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Correlation between the levels of survivin and survivin promoter-driven gene expression in cancer and non-cancer cells

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SERUM DECREASES THE SIZE OF METAFECTENE- AND GENEJAMMER-DNA COMPLEXES BUT DOES NOT AFFECT SIGNIFICANTLY THEIR TRANSFECTION ACTIVITY IN SCCVII MURINE SQUAMOUS CELL CARCINOMA CELLS

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Abstract: Cationic liposome-DNA (lipoplexes) or polymer-DNA (polyplexes) complexes have been used to deliver therapeutic genes, both *in vitro* and *in vivo*. However, gene transfer by these non-viral vectors is usually inhibited by biological milieu. A relatively high efficiency of transfection could be achieved in human oral cancer cells transfected with the polycationic liposome, Metafectene, and the polyamine reagent, GeneJammer, in the presence of 60% fetal bovine serum (FBS) (Konopka *et al.,* **Cell. Mol. Biol. Lett.** 10 (2005) 455-470). Here, we examined the efficacy of these vectors to deliver *β*–galactosidase (*β*-gal), luciferase and Herpes Simplex Virus thymidine kinase (HSV-*tk*) genes to SCCVII murine squamous cell carcinoma cells, which are used to generate an orthotopic murine model of oral cancer. We also evaluated the hydrodynamic size and zeta potential of the vectors and the effect of FBS and mouse serum (up to 60%) on the size of Metafectene and GeneJammer complexes with the pCMV.Luc plasmid. Our results indicate that Metafectene and GeneJammer are highly effective in transfecting SCCVII cells. Approximately 60-70% of SCCVII cells transfected with pCMV.lacZ were positive for *β*-gal staining. The expression of *β*-galactosidase was essentially not affected by serum. Mouse

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Abbreviations used: *β*-Gal - *β*–galactosidase; DMEM - Dulbecco's modified Eagle's MEM medium; FBS - fetal bovine serum; GCV - ganciclovir; HBS - HEPES-buffered saline; HNSCC - head and neck squamous cell carcinoma; HSV-*tk* - Herpes Simplex Virus thymidine kinase gene; OSCC - oral squamous cell carcinoma; RLU - relative light units

serum (20-60%) reduced both Metafectene- and GeneJammer-mediated luciferase expression by ~30-45%, while FBS did not affect transfection efficiency. The delivery of the HSV-*tk* gene by Metafectene or GeneJammer in the presence of 0% or 60% FBS, followed by GCV treatment for 6 days, resulted in over 90% cytotoxicity. The mean diameters of the DNA complexes of Metafectene and GeneJammer decreased significantly as a function of the serum concentration. The reduction in the size of the lipoplexes and polyplexes by serum was essentially not inhibitory to transfection of SCCVII cells. This is in contrast to previous hypotheses that serum-induced decrease in the size of lipoplexes is the primary cause of serum inhibition of transfection.

Key words: Transfection, Metafectene, GeneJammer, Serum inhibition, HSV-*tk* gene, SCCVII murine squamous cell carcinoma cells

INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC) afflicts about 50,000 new patients each year in the U.S. and more than 500,000 worldwide [1, 2]. Fifty two % of positively diagnosed patients have a mean survival time of only five years [3], a statistic that has not changed considerably over the past 20 years. Patients who survive a first cancer of the oral cavity have a 20-fold increased risk of developing a second primary oral cancer [4]. About 67% of patients dying of oral cancer have no evidence of symptomatic distant metastases; thus control of the localized or regional disease via effective therapies is of utmost importance. Despite advances in surgery, chemotherapy and radiation of HNSCC, no major improvement in efficacy and survival has occurred. The treatments cause many side effects, including jaw pain, mouth sores, dysfunctional salivary glands, difficulties in chewing, swallowing and talking [5].

Gene therapy approaches may be able to target cancer cells while sparing normal cells, and may be useful in the treatment of recurrent disease [6-8]. Oral cancer is a particularly appropriate target for gene therapy, since direct injection of most primary and recurrent lesions is possible with this approach. Gene delivery systems include viruses and non-viral vectors. 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 [8, 9].

Synthetic cationic liposome-DNA (lipoplexes) or polymer-DNA complexes (polyplexes) provide a simple means of transferring DNA into target cells [9-11]. Cationic liposomes have been utilized in numerous studies of gene delivery *in vivo* [12-17]. It is thought that DNA delivered into the cytoplasm via lipoplexes is transported inefficiently through the nuclear membrane [18, 19]. The breakdown of the nuclear membrane during mitosis appears to be crucial to efficient entry of the transfected DNA into the nucleus [20]. Therefore, cell proliferation may be an important factor in the efficiency of gene transfer, and

rapidly dividing, transformed cells in HNSCC are likely to be more susceptible to transgene expression via non-viral vectors compared to normal, non-dividing cells.

We have focused on establishing a highly efficient and tumor-specific non-viral gene delivery system for the treatment of oral SCC tumors in immunocompetent C3H/HeJ mice. This syngeneic orthotopic murine model for HNSCC, that involves the injection of SCCVII murine squamous cell carcinoma cells in the floor of the mouth, was developed by O'Malley *et al.* [21, 22] and has been used previously in evaluating the efficacy of immunomodulator genes delivered by adenoviral vectors [23, 24]. The delivery of the murine interleukin-2 (IL-2) gene via cationic liposomes in combination with the murine IL-12 gene via a polymeric vector also resulted in significant antitumor effects compared to the individual cytokine gene treatments and no treatment [25].

