Quiescence, cell viability, apoptosis and necrosis of smooth muscle cells using different growth inhibitors

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Abstract. Smooth muscle cells and endothelial cells play an important role in cardiovascular diseases and may therefore be a potential target for gene therapy. Most *in vitro* experiments are performed using proliferating cell cultures. Nevertheless, non-dividing cells would represent more realistic *in vivo* conditions for gene therapy. Therefore, a simple method to achieve physiologically quiescence in cell cultures is needed for experiments. Growth to confluence is sufficient for endothelial cells to reach quiescence, in contrast to smooth muscle cells. Alternative techniques were investigated to achieve quiescence for smooth muscle cells. N-acetyl-cysteine, heparin, aphidicolin and serum-free medium are known inhibitors of smooth muscle cell proliferation and were tested for cell viability, necrosis and apoptosis. The inhibition status was evaluated counting cells in a cell counter. Toxicity, necrosis and apoptosis were determined using FACS analysis. Then, smooth muscle cells and endothelial cells were transfected with plasmid containing the β -galactosidase gene using liposomes. Analysis of gene expression in transfected cells included a quantitative β -galactosidase assay and X-gal staining. Growth inhibition was achieved with all agents tested. Using N-acetyl-cysteine, only slightly reduced growth rates were observed. Aphidicolin stopped cell growth almost immediately, but demonstrated enhanced toxicity. The amount of apoptotic and necrotic cells was lowest using heparin in the presence of foetal calf serum. Transfection experiments using stationary cultures of smooth muscle cells using heparin or aphidicolin demonstrated 5–10-fold lower transfection rates compared to transfected proliferating cell cultures serving as controls. Transfection experiments using stationary cultures of endothelial cells using growth inhibition through confluence demonstrated 40-fold lower transfection rates than transfected proliferating cell cultures. Transfer efficiency was much lower in endothelial cells compared to smooth muscle cells. In conclusion, quiescent cells simulate more realistically the *in vivo* situation and may therefore represent a better model for future *in vivo* experiments based on *in vitro* findings.

INTRODUCTION

The vessel wall, endothelial cells (ECs) and smooth muscle cells (SMCs) play an important role in a number of cardiovascular disorders (Shi *et al.* 1996). They may therefore serve as a potential target for the treatment of such disorders, including gene therapy (Huehns *et al.* 1999; Nikol *et al.* 1999; Yla-Herttuala & Martin 2000). SMCs in intact vessel walls *in vivo* exist mainly in a quiescent state. The proliferative index of SMCs in primary arteriosclerotic lesions is $3.6 \pm 3.5\%$ (Isner *et al.* 1994, 1996).

This is comparable to the proportion of proliferating cells in stationary cultures of SMCs (1–3%). SMCs do not demonstrate increased proliferative activity unless it is induced by stimuli such as vessel injury at angioplasty, including balloon dilatation or stent implantation. This excessive proliferation results in what is termed restenosis, or renarrowing, of the affected segment of the vessel. Gene transfer that aims to target SMCs and prevent restenosis, however, is preferentially conducted during the same sitting, directly following the angioplasty intervention. At this time point, most SMCs are quiescent; proliferation only reaches its maximum at around 7 days post-angioplasty (Nikol *et al.* 1996). An *in vitro* model with SMCs with a similar proliferative index to that in the vessel wall immediately following angioplasty, that is, with over 95% of SMCs quiescent, would be the most appropriate when optimizing gene transfer conditions before using an experimental model. Several different methods of preventing cell proliferation have been described. One of the most commonly used techniques is the withdrawal of foetal calf serum (FCS) (Wilke *et al.* 1996). However, this technique is not suitable for all cell types, particularly for some cells in primary culture, because the medium may have significant cytotoxicity in the absence of the growth factors from the FCS. Another common way of achieving quiescence is growth to confluence. This seems to work better for ECs than for SMCs. Several growth inhibitory agents that are easy to handle have previously been compared with withdrawal of FCS and growth to confluence for the prevention of SMC and EC proliferation (Dethlefsen *et al.* 1994; Matsuno *et al.* 1995; Ranganna *et al.* 1995; Escobales *et al.* 1996; Igarashi *et al.* 1997; Raiteri *et al.* 1997). N-acetyl-cysteine (NAC) may halt cell growth in a reversible manner. Removal of NAC stimulates SMCs to re-enter the cell cycle synchronously (Lee *et al.* 1998). Another antiproliferative agent is the aphidicolin with antiviral and antimitotic properties produced by *Nigrospora sphaerica* or *Cephalosporium aphidicola* (Starratt & Loschiavo 1974; Spadari *et al.* 1985). This antibiotic specifically inhibits DNA polymerase α , responsible for DNA replication. Heparin, a glycosaminoglycan, that is synthesized by subendothelial cells, also inhibits SMC proliferation and migration *in vitro* and *in vivo* (Buchwald *et al.* 1996; Geary *et al.* 1995; Herbert *et al.* 1996; Skaletz-Rorowski *et al.* 1996; Daum *et al.* 1997).

