicted to develop head and neck cancer in 2005.<sup>1</sup> The five-year survival rate in patients with oral and pharyngeal cancers has remained at 53 percent between 1974 and 1994, the rates for African Americans and whites being 32 percent and 55 percent, respectively.<sup>2</sup> Current treatments for head and neck cancers include surgery, radiation therapy, and chemotherapy, all of which have severe side effects. The genetic approach to the treatment of HNSCC is based on the hypothesis that expression of therapeutic genes in target cells will cause a cytotoxic effect or mediate apoptosis, or

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**Acknowledgments** / This work was supported by research funds from the University of the Pacific Arthur A. Dugoni School of Dentistry. The authors have no commercial ties related to this work.

that the defective genes can be replaced with normal ones.<sup>3</sup> Oral cancer is a particularly appropriate target for gene therapy, since direct injection of genes into most primary and recurrent lesions is possible. The gene therapy approach is also amenable to cancer cell-specific gene delivery and expression, which will alleviate the problem of destroying normal cells during therapy.

SCCVII is an aggressive squamous cell carcinoma (SCC) cell line established from a carcinoma that developed spontaneously in C3H/HeJ mice. Injection of SCCVII cells into the floor of the mouth in C3H/HeJ mice results in the development of oral tumors, serving as a useful immunocompetent animal model of HNSCC.<sup>4</sup> Successful suicide gene therapy in this system, via direct injection of the HSV-tk gene into the tumor, followed by GCV treatment, would be expected to reduce the tumor size. This approach could be used in conjunction with immunostimulatory gene therapy.

Although generally efficient in transducing cells, viral vectors suffer from problems of immunogenicity, toxicity, limits in the size of exogenous DNA, and the risk of inducing tumorigenic mutations and generating active viral particles through recombination. Synthetic cationic polymer-DNA complexes (polyplexes) and cationic liposome-DNA complexes (lipoplexes) constitute a promising alternative to the use of viral vectors and provide a simple means of transferring DNA into target cells ("transfection").<sup>5-8</sup> One of the limitations of transfection mediated by nonviral vectors is that it is usually inhibited by serum components.<sup>8-14</sup> Thus, gene delivery in vivo is expected to be far from efficient. It is therefore impor-

tant to identify nonviral vectors that are resistant to the inhibitory effects of serum. Previous studies in the authors' laboratory had indicated that both the cationic polymer, GeneJammer, and the cationic liposome, Metafectene, are effective in transfecting human SCC cells even in the presence of high concentrations of fetal bovine serum.<sup>15</sup>

In this study, the authors examined the effect of mouse serum on the delivery of the genes encoding luciferase and the Herpes Simplex Virus thymidine kinase (HSV-tk), to SCCVII cells,

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by Metafectene and GeneJammer. The luciferase gene is expressed from the pCMV.Luc plasmid under the control of the cytomegalovirus promoter, and is used as a reporter gene to monitor the efficiency of gene transfer. Delivery of HSV-tk to these cells, followed by ganciclovir (GCV) treatment is expected to cause cell death, in an approach termed "suicide gene therapy."<sup>3,16-18</sup> In previous studies, the authors demonstrated that nonviral vectors, including Fugene and transferrin-lipoplexes, could mediate the delivery of HSV-tk to human SCC cells in serum-free medium, thereby causing extensive cytotoxicity in the presence of the prodrug GCV.<sup>19</sup>

### Materials and Methods

**Cells.** SCCVII murine squamous cell carcinoma cells were a gift of Dr. D. Li and Dr. B. O'Malley (University of Pennsylvania). They were propagated in

Dulbecco's modified Eagle's MEM medium (DMEM; Irvine Scientific, Santa Ana, Calif.), supplemented with 10 percent (v/v) fetal bovine serum (FBS) (Sigma, St. Louis, Mo.), penicillin (100 U/ml), streptomycin (100 µg/ml) and L-glutamine (4 mM) (DMEM/10). Mouse serum was obtained from Equitech-Bio, Inc. (Kerrville, Texas).