As a primer to investigating the use of non-viral vectors in an animal model of HNSCC, we examined the effect of high concentrations (20-60%) of fetal bovine serum (FBS) and mouse serum on the delivery of luciferase, β-galactosidase and Herpes Simplex Virus thymidine kinase (HSV-*tk*) genes to SCCVII cells, by the polycationic liposome, Metafectene, and the polyamine reagent, GeneJammer. We also evaluated the physicochemical properties of Metafectene and GeneJammer complexes with DNA in terms of their size and zeta potential. We determined the effect of serum on the size of Metafectene and GeneJammer complexes with the pCMV.Luc plasmid. We then examined whether the HSV-*tk* gene delivered in the presence of high concentrations (up to 60%) of serum, could induce cell death following treatment with GCV. Some of our results have been presented earlier in preliminary form [26].

MATERIALS AND METHODS

Materials

Metafectene^{TM}, a polycationic liposomal transfection reagent, containing a polyamine-lipid and dioleoyl phosphatidylethanolamine (DOPE), was purchased from Biontex Laboratories GmbH (Munich, Germany). GeneJammer transfection reagent, a proprietary formulation of polyamine and other components in 80% ethanol, was obtained from Stratagene (La Jolla, CA). Alamar Blue dye (alamarBlue^{TM}) was purchased from Biosource International, Inc. (Camarillo, CA). The β-Gal Staining Kit was obtained from Invitrogen (San Diego, CA). The penicillin, streptomycin and L-glutamine solutions were obtained from Irvine Scientific (Santa Ana, CA). Ganciclovir (GCV) was a gift from Hoffmann-La Roche, Inc. (Nutley, NJ).

Plasmids

The plasmids, pCMV.Luc (VR-1216; a gift of Dr. P. Felgner (Vical, San Diego, CA)) encoding luciferase, and pCMV.lacZ (Clontech, Palo Alto, CA) encoding β-galactosidase, were used for evaluating transfection efficiency. The plasmid pUMVC1-*tk* (previously designated pNGVL1-*tk*) expressing thymidine kinase

was obtained from the National Gene Vector Core Laboratory at the University of Michigan (Ann Arbor, MI) and propagated by Aldevron (Fargo, ND) (endotoxin level: <100 EU per mg).

Physicochemical characterization of Metafectene and GeneJammer complexes with DNA

Complexes were characterized with respect to their size and zeta potential using a Coulter N4 Plus Submicron Particle Analyzer and a Coulter DELSA 440 (Coulter Corporation, Miami, FL), respectively. The Coulter N4 Plus uses a photon correlation spectroscopy-based technique, employing autocorrelation spectroscopy of scattered laser light to determine its time-dependent fluctuations resulting from the Brownian motion of particles in suspension. The detection angle was fixed at 90° with 5 min of calibration and 7 min of run time. The mean particle size of complexes was determined, both in the absence and the presence of serum. Complexes of Metafectene and GeneJammer with the pCMV.Luc plasmid were prepared in DME medium. Metafectene was used at 2 µl:1 µg or 4 µl:1 µg DNA and GeneJammer at 3 µl:0.5 µg or 6 µl:0.5 µg DNA. Where indicated, increasing concentrations of serum were included in the medium. To evaluate if the presence of serum affected measurements of the size distribution obtained by the light-scattering method, the size of control latex beads was determined in the presence of serum. The size distribution was also determined for DME medium alone containing 0, 20, 40 and 60% FBS or mouse serum. Measurements were carried out in duplicate 15 min after preparation of complexes. The Delsa 440 is a laser-based multiangle particle electrophoresis analyser that measures the electrophoretic mobility and zeta potential distribution simultaneously with the hydrodynamic size of particles in suspension. Complexes of Metafectene and GeneJammer with the pCMV.Luc plasmid were prepared in HEPES-buffered saline (HBS) (20 mM HEPES pH 7.4 and 100 mM NaCl). Samples of the prepared complexes were placed in the measuring cell, whose position was adjusted to cover a previously determined stationary level, and an electronic current of 3.0 mA was applied. Measurements were recorded and the zeta potential was calculated for each scattering angle (8.7°, 17.4°, 26.8° and 34.7°); the values represent the mean from the different angles of two measurements. Measurements were carried out 15 min after preparation of complexes.

Cell culture and transfection

SCCVII is an aggressive murine squamous cell carcinoma cell line that was established from the squamous cell carcinoma that developed spontaneously in C3H/HeJ mice and has been propagated subsequently in vitro [27]. For our studies, SCCVII cells were kindly provided by Drs. D. Li and B. O'Malley (University of Pennsylvania, Philadelphia, PA). The cells were cultured in Dulbecco's modified Eagle's MEM (DMEM) (Irvine Scientific) supplemented with 10% 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, TX). For transfection, between 1.0-1.8 x 10^5 cells were seeded in 1 ml of DMEM in 48-well culture plates one day before transfection, and used at approximately 60-80% confluence. Cells were pre-washed 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 100 µl 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. Metafectene or GeneJammer complexes with DNA 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. For Metafectene and GeneJammer, the optimal ratio of transfection reagent to DNA was determined as described previously [28]. It was not possible to determine a +/- (cationic component/DNA) charge ratio for these reagents, since the chemical structures, the concentration of the cationic component and the molecular charges have not been disclosed by the manufacturers. When transfection was performed in the presence of serum, FBS or mouse serum was added to the cells to a final concentration as indicated. In experiments involving high concentrations of serum during the 4-h transfection, the protocol necessitated the use of 30% FBS in the subsequent incubation phase.