This paper reports the establishment of quiescent cultures of SMCs and ECs with a proliferation activity comparable to what is found in arterial wall cells *in vivo* with a maximum of cell viability and minimum of apoptosis or necrosis.

MATERIALS AND METHODS

Cell culture

Primary SMCs and ECs were isolated from explants of porcine aortic vessels and cultured in medium [HAM:Waymouth = 1:1; HAM (Biochrom, Berlin, Germany); Waymouth (Gibco, Eggenstein, Germany)] with 10% FCS (Gibco, Eggenstein, Germany), 4 mM glutamine, 100 U penicillin,

100 µg/ml streptomycin and 2.5 mg/ml amphotericin B (Biochrom, Berlin, Germany) as previously described (Nikol *et al.* 1999). Both cell types were used during the first four passages. The outgrowing SMCs were tested by immunohistochemistry for α -actin expression (Huehns *et al.* 1999). The typical cobblestone appearance of endothelial cells was seen.

Inhibition assays

100 000–150 000 cells were seeded into six well plates or 50 000–70 000 cells into 12 well plates overnight in serum-free medium supplemented with 10% FCS. Primary SMCs and ECs were grown to confluence. In addition, SMCs were incubated with different concentrations of heparin (100–1000 µg/ml) (B. Braun, Melsungen, Germany) and aphidicolin (0.1–5 µg/ml) (ApC; Sigma, Deisenhofen, Germany), 10 mM N-acetyl-cysteine (NAC; Sigma, Deisenhofen, Germany) or FCS (0–20%) for growth inhibition assays. Cell growth was monitored by triplicate counting of the cells in suspension with the cell analyser system (Schaerfe Systems, Reutlingen, Germany). A live gate was used for particle sizes between 9 and 35 µm.

Proliferation assays

Bromodeoxyuridine (BrdU, 10 µM final concentration; Roche, Mannheim, Germany) was added to the cell cultures 4 h before the end of the culture period to determine the number of proliferating cells. Cells were fixed according to the manufacturer's protocol and anti-BrdU-peroxidase (BrdU-POD) solution (Roche, Mannheim, Germany) was added. Two hours later the cells were washed with phosphate-buffered saline (PBS). Two different detection methods were used to determine the number of proliferating cells. For one method, the substrate solution was added according to the manufacturer's protocol (Roche) and 20 min later the reaction was stopped using 1 M H₂SO₄. The absorbance of the sample was measured in an ELISA reader (Titertek Multiscan MCC/340, EFLAB, Finland) at 450 nm. In the alternative method, the AEC substrate for POD (Vector Laboratories, Burlingame, CA, USA) was added to the cells and 20–40 min later, following the development of appropriate colour staining, the proportion of proliferating cells was determined using light microscopy and compared with the first method using an ELISA reader.

Flow cytometry

Analysis of apoptosis was performed using the Annexin-V-FLUOS staining kit (Roche, Mannheim, Germany). 10⁶ cells were trypsinized, washed with PBS and suspended in 100 µl staining solution (Annexin-V-FLUOS/Propidium iodide). Following a 15 min incubation at room temperature, 10 000 cells were analysed using the flow cytometer FACScan (Becton Dickinson, Heidelberg, Germany). Necrotic cells were distinguished from the Annexin-V positive cells by double-staining with propidium iodide.