**Plasmids and reagents.** Metafectene, a polycationic transfection reagent based on liposome technology, containing a polyamino-lipid and dioleoylphosphatidylethanolamine (DOPE) was obtained from Biontech Laboratories GmbH (Munich, Germany). GeneJammer, a proprietary formulation of polyamine and other components in 80 percent ethanol, was purchased from Stratagene (La Jolla, Calif). The plasmids pCMV.Luc (VR-1216), encoding luciferase was a gift of Dr. P. Felgner (Vical, San Diego, Calif). The plasmid pCMV.lacZ, encoding β-galactosidase was from Clontech (Palo Alto, Calif.), and pCMV.HSV-tk (pNGVL1-tk), expressing the Herpes Simplex Virus thymidine kinase was obtained from the National Gene Vector Laboratory (University of Michigan, Ann Arbor, Mich.).

**Transfection.** For transfection, 2 x 10<sup>5</sup> cells were seeded in 1 ml of DMEM in 48-well culture plates one day before transfection, and used at approximately 80 percent confluence. Metafectene- and GeneJammer-mediated transfection procedures were performed according to the manufacturers' recommendations. Cells were prewashed with serum-free DMEM medium and then covered with 0.4 ml of the same medium. Complexes were prepared by mixing Metafectene or GeneJammer with 0.1 ml of serum-free DMEM medium, followed by the addition of plasmid DNA. The mixture

was incubated for 15 min at room temperature after the addition of the transfection reagent, and another 15 min after addition of DNA. Lipid/DNA complexes were added in a volume of 0.1 ml per well, the cells were incubated for 4 h at 37°C, and then 0.5 ml of serum-containing medium was added. Luciferase activity was assayed 48 hours after transfection, using the Luciferase Assay System (Promega, Madison, Wisc.), and a TD-20/20 luminometer (Turner Designs, Sunnyvale, Calif.). The data were expressed as relative light units (RLU) per ml of cell lysate. These values are designated "transfection activity." Transfection efficiency, i.e. the percentage of transfected cells in the culture, was examined by transfecting pCMV.lacZ followed by staining for  $\beta$ -galactosidase, using the X-gal (5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside) reagent as a substrate for the expressed enzyme.<sup>19,20</sup>

**HSV-tk + ganciclovir treatment and cytotoxicity.** Cells transfected with the HSV-tk plasmid (pCMV.HSV-tk) were incubated in the absence or the presence of GCV (20  $\mu$ g/ml) for the indicated periods of time. Ganciclovir was a gift from Hoffmann-La Roche, Inc. (Nutley, N.J.). GCV-mediated cytotoxicity was assessed by the Alamar Blue (Accumed International Companies, Westlake, Ohio) assay, as described by Konopka et al.<sup>21</sup>

## Results

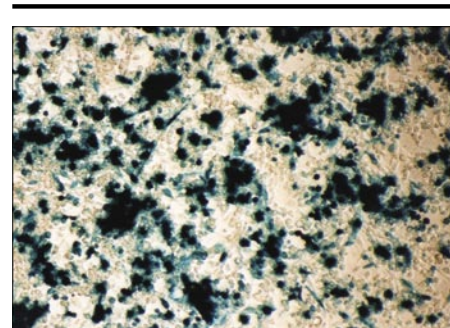
To achieve the highest levels of gene expression without causing significant toxicity to the cells when using nonviral vectors, it is essential to determine the optimal ratio of the transfection reagent to plasmid DNA, and the amount of DNA for every cell line.<sup>22</sup> Therefore, in preliminary experiments, transfection conditions were optimized using different ratios of reagent:pCMV.

Luc. The optimal conditions for SCCVII cells were 2  $\mu$ l Metafectene:1  $\mu$ g DNA per well, and 3  $\mu$ l GeneJammer:0.5  $\mu$ g DNA per well (data not shown).

While luciferase expression, using the pCMV.Luc plasmid, indicates the level of transgene expression in the entire culture, the pCMV.lacZ plasmid can be used to evaluate the percentage of cells that can be visibly transfected. With the latter plasmid, cells expressing  $\beta$ -galactosidase turn blue after the addition of the specific substrate, X-gal. SCCVII cells were transfected using Metafectene. **Figure 1** shows that approximately 60 percent to 70 percent of the cells stained positive for  $\beta$ -gal.