Transfection efficiency and activity

Transfection efficiency was examined by β-Gal staining as described previously [29]. Luciferase activity was assayed 48 h after transfection, using the Luciferase Assay System (Promega, Madison, WI), and a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA). The data were expressed as relative lights units (RLU) per ml of cell lysate. These values are designated “transfection activity”. The protein content of the lysates was measured by the DC Protein Assay reagent (Bio-Rad, Hercules, CA) using bovine serum albumin Fraction V (Sigma) as the standard.

Cell viability assay

Cell morphology was evaluated by inverted phase contrast microscopy at 25x magnification. The number of viable cells used for the experiments was determined by Trypan Blue exclusion. Cell viability was quantified by a modified Alamar Blue assay [30, 31]. Cell viability (as a percentage of mock-treated control cells) was calculated according to the formula [(A_{570} - A_{600}) of test cells] x 100 / [(A_{570} - A_{600}) of control cells].

Ganciclovir-mediated cytotoxicity

Cells transfected with the HSV-*tk* plasmid were incubated in the presence of GCV (20 µg/ml) for the indicated periods of time. Cell viability was quantified as described above. After removal of the Alamar Blue/medium mixture, fresh DMEM/10 medium with GCV was added and cells were incubated further at

37°C. Mock-transfected cells incubated either in the presence or the absence of GCV served as controls.

Statistical analysis

The statistical evaluation was performed by the unpaired Student's t-test, using StatView software (BrainPower, Inc., Calabasas, CA). A probability value (P) of less than 0.05 was considered significantly different.

RESULTS

The effect of serum on Metafectene- and GeneJammer-mediated gene transfer to SCCVII cells

For many transfection reagents, the presence of serum can interfere with gene delivery and transfection efficiency [28, 32-38]. We examined the effects of FBS or mouse serum on transfection of SCCVII cells by the polycationic liposome, Metafectene, and the polyamine reagent, GeneJammer. SCCVII cells were chosen since they are employed in the generation of OSCC tumors in an oral cancer model in C3H/HeJ mice [21, 22]. The ratios of transfection reagent/DNA (v/w) were selected based on the manufacturers' recommendations. Experiments were performed under the optimal conditions. It was not our intention to compare Metafectene and GeneJammer under identical conditions. Moreover, this would have been impossible, given the completely different nature of the two reagents. Metafectene was used at 2 µl:1 µg or 4 µl:1 µg DNA and GeneJammer at 3 µl:0.5 µg or 6 µl:0.5 µg DNA with the reporter pCMV.Luc plasmid. The optimal conditions for SCCVII cells were 2 µl Metafectene:1 µg DNA per well, and 3 µl GeneJammer:0.5 µg DNA per well (data not shown), and the subsequent experiments were performed under these conditions. SCCVII cells were readily transfectable and the expression of luciferase was very high, both with Metafectene and GeneJammer. The luciferase activity was strongly dependent on the number of cells seeded before transfection and the cell confluency at the time of transfection. Both with Metafectene and GeneJammer, the average value of luciferase activity (RLU/ml) in the absence of serum was $1{,}13 \times 10^6 \pm 0.16 \times 10^6$, but in some experiments the values were even higher, in the range of $4\text{-}6 \times 10^6$ RLU/ml. The high transfection efficiency was also confirmed by β-gal staining. When SCCVII cells were transfected with the pCMV.lacZ plasmid using Metafectene or GeneJammer, approximately 60-70% of the cells were positive for β-gal staining. The β-galactosidase expression was essentially not affected by FBS or mouse serum (up to 60%) (data not shown). The expression of luciferase was also examined as a function of serum concentration. Mouse serum (20-60%) reduced both Metafectene- and GeneJammer-mediated luciferase expression by ~30-45%, while FBS did not affect transfection efficiency. It should be noted that the inhibitory effect of mouse serum was essentially independent of its final concentration (Tab. 1). When compared to mock-transfected controls, both Metafectene- and

GeneJammer-mediated transfection with pCMV.Luc plasmid did not result in the reduction of cellular protein (data not shown).

Tab. 1. The effect of fetal bovine serum and mouse serum on luciferase gene expression by SCCVII murine squamous cell carcinoma cells transfected with Metafectene- or GeneJammer-complexed pCMV.Luc plasmid.

Serum	Transfection reagent	% Serum			
		0	20	40	60
FBS	Metafectene	100.0 ± 4.3	126.1 ± 4.9[x]	102.2 ± 12.1	88.4 ± 12.4
FBS	GeneJammer	100.0 ± 7.6	118.2 ± 14.5	88.7 ± 6.8	99.1 ± 3.9
Mouse	Metafectene	100.0 ± 15.1	30.7 ± 2.4[x]	41.6 ± 7.8[x]	30.8 ± 7.0[x]
Mouse	GeneJammer	100.0 ± 7.3	45.9 ± 8.7[x]	46.9 ± 2.5[y]	35.4 ± 7.0[y]

The actual values of luciferase activity (RLU/ml) for 0% serum are given in the text. Values represent means ± S.D. from two separate experiments performed in triplicate, expressed as percentage of luciferase activity (RLU/ml) obtained in the absence of serum. Values obtained in the presence of 20, 40 and 60% serum significantly different from the control: x - ($P<0.005$); y - ($P<0.0005$).