Cell transfections

Porcine SMCs and ECs were seeded into six well plates 48 h before transfection (50 000–200 000 cells per well to achieve proliferating cells and 200 000–500 000 cells to achieve stationary cell cultures). Heparin (200 µg/ml) was added 24 h later and cells were incubated again for 24 h. Aphidicolin (1 µg/ml) was added 4 h before transfection. For transfection without serum the cells were incubated 24 h before transfection in serum-free medium. The vector pCMV β , containing the β -galactosidase gene (Clontech, Heidelberg, Germany), together with the cationic lipid DOCSPER (1,3-dioleoyloxy-2-(N⁵-carbamoyl-spermine)-propane) (Groth *et al.* 1998) or LipofectAMINE (Gibco, Eggenstein, Germany) were used for the transfection procedures. For each transfection, 0.5–5 µg plasmid-DNA and 1–12 µg liposomes were diluted in separate tubes in 100 µl of Opti-MEM (Gibco, Eggenstein, Germany), complexed and incubated for 30 min at room temperature.

The cells were washed twice with PBS and 800 µl Opti-MEM was added. Transfection complex was added drop-wise on to the cells. After 4 h incubation time at 37 °C/5%CO₂, the medium was replaced with fresh medium with 10% FCS, or with medium containing 10% FCS and heparin, 10% FCS and aphidicolin, or with serum-free medium alone.

Beta-galactosidase assay

For quantification of β-galactosidase gene expression, cells were trypsinized 48 h after transfection, counted and resuspended in 50 µl lysis buffer (100 mM potassium phosphate pH 7.8, 0.2% Triton X-100). 50 µl samples containing 100 µg protein were incubated for 2 h with 150 µl substrate solution, consisting of 1 mg/ml CPRG (Roche, Mannheim, Germany) in Hanks' balanced salt solution. The optical density was measured at 540 nm with reference at 690 nm on the ELISA reader. The activity was calculated using known β-galactosidase concentrations (Promega, Munich, Germany) and corrected for the activity of non-transfected cells.

X-gal staining

For histochemical detection of porcine SMCs and ECs expressing β-galactosidase, cells were fixed with 2.5% glutaraldehyde for 5 min, washed with PBS and incubated overnight at 37 °C with the X-gal solution (1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, 5 mM K₄[Fe(CN)₆], 5 mM K₃[Fe(CN)₆], 2 mM MgCl₂ in PBS; Roche, Mannheim, Germany) (Couffinhal *et al.* 1997). Transfection efficiency was determined by counting all blue cells per well and comparing this value to the total cell number at the time of transfection. Non-transfected cell cultures were used as a negative control.

Statistical analysis

All values are expressed as mean ± standard error of the mean (mean ± SEM) of three experiments for each condition. Multivariate ANOVA was used for comparison of differences among the groups (SPSS Inc, Chicago, IL). The statistical significance was defined as $P < 0.05$.

RESULTS

Growth inhibition and cell viability of porcine SMCs and ECs

Proliferation of porcine SMCs under different conditions is demonstrated in Fig. 1. In the presence of 10% FCS the number of SMCs in culture doubled approximately once per day (Fig. 1a). Serum-free medium led to a reduction of the number of adherent cells to almost half during the first day, with slightly decreasing cell numbers during the following days. Cells were seen to detach from the surface of the cell culture plates. The effect of NAC alone was comparable to the effect of the withdrawal of FCS with regard to proliferation activity and cell viability. In the presence of FCS, NAC (10 mM) inhibited cell proliferation in part, but was not able to stop the cell growth entirely (Fig. 1a). Higher concentrations of NAC demonstrated markedly increased toxicity (data not shown).

Aphidicolin stopped the proliferation of SMCs completely immediately following the addition of antibiotics (Fig. 1b). At a concentration of 0.1 µg/ml aphidicolin, cells were still able to replicate. At concentrations of 0.5 and 1.0 µg/ml aphidicolin, relatively stable cell numbers were detectable during the first 3 days. Concentrations of 5.0 µg/ml aphidicolin, or higher, demonstrated signs of toxicity (Fig. 1b).