To mimic the effect of physiological, or in vivo, conditions on gene transfer and expression in SCCVII cells mediated by nonviral vectors, cells were transfected with Metafectene-pCMV.Luc complexes in the presence of varying concentrations of mouse serum. At 20 percent serum, transfection was inhibited by 70 percent, but the inhibitory effect of serum was not as pronounced at 40 percent, and at 60 percent serum luciferase expression was inhibited by only about 23 percent (**Figure 2**). GeneJammer-mediated luciferase expression was reduced by about 56 percent in 20 percent and 40 percent serum, and about 67 percent in 60 percent serum (**Figure 3**).

To establish whether the relatively serum-resistant nonviral vector Metafectene could also mediate the delivery of a therapeutic gene to murine SCC cells, the pCMV.HSV-tk plasmid was complexed with this reagent and incubated with SCCVII cells in the absence or presence of 60 percent mouse serum. Treatment with GCV for three days, resulted in 36 percent and 0 percent cytotoxicity, respectively. After seven days of GCV treatment, however, 90 percent and 82 percent cytotoxic-



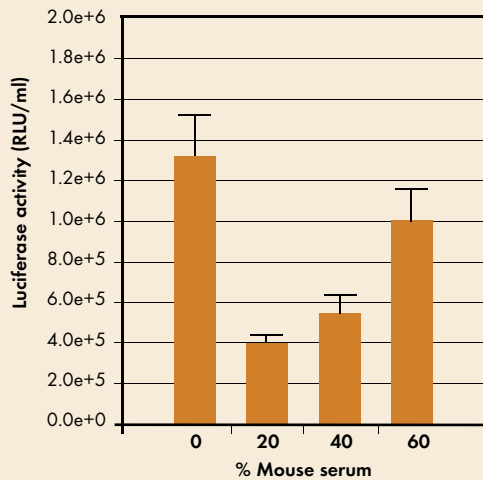
**Figure 1.** Gene expression in SCCVII cells transfected with the pCMV.lacZ plasmid using Metafectene. Cells expressing  $\beta$ -galactosidase turn blue after the addition of the specific substrate, X-gal.

ity was observed in the presence of 0 percent and 60 percent mouse serum, respectively (**Figure 4**). No significant nonspecific cytotoxicity was observed in the absence of GCV.

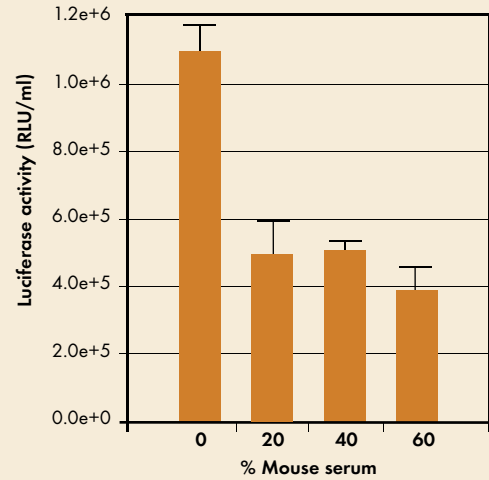
## Discussion

The inhibitory effect of serum on transfection by lipoplexes is most likely mediated by negatively charged proteins that bind to the cationic components of the vector, resulting in the inhibition of electrostatic interactions with cellular membranes and release of the DNA. Serum nucleases can also degrade the exposed segments of plasmid DNA. Most transfection protocols involving cultured cells require that the reagents are added either in the absence of serum, or with only 10 percent serum normally used in culture media, the latter only with a few reagents. In vivo applications of nonviral vectors include intravenous or intratumoral injection, both of which involve exposure to physiological milieu. To identify transfection reagents that are likely to be effective when delivered in vivo, particularly in murine models of oral or other cancers, the effect of high concentrations of mouse serum was investigated.

Two transfection reagents that have



**Figure 2.** Luciferase expression in SCCVII cells transfected with Metafectene-pCMV.Luc complexes in the presence of varying concentrations of mouse serum. Data represent the mean  $\pm$  SD obtained from triplicate wells.



**Figure 3.** Luciferase expression in SCCVII cells transfected with GeneJammer-pCMV.Luc complexes in the presence of varying concentrations of mouse serum. Data represent the mean  $\pm$  SD obtained from triplicate wells.