The effect of serum on Metafectene- and GeneJammer-mediated delivery of HSV-*tk* "suicide gene" to SCCVII cells

We next investigated the effect of serum on the expression of the HSV-*tk* "suicide gene" in SCCVII cells. Delivery of HSV-*tk* to target cells results in expression of viral thymidine kinase, which selectively phosphorylates GCV. Monophosphorylated GCV is further phosphorylated by cellular enzymes into an active triphosphate compound. The end product is incorporated into cellular DNA causing chain termination and inhibition of DNA replication, thus leading to a cytotoxic effect and cell death. The delivery of the HSV-*tk* gene to SCCVII cells by Metafectene in the absence of serum, followed by GCV treatment for 3 and 6 days, resulted in ~68 and 100% GCV-mediated cytotoxicity, respectively. When transfection was performed in the presence of 60% FBS, about 36 and 93% cytotoxicity was determined after 3 and 6 days, respectively (Fig. 1A). GeneJammer-mediated delivery of the HSV-*tk* gene to SCCVII cells, followed by GCV treatment for 3 and 6 days, resulted in ~60% and 100% cytotoxicity, respectively, both in the absence and in the presence of 60% FBS (Fig. 1B).

To mimic the effect of physiological, or *in vivo*, conditions on gene transfer, the delivery of the HSV-*tk* gene to SCCVII cells was also performed in the absence or presence of 60% mouse serum. In the absence of serum, followed by GCV treatment for 3 and 6 days, Metafectene-mediated transfection resulted in ~60 and ~88% GCV-mediated cytotoxicity, respectively. When transfection was performed in the presence of 60% mouse serum, about 32 and 76% cytotoxicity was determined after 3 and 6 days, respectively (Fig. 2A). GeneJammer-mediated delivery of the HSV-*tk* gene to SCCVII cells, followed by GCV

treatment for 3 and 6 days, resulted in ~45% and ~90% cytotoxicity, respectively, both in the absence and the presence of 60% mouse serum (Fig. 2B).

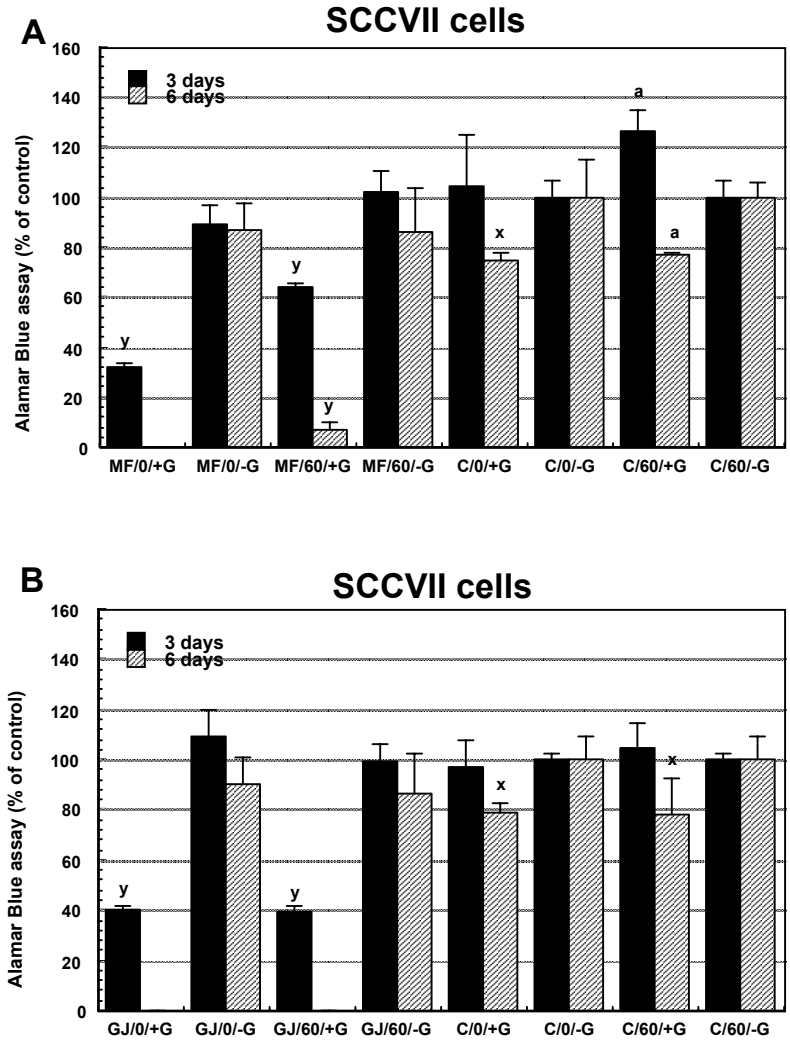

Fig. 1. The effect of 60% FBS on the sensitivity to GCV of SCCVII cells transfected with HSV-*tk* using Metafectene (MF) (A) or GeneJammer (GJ) (B). The cells were transfected with 2 µl Metafectene and 1 µg plasmid DNA or with 3 µl GeneJammer and 0.5 µg plasmid DNA. Cell viability was measured by the Alamar Blue assay on days 3 and 6 post-transfection. Results are expressed as a percentage of mock-transfected controls not treated with GCV (C/0/-G and C/60/-G). Data represent the mean ± S.D. obtained from triplicate wells, and are representative of two independent experiments. a - significantly different from the control ($P<0.01$); x - ($P<0.05$); y - ($P<0.0005$).