In the presence of FCS and heparin, SMCs continued to multiply during the following 24 h. Inhibition occurred first at the second day. SMCs grown in medium containing 100 µg/ml heparin

Figure 1. Growth of SMCs under different conditions. (a) Medium with 10% FCS (FCS), medium with 10% FCS containing 10 mM NAC (FCS/NAC), serum-free medium containing 10 mM NAC (NAC) or serum-free medium only (serum-free) were added to cultures of growing SMCs. Cells were counted after 24 h in triplicate experiments. The effect of NAC alone on proliferation was comparable to the effect of serum-free medium. In the presence of FCS, NAC did not stop cell growth entirely. (b) Aphidicolin concentration ranging from 0.1 to 5 µg/ml inhibited cell growth in the presence of FCS (10%), with incomplete inhibition of cell growth at a concentration of 0.1 µg/ml and with toxic effects using higher concentrations than 1 µg/ml. (c) Inhibition of growing cultures of SMCs in the presence of heparin. Proliferation correlated inversely with heparin concentrations used, corresponding to a strong concentration-dependent anti-proliferative activity of heparin. At heparin concentrations of 200–300 µg/ml with 10% FCS, SMCs remained quiescent without significant toxic effects. Cells were counted after 24 h in triplicate experiments. Data are mean $\pm$ SEM.

remained alive, with an estimated 10–20% of cells still dividing. At concentrations of 200 and 300 µg/ml, the number of cells remained widely constant. At concentrations of 500 µg/ml or more, heparin was toxic and the number of surviving cells decreased after the initial growth during the first day of culture (Fig. 1c).

Figure 2. Long-term effect of heparin on primary cultures of SMCs. Cells were seeded 24 h before addition of heparin in 12 well plates in triplicates. Medium containing 10% FCS and heparin (200 or 300 µg/ml) was changed every 3 days. In the first experiment, heparin was added for 20 days (200 µg/ml heparin, diamonds; 300 µg/ml heparin, triangles). Quiescence was achieved for at least 20 days under these conditions. In the second experiment, heparin was removed at day 14 following the seeding of cells (200 µg/ml heparin, crosses; 300 µg/ml heparin, squares). The inhibitory effect of heparin was completely reversible after 14 days (values are mean $\pm$ SEM).

Figure 3. Inhibition of cell growth using heparin depends on the concentration of FCS. Primary SMCs in growth medium containing 2, 10 or 20% FCS with or without heparin (200 µg/ml) were compared for proliferation rates. With increasing concentrations of FCS, higher concentrations of heparin were necessary to stop proliferation of SMCs. Medium containing 2% FCS and heparin resulted in increased toxicity. Data are mean $\pm$ SEM with measurements performed in triplicate.

In order to induce stationary cultures for a longer period of time, medium containing FCS and heparin was changed every 3 days. Quiescence for at least 20 days was achieved under these conditions. The described effect observed using heparin was reversible after 14 days following the seeding of the cells (Fig. 2).

The inhibition of cell growth by heparin also depended on the concentration of FCS. The same amount of heparin that inhibited the growth of SMCs at 10% FCS had only a small effect on the proliferation of cells at 20% FCS. Cultures with 200 µg/ml heparin and 20% FCS had growth curves comparable with 2% FCS without heparin. The culture of SMCs in medium containing 2% FCS and heparin resulted in an increased toxicity (Fig. 3).

Figure 4. Proliferation assay for SMCs and ECs. BrdU incorporation for the determination of proliferating cells 1, 2 and 3 days after the addition of different growth inhibitors under different growth conditions. Using serum-free medium, heparin (Hep200: 200 µg/ml; Hep300: 300 µg/ml) and aphidicolin (ApC: 1 µg/ml), 0.5–3% of SMCs were proliferating compared to 30–40% of replicating cells in growth medium containing 10% FCS. Use of NAC with serum (10% FCS/10 mM NAC), 1% FCS or growth to confluence did not stop entirely the proliferation of SMCs. For ECs both serum-free medium and growth to confluence were sufficient to introduce the stationary state with a proliferating rate of 0.5–3% compared to 40–50% of replicating ECs under normal growth conditions. Data are mean ± SEM.