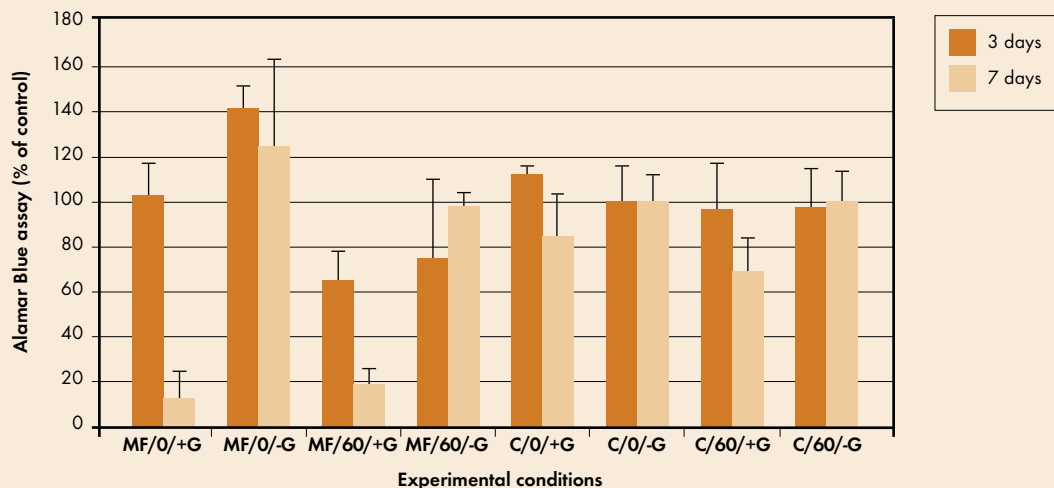
become available recently, the polycationic liposome, Metafectene, and the polyamine reagent, GeneJammer were examined. Metafectene has been used for gene delivery to a variety of cells in culture, including prostate cancer cells, human cervical carcinoma cells, and mouse fibroblasts.<sup>23-25</sup> Significantly, Metafectene has been employed in delivering the gene encoding bone morphogenetic protein, BMP-2, to tissues surrounding implants.<sup>26</sup> GeneJammer has been used for transfection of zebrafish embryos.<sup>27</sup>

GeneJammer- and Metafectene-plasmid complexes were shown to transfect murine SCCVII cells efficiently both in the absence and presence of mouse serum. These cells were chosen since they are employed in the generation of SCC tumors in an oral cancer model in C3H/HeJ mice.<sup>4</sup> Using the adenovirus HSV-tk vector for intratumoral injection

and systemic GCV administration, Sewell et al. have demonstrated tumor regression and improved animal survival in this model.<sup>4,28</sup> The results also demonstrated that the delivery of the HSV-tk gene by Metafectene, followed by GCV treatment, causes extensive cytotoxicity even in the presence of 60 percent mouse serum. Thus, it is likely that Metafectene will be useful for the delivery of genes in biological milieu, either for intravenous injection in the treatment of disseminated cancers or for intratumoral injection in the therapy of oral SCC. Although GeneJammer facilitated serum-resistant gene delivery to SCCVII cells, the presence of ethanol in the formulation of this reagent may preclude its use in vivo. Current studies in the authors' laboratory are directed toward cancer cell-specific delivery and expression of reporter (Luciferase and  $\beta$ -galactosidase) and suicide (HSV-tk)

genes. Cancer cell-specific cytotoxicity is likely to leave neighboring normal cells intact, and to enhance the eradication of tumors, especially when combined with immunotherapy. This approach to the treatment of head and neck cancers is expected to have minimal side-effects, in contrast to current therapies. **CDA**

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**Figure 4.** Cytotoxicity of HSV-tk + ganciclovir in SCCVII cells in the absence (MF/0) or presence (MF/60) of 60 percent mouse serum. The cells were transfected with the pCMV.HSV-tk plasmid using Metafectene. Cell viability was measured by the Alamar Blue assay on Days 3 and 7 post-transfection. Results are expressed as a percentage of mock-transfected controls not treated with GCV (C/0-G and C/60-G), respectively. Data represent the mean  $\pm$  S.D. obtained from triplicate wells.

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