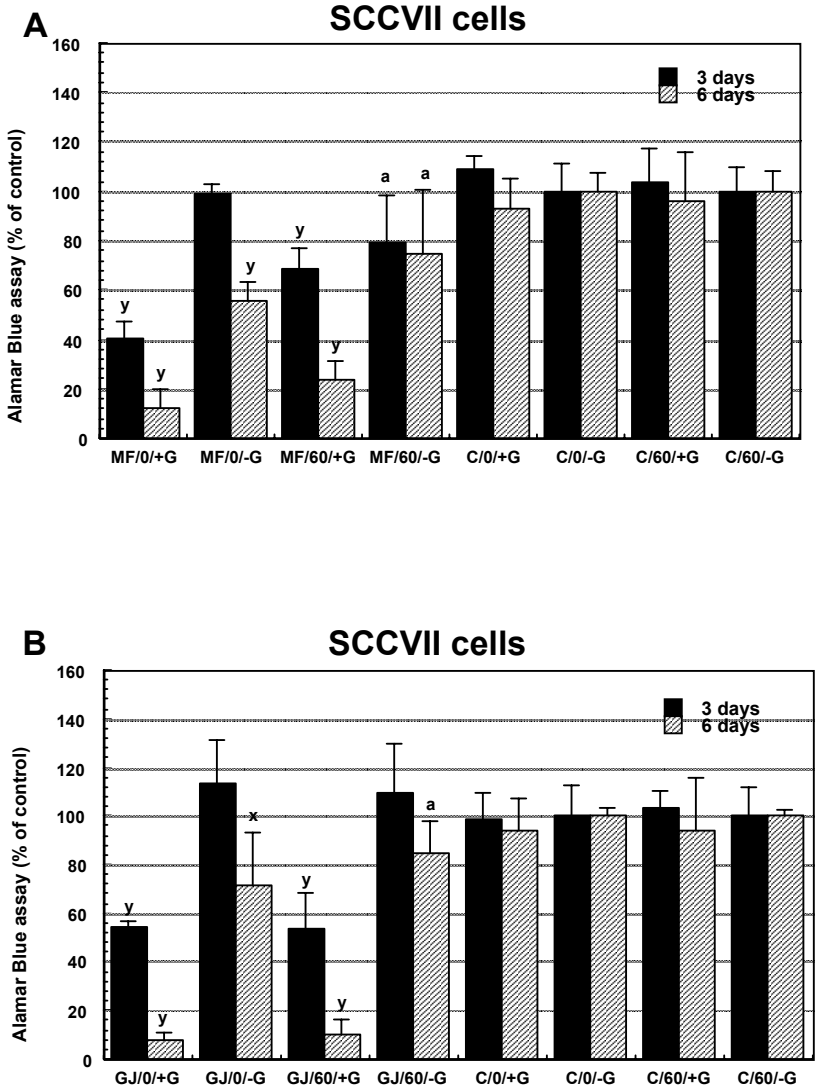

Fig. 2. The effect of 60% mouse serum on the sensitivity to GCV of SCCVII cells transfected with HSV-*tk* using Metafectene (MF) (A) or GeneJammer (GJ) (B). The cells were transfected with 2 µl Metafectene and 1 µg plasmid DNA or with 3 µl GeneJammer and 0.5 µg plasmid DNA. Cell viability was measured by the Alamar Blue assay on days 3 and 6 post-transfection. Results are expressed as a percentage of mock-transfected controls not treated with GCV (C/0/-G and C/60/-G). Data represent the mean ± S.D. obtained from triplicate wells, and are representative of two independent experiments. a - significantly different from the control ($P<0.025$); x - ($P<0.005$); y - ($P<0.0005$).

In the experiments performed with FBS, Metafectene and GeneJammer were not toxic in the absence of GCV. In the presence of GCV some cytotoxicity was observed in the mock-transfected cells after 6 days ($P<0.05$ compared to -GCV controls) (Fig. 1A, B). For SCCVII cells transfected at 0% or 60% mouse serum, a cytotoxic effect of transfection was observed after 6 days in the cells not treated with GCV. For Metafectene this effect was higher at 0% serum ($P<0.0005$ compared to mock-transfected cells -GCV) than at 60% serum ($P<0.025$). GeneJammer-mediated transfection was less toxic ($P<0.005$ at 0% and $P<0.025$ at 60% mouse serum). In these experiments, no obvious cytotoxicity was observed in control wells (Fig. 2A, B). For each condition the transfection experiment was performed in triplicate wells and it was repeated at least two times as indicated in the figure legends. While there was little variability among the triplicates in a particular experiment, there was noteworthy variability between different experiments. It is likely that the fast growth rate of SCCVII cells and their tendency to overgrow the culture wells during the long incubations (6 days), contribute to this variability. Nevertheless, GCV treatment resulted in a highly significant increase in toxicity compared to cells not treated with GCV.

Effect of serum on the size of Metafectene and GeneJammer complexes with DNA

The zeta potentials and mean diameters of Metafectene and GeneJammer complexes with pCMV.Luc are shown in Tab. 2. The overall charge of the complexes was negative. Complexes prepared from GeneJammer exhibited a surface charge close to neutrality. Surprisingly, a more negative zeta potential was observed when Metafectene/DNA complexes were prepared with a higher amount of the cationic reagent (Tab. 2). One possible explanation is that the increase in Metafectene reagent (from 2 µl:1 µg DNA to 4 µl:1 µg DNA) caused structural alterations in the lipoplex, which enabled the additional cationic charge to be neutralized by some of the DNA that was not exposed previously. The resulting particles, then exposed an equivalent negative charge at the shear plane of the hydrodynamic particle, as observed by the electrophoretic mobility measurements. It is interesting to note that particle size did not change appreciably from one charge ratio to the other (Tab. 2).