Growth to confluence was sufficient to inhibit proliferation of porcine ECs. Cells doubled their numbers in approximately 24 h until they reached confluence and cell–cell contact inhibition was achieved. For several days following confluence the cell number remained constant without any detectable toxic effects (data not shown).

Proliferation assays

The proliferative state of cells was also determined using the BrdU method (Fig. 4) with 20–40% BrdU-positive SMCs and 40–50% BrdU-positive ECs in growth medium with 10% FCS. $23 \pm 6\%$ of SMCs replicated in medium containing 10% FCS and NAC. Using heparin (200 and 300 µg/ml), aphidicolin (1 µg/ml) and serum-free medium, the proportion of proliferating SMCs was 1.5–3%. Using serum-free medium, only 0.5–1% of SMCs were proliferating. Of the ECs at confluence, 2–3% of cells were proliferating, in contrast to confluent SMCs, where $8 \pm 3\%$ were proliferating. Low serum concentrations, with 1% FCS, did not stop the proliferation of SMCs. There remained about 20% replicating SMCs (Fig. 4).

Toxicity, necrosis and apoptosis of SMCs and ECs

Toxicity correlates with the number of cells in necrosis and apoptosis. Therefore, the content of apoptotic and necrotic SMCs was compared in different cultures at day 3, using staining with Annexin-V-FLUOS and propidium iodide for flow cytometry analysis. The amount of apoptotic cells in growing cell cultures with 10% FCS was 0.3%, with 2.4% of the cells in necrosis (Fig. 5a). In the presence of 200 µg/ml heparin, 3.40% of SMCs were apoptotic and 0.44% cells were necrotic (Fig. 5b). In contrast, aphidicolin (1.0 µg/ml) demonstrated relatively high toxicity with 10.9% apoptotic and 24.6% necrotic cells (Fig. 5c). In cultures without FCS the number of apoptotic cells was as high as 15.7%, with 36.3% necrotic cells (Fig. 5d). The percentages of

Figure 5. Flow cytometric analysis of apoptotic cells. SMCs at day 3 were stained with the Annexin-V-FLUOS staining kit and sorted using FACS analysis. (a) Cells with 10% FCS; (b) cells cultured with 10% FCS and 200 µg/ml heparin; (c) cells with 10% FCS and 1.0 µg/ml aphidicolin; (d) cells without FCS. Vital cells are shown in the lower-left panels (Annexin-V-negative, propidium-iodide-negative), apoptotic cells are shown in the lower-right panels (Annexin-V-positive, propidium-iodide-negative) and necrotic cells are shown in the upper-right panels (Annexin-V-positive, propidium-iodide-positive). The percentage of apoptotic cells in cultures with 10% FCS and heparin was comparable to the proportion of apoptotic cells in commonly used medium with 10% FCS only. Apoptosis was far more prominent in cultures with aphidicolin and without FCS.

necrotic and apoptotic cells using different concentrations of growth inhibitors (aphidicolin, heparin, NAC and serum-free medium) are summarized in Table 1. Confluent ECs showed no significant increase in apoptotic or necrotic cells and therefore did not differ from proliferating non-confluent ECs.

Visual examination of the cell culture flasks/wells did not indicate a larger number of floating cells compared with untreated culture flasks.

Optimization of liposomal transfection in SMCs and ECs

Optimization of transfection conditions was performed to achieve highest transfer efficiencies with minimum toxicity. Transfection was optimized for lipid and DNA concentrations and the number of transfected cells. To determine the optimal concentration of liposomes for maximum transfection efficiency with a constant DNA concentration of 1 µg per well in a six-well plate with 200 000 cells, the amount of LipofectAMINE and DOCSPER was varied between 1 and 10 µg per well for proliferating and 1 and 12 µg per well for stationary cell cultures (Fig. 6). The same procedure was performed with constant concentrations of liposomes and varying concentrations of DNA. The optimized DNA/liposome ratio for proliferating SMCs was 1:6 for both LipofectAMINE and DOCSPER. Higher concentrations of liposomes were needed in stationary SMCs to achieve maximum transfection efficiencies. The optimal DNA/liposome ratio was 1:10 for both DOCSPER and LipofectAMINE. The highest transfer efficiency was achieved with a DNA/liposome ratio of 1:8 for proliferating ECs and 1:10 for stationary ECs using both liposome preparations.

Table 1. Necrosis, apoptosis and transfection efficiency of SMCs under different growth conditions at day 3 following the addition of the growth inhibitors

Growth conditions	Necrosis ^a (%)	Apoptosis ^a (%)	β -galactosidase activity ^a (mu/100 μ g protein)
<i>Porcine SMCs</i>			
FCS (10%)	2.40	0.30	430
serum-free medium	36.30	15.70	31
FCS/aphidicolin (0.1 μ g/ml)	–	–	200
(0.5 μ g/ml)	21.8	10.4	97
(1.0 μ g/ml)	24.6	10.9	90
(5.0 μ g/ml)	29.7	12.3	74
FCS/heparin (100 μ g/ml)	2.84	0.38	85
(200 μ g/ml)	3.37	0.44	63
(300 μ g/ml)	3.41	0.43	56
(500 μ g/ml)	3.56	0.45	29
FCS/NAC (10 mM)	5.96	0.93	–
NAC (10 mM)	26.40	11.14	–
<i>Porcine ECs</i>			
FCS (10%)	2.11	0.41	32
confluent cells	2.34	0.44	0.8

^aTransfection efficiency expressed as β -galactosidase activity, necrosis and apoptosis from FACS analysis.

Figure 6. Beta-galactosidase assay for the optimization of DNA/liposome ratio for SMCs and ECs using DOCSPER or LipofectAMINE. (a) Proliferating SMCs; (b) stationary SMCs; (c) proliferating ECs; (d) stationary ECs. For proliferating SMCs the optimal DNA/liposome ratio was 1:6 using both liposomes; for stationary SMCs it was 1:10. For proliferating ECs the optimal DNA/liposome ratio was 1:8; for stationary ECs it was 1:10 for both liposomes. In addition, the transfer efficiency in stationary cells was markedly lower than in the proliferating cell cultures. Data are mean $\pm$ SEM with measurements performed in triplicate.

Figure 7. Beta-galactosidase assay for the optimization of DNA/liposome ratio for different numbers of transfected proliferating SMCs and ECs. (a) SMCs and DOCSPER; (b) SMCs and LipofectAMINE; (c) ECs and DOCSPER; (d) ECs and LipofectAMINE. In all experiments, the optimal DNA/liposome ratio depended upon the amount of transfected cells. For SMCs and DOCSPER100 the ratio changed from 1:5 for 50 000 cells to 1:6 for 200 000 cells. For SMCs and LipofectAMINE the ratio increased from 1:4 for 50 000 cells to 1:6 for 200 000 cells. For ECs the DNA/liposomes ratio changed for both liposomes from 1:6 for 50 000 cells to 1:8 for 200 000 cells. Measurements were made in triplicate (mean $\pm$ SEM).

The optimization of transfection conditions to find the optimal number of cells for transfection was performed using 50 000, 100 000 or 200 000 cells in proliferating cell cultures and 150 000, 300 000 or 500 000 cells in stationary cell cultures (Figs 7, 8). In proliferating SMCs and ECs, the optimized DNA/liposome ratio increased with the number of transfected cells (Fig. 7). For SMCs, the optimized ratio changed for DOCSPER from 1:5 (50 000 cells) to 1:6 (100 000 cells) and was slightly enhanced, with a ratio of 1:8, for 200 000 cells (Fig. 7a). For LipofectAMINE the ratio was 1:4 for 50 000 cells and 1:6 for 200 000 cells (Fig. 7b). For 50 000 ECs the optimized ratio was 1:6, for 100 000 cells 1:7 and for 200 000 transfected ECs 1:8 for both liposome preparations.

There was no change of the optimal DNA/liposome ratio (1:10), independent of the number of transfected cells in stationary SMCs and ECs. In all experiments, ECs showed significantly lower transfer efficiencies compared with SMCs by a factor of 150–200 for proliferating cells and by a factor of 50–80 for stationary cells.

Transfection of proliferating versus stationary cells

Optimized conditions for liposomal transfection with pCMV β and DOCSPER or LipofectAMINE that had been established for proliferating cultures of porcine SMCs and ECs were compared in stationary cultures induced by heparin, aphidicolin or withdrawal of FCS for SMCs and growth to confluence for ECs.