For liposomes containing the cationic lipid DC-6-14, cholesterol and DOPE (1:7.5:7.5 molar ratio), increasing the serum concentration up to 100% results in a significant decrease both in lipoplex size and lipofection efficiency [37]. We therefore investigated the effect of serum on the size of complexes of pCMV.Luc with Metafectene or GeneJammer. We first assessed the potential contribution of serum itself to size determination. Samples of 160 µl of DME containing 0, 20, 40 and 60% FBS were diluted in 3 ml water. Under these conditions, a mean particles size of 8 nm was obtained for DME/60% FBS, while the particle counts at 20 and 40% FBS were too low to produce size measurements (data not shown). Subsequently, the mean diameter of control

latex beads was determined in the absence or the presence of serum to evaluate the contribution of serum to the size determination of standard particles of defined and constant size. DME medium with 0, 20, 40 and 60% FBS or mouse serum in a volume of 160 µl were diluted in 3 ml water before adding the control beads of 500 nm. As shown in Figure 3, the increase in serum concentration in the medium resulted in a linear decrease in the measurements obtained for the control beads. A strong linear correlation was found between the concentration of FBS (R^2=0.998) and the mouse serum (R^2=0.956), and the results. At 60% FBS and mouse serum, the apparent mean diameter of 500 nm control beads was

Tab. 2. The zeta potential and mean diameter of Metafectene and GeneJammer complexes with DNA

Complexes	Zeta potential (mV)	Mean diameter (nm)
Metafectene/DNA (v/w) (2:1)	- 23.4 ± 3.9	2561 ± 477
Metafectene/DNA (v/w) (4:1)	- 30.7 ± 5.2	2327 ± 685
GeneJammer/DNA (v/w) (3:0.5)	- 8.3 ± 1.8	370 ± 40
GeneJammer/DNA (v/w) (6:0.5)	- 3.5 ± 1.4	571 ± 206

Complexes were prepared as described in Material and Methods. The size distribution of the complexes was assayed in DME medium in the absence of serum. The zeta potential of the complexes was measured in HBS. Values represent means ± S.D. and are representative of two separate experiments performed in duplicate.

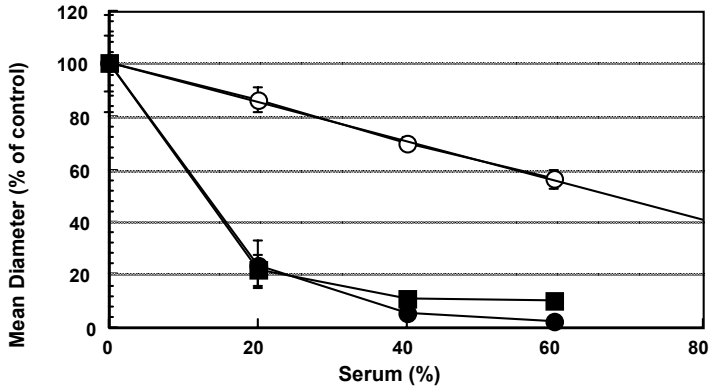

Fig. 3. Effect of FBS concentration on the size of complexes containing Metafectene (filled circles) or GaneJammer (filled squares) and the pCMV.Luc plasmid, and on the mean diameter of control latex beads (open circles). The 100% values the latex beads, Metafectene lipoplexes, and GeneJammer polyplexes were 501 ± 8, 2561 ± 477, and 370 ± 40 nm, respectively. Data represent the mean ± S.D. obtained for two measurements and are representative of two independent experiments.

in the range of 281 ± 17 and 289 ± 8 nm, respectively, which reflects a reduction to ~58 ± 2% of controls in the absence of serum. Thus, the presence of serum affected the apparent size distribution of the control particles. When measurements were performed with Metafectene- or GeneJammer-DNA complexes, the decrease in the mean size of the complexes in the presence of FBS was considerably larger than that of the control beads (Fig. 3). The mean sizes of the 2 µl Metafectene:1 µg DNA and 3 µl GeneJammer:0.5 µg DNA complexes were 2561 ± 477 nm and 370 ± 40, respectively (Tab. 2). At 60% FBS, the mean diameters of the Metafectene/DNA and GeneJammer/DNA complexes were 39 ± 2 nm and 38 ± 8 nm, respectively. This reflects a reduction of 98.5% for Metafectene and 90% for GeneJammer. Although in the absence of serum the mean size of Metafectene-containing complexes was much larger than that of GeneJammer, extensive condensation in the presence of 60% FBS resulted in a similar, small size of complexes. Similar results were obtained with complexes at different reagent:DNA ratios (Metafectene, 4 µl:1 µg DNA; GeneJammer, 6 µl:0.5 µg DNA; data not shown). Similar results were also observed with mouse serum, but the percentage of size reduction was lower than that with FBS. The starting sizes of the 2 µl Metafectene:1 µg DNA and 3 µl GeneJammer:0.5 µg DNA complexes were 1308 ± 244 nm and 868 ± 83 nm, respectively. For these complexes, the percentage of size reduction was ~70% and ~60%, respectively, independent of the concentration of mouse serum (20, 40 and 60%). The observation that the inhibitory effect of serum for transfection of SCCVII cells was very limited, if any, especially in the case of FBS, suggests that the lipoplexes or the polyplexes were not dissociating under these conditions. If they were to dissociate, the complexes would have lost their ability to transfect the cells. This argument points to condensation being the cause of reduced particle size. The decrease in the transfection activity with mouse serum my indeed be caused by dissociation. It should be pointed out, however, that under these conditions the transfection efficiency (measured by *β*-gal expression) was not affected appreciably.