Gene transfer in stationary cultures of SMCs induced by heparin or aphidicolin in the presence of serum demonstrated similar transfer efficiencies. The efficiency of transfection, quantified using a β -galactosidase assay, was 5–10 times lower in stationary SMCs and 40 times lower in EC than in the proliferating controls (Fig. 9a). The evaluation of transfer efficiencies using X-gal

Figure 8. Beta-galactosidase assay for the optimization of DNA/liposome ratio for different numbers of transfected stationary SMCs and ECs. (a) SMCs and DOCSPER; (b) SMCs and LipofectAMINE; (c) ECs and DOCSPER; (d) ECs and LipofectAMINE. In stationary cell cultures the optimal DNA/liposome ratio was independent of the amount of transfected cells. The optimal ratio stayed at 1:10 in all experiments made. Data are mean $\pm$ SEM from experiments performed in triplicate.

Figure 9. Transfection using cationic liposomes in proliferating versus stationary porcine SMC and EC cultures. Two different liposomes, DOCSPER and LipofectAMINE, were used to test gene transfer efficiencies using optimal conditions in stationary versus proliferating cultures, induced by the addition of various agents. Transfection efficiencies were quantified using β -galactosidase assay to determine enzyme activity 48 h following transfection of SMCs (a) or ECs (b). Transfection efficiencies using X-gal staining 48 h following transfection of SMCs (c) or ECs (d). The transfection efficiency using either DOCSPER or LipofectAMINE was significantly higher in proliferating than in stationary cultures. In addition, transfer efficiencies in ECs were markedly lower than in SMCs. The transfection efficiency was 150–200 times higher in proliferating SMCs than in proliferating ECs. In stationary SMCs the transfection efficiency was 50–80 times higher than in stationary ECs. Data are mean $\pm$ SEM from experiments performed in triplicate.

staining confirmed this observation (Fig. 9b). Gene transfer efficiencies using SMCs correlated inversely with the concentrations of heparin and aphidicolin used (Table 1).

DISCUSSION

Many successful gene transfection experiments performed *in vitro* fail when similar conditions are applied *in vivo*. One aim in experimental cellular biology is therefore constantly to improve established *in vitro* models to achieve working conditions as close as possible to conditions found *in vivo*. The aim of these investigations was to achieve quiescence of SMCs with a maximum of cell viability and a minimum of apoptosis or necrosis.

Our results demonstrated that growth to confluence is sufficient for ECs to achieve quiescence, with only 1–3% of proliferating cells at confluence. No toxic effects were detectable. Results are comparable with data published by other investigators (Limanni *et al.* 1988; Steward *et al.* 1990; Vinals & Pouyssegur 1999). No further methods were investigated for growth inhibition of ECs. In contrast, quiescence was much more difficult to achieve in primary SMCs. These cells are those most likely to be targeted by gene therapy in the treatment of conditions such as restenosis after angioplasty.

In the SMC culture experiments, withdrawal of FCS resulted in a very high percentage of necrotic (36.3%) and apoptotic (15.7%) cells, probably because of the absence of growth factors from the FCS that are important for cell viability and growth. In addition, cells were seen to detach from the surface of cell culture plates. This method may therefore not be a suitable one to reduce replication *in vitro* when experiments to optimize therapeutic gene transfer are carried out. We found that growth to confluence did not result in efficient inhibition of cell proliferation. About $8 \pm 3\%$ of SMCs continued to replicate, building the characteristic 'hill and valley' pattern, formed cords and grew on top of each other in a similar manner to that described by other investigators (Ricciardelli *et al.* 1989). In addition, analysis of cell transfection efficiency is difficult at this stage. Different methods and reagents were therefore investigated with the aim of achieving complete growth inhibition of primary smooth muscle cells, avoiding detachment of cells from the surface, as well as the 'hill and valley' patterns, whilst featuring minimal toxicity, apoptosis and necrosis.

Experiments using NAC (10 mM) in the presence of FCS resulted in only minimally reduced growth rates. This is in contrast to results reported by