DISCUSSION

Non-viral gene therapy has been gaining increased interest, because it can overcome some problems inherent to viral therapies, including immune and toxic reactions and the potential for viral recombination [8, 9]. Non-viral gene delivery systems, however, also have several limitations: (i) they can be toxic; (ii) they have a relatively large particle size; (iii) their transfection efficacy is usually low and often reduced by serum [28, 32-38] (iv) successful transfection is strongly cell-type dependent [9-11, 19, 28, 29, 39, 40]. Using two novel transfection reagents, Metafectene and GeneJammer, we showed previously a significant level of HSV-*tk*/GCV-mediated cytotoxicity in HSC-3 human OSCC cells, in the presence of 60% FBS. In contrast, very low transfection activity and a much higher inhibitory effect of serum were observed in a different OSSC cell line, H357 [28]. The reasons for the various

susceptibilities to transfection displayed by relatively comparable cells derived from OSCC tumors are basically unknown. The rationale might be quite complex, including differences in endocytosis, intracellular transport and transcription activity, and in the experimental conditions (including size and charge ratio of transfection vectors, cell confluency and time of transfection) [39-43]. Here, we examined Metafectene- and GeneJammer-mediated transfection in murine SCCVII cells that are used to generate a model of oral cancer in C3H/HeJ mice [21, 22].

SCCVII cells were readily transfectable in serum-free medium. This high transfection activity was not affected by FBS, while it was reduced by ~30-45% in the presence of mouse serum (up to 60%). The distinct effect of various sera on transfection efficiency has been reported and it has been suggested that mouse serum more accurately mimics human serum conditions compared to FBS [38, 44]. Li *et al.* [44] reported that the addition of mouse serum to DOTAP:Chol/DNA or DOTAP:DOPE/DNA lipoplexes, at a ratio 1:2 (v/v), results in immediate aggregation, followed by disintegration of lipoplexes associated with DNA release. The disintegration rate is affected by lipid composition and is dependent on the species origin of the serum. Disintegration of lipoplexes is a much slower process in either fetal bovine serum or human serum than in mouse serum [44]. It has been suggested that this might be due to the differences in the amount of active components responsible for vector disintegration, although it is not known which serum proteins are the major components responsible for disintegration. These data might explain the higher inhibition of transfection efficiency by mouse serum observed in our study. The removal of low-density lipoproteins from mouse serum does not affect its interaction with lipoplexes [44]. In contrast, Tandia *et al.* [45, 46] reported that interactions between lipoplexes and low- or high-density lipoproteins, isolated from human plasma, lead to inhibition of the transfection activity. The ability of lipoproteins to inhibit the transfection efficiency of lipoplexes is well correlated with their potential to undergo lipid mixing with the cationic lipid bilayer [46]. In addition, the effect of serum on transfection efficacy is cell-type specific [28]. For non-viral vectors to be used successfully *in vivo*, it is important to establish the molecular basis for the differences in transfection of different cell types, and the distinct effects of different sera.

Binding of serum components to lipoplexes or polyplexes *in vivo* may cause an increase in particle size and entrapment in capillary beds, poor accessibility to cells or increased clearance of complexes by the reticuloendothelial system (RES). *In vitro*, however, larger lipoplexes may result in a greater cellular association and uptake than smaller lipoplexes [32, 37, 47]. Almofti *et al.* [37] have shown that increasing the serum concentration causes a decrease in binding, uptake and lipofection efficiency, corresponding to a significant decrease in lipoplex size, using lipoplexes containing the cationic lipid DC-6-14, cholesterol and DOPE (1:0.75:7.5 molar ratio), complexed with DNA at a 1.5/1 (+/-) charge ratio. At 50 and 100% FBS the lipoplex size decreases by

87.5%. The decrease in the size of DOTAP:DOPE/DNA [47] and lipopolyamine/DNA [32] complexes observed in the presence of FBS (up to 15%) has been considered as the major determinant of the inhibitory effect of serum, and the use of larger lipoplexes to overcome the inhibitory effect of serum has been recommended. Faneca *et al.* [48] reported that the effect of serum on the size of DOTAP:Chol/DNA complex is affected both by the charge ratio and by the inclusion of human serum albumin (HSA) in the lipoplexes. For plain lipoplexes, incubation with 60% FBS for 30 minutes results in no size changes at the charge ratio 4/1 (+/-) and in a decrease by 60% for the ratio 2/1 (+/-). For HSA-lipoplexes, however, a decrease by 72% for the ratio 4/1 (+/-) is observed. The presence of serum (up to 20%) does not affect the size of DOTAP/DNA complexes at a charge ratio of 4/1 (+/-); but a substantial aggregation of liposomes alone occurs even in the presence of 3% serum [49]. In all of these studies control experiments evaluating the effect of serum on the size of latex particles were not performed.

Regarding the possibility that the inhibitory effect of serum on transfection is mediated by the serum-induced decrease in lipoplex size [32, 37, 47], we have found that the average diameters of both the Metafectene and GeneJammer complexes with DNA decrease drastically in the presence of serum. We observed a similar decrease in size for DOTAP:DOPE/DNA and DOTAP:Chol/DNA lipoplexes at a 1:1 (+/-) charge ratio in the presence of 60% FBS (K. Konopka, N. Overlid, N. Düzgünes, unpublished data). We have observed, however, that the apparent size of control latex particles also decreases, but in a linear fashion, indicating that the dynamic light scattering measurements themselves are affected by the presence of serum.

The Coulter N4 Plus Submicron Particle Analyzer determines the particle size by measuring the rate of diffusion of particles through a diluent. The rate of diffusion depends on temperature, viscosity and the refractive index of the diluent, and the particle size. The increase in the viscosity caused by serum should slow the intensity of particle fluctuations resulting in a lower diffusion coefficient, which is inversely related to the particle size according to the Stokes-Einstein equation. As a consequence, the presence of serum should result in a larger measured mean size of the control latex particles. Therefore, the observed reduction of size of the control particles is likely to be a consequence of the change in the refractive index of the diluent according to the theory. The increase of the refractive index will magnify the decay constant (Γ) and will reduce the apparent size of the control particles. An additional factor could be the binding of serum proteins to the polystyrene particles; but this is expected to increase the actual size of the particles. The small particles present in serum do not provide enough scattering for signal to be detected, since a mean particle size of 8 nm was obtained only for DME/60% FBS, while the particle counts at 20 and 40% FBS were too low to produce size measurements (data not shown). Clearly, further studies are needed to explain the apparent reduction in size of the control latex particles.

The observed effect of serum on the size of lipoplexes may be the result of a number of factors, including viscosity, refractive index, serum protein binding and the partial dissociation of the lipoplexes. While the sizes of the lipoplexes and polyplexes decrease exponentially as a function of the serum concentration, they retain substantial transfection activity in SCCVII cells. Our previous observations with Metafectene and GeneJammer using different types of cells have shown that serum is highly inhibitory in the case of H357 cells and less inhibitory in HSC-3 cells, both of which were derived from human OSCC cells [28]. It appears, therefore, that cells have different ways of dealing with lipoplexes or polyplexes of different size, assuming that the size is a major determinant of transfection [37, 47]. One interesting observation is that if the efficiency of transfection is high, as in the case of SCCVII cells, serum is not as inhibitory as for cells that are difficult to transfect (e.g. H357 cells). The fact that FBS reduces the complex size to a greater extent than mouse serum while not being as inhibitory for transfection of SCCVII cells suggest that at least for these cells the complex size is less important. These observations suggest that the serum-induced decrease in size may not be the primary cause of the serum inhibition of transfection by non-viral vectors. Thus, our results do not support the hypothesis that lipoplex size is the major determinant of *in vitro* lipofection efficiency.

Although lipoplexes with a high negative zeta potential may be expected to have a small mean diameter, conflicting results have been reported. Faneca *et al.* [43] have reported that DOTAP:DOPE/DNA complexes at a 1/1 (+/-) charge ratio exhibit a mean diameter of ~250 nm and a zeta potential of –30 mV. On the other hand, DC-6-14:Chol:DOPE/DNA complexes prepared at a 0.75/1 (+/-) charge ratio exhibit a mean diameter of 1350 nm and a zeta potential of –26 mV [42]. Judging from these data our results are not entirely unexpected. It was not possible to determine the +/- charge ratio for Metafectene/DNA complexes because the molecular structure, concentration and molecular charge of Metafectene have not been disclosed.

Our studies are aimed at establishing an efficient and tumor-specific non-viral gene delivery system for the treatment of OSCC tumors in immunocompetent C3H/HeJ mice. This syngeneic orthotopic murine model involving SCCVII cells has the histological characteristics of squamous cell carcinoma, the most common tumor in the head and neck region [21, 22]. The anatomical site and the initial regional aggressiveness of this tumor, with early direct extension into the neck and later cervical lymph nodes, and pulmonary metastases, resemble the biological behavior of tumor progression in HNSCC [22].

Viral vectors have been employed to deliver HSV-*tk* to HNSCC cells, both *in vitro* and *in vivo*. Adenovirus-mediated transfer of the HSV-*tk* gene into human laryngeal epidermoid cancer cells, HEp-2, and human hypopharyngeal carcinoma cells, FaDu, followed by exposure to GCV results in significant cell killing. Injection of the adenovirus HSV-*tk* vector (AdHSVtk) into tumors derived from FaDu and HEp-2 cells in SCID mice, followed by GCV treatment,

results in a reduction of tumor growth [50, 51]. Similar results were obtained with an adeno-associated virus HSV-*tk* vector and four human OSCC cells (SAS, HSC-2, HSC-3, HSC-4) [52]. The conjugation of AdHSVtk with cationic liposomes enhances the transduction efficiency both *in vitro* and *in vivo* [53]. Tumor regression and improved animal survival have been demonstrated in the oral cancer model described above using AdHSVtk and systemic GCV administration [54, 55]. The effect of the HSV-*tk*/GCV treatment is enhanced by the delivery of the IL-2 gene [23].

Our results indicate that with the non-viral vectors, Metafectene and GeneJammer, extensive HSV-*tk*/GCV-mediated cytotoxicity can be achieved in SCCVII cells even in the presence of high concentrations of FBS or mouse serum. We are currently working on the translation of our *in vitro* results with SCCVII cells to the immunocompetent murine OSCC model.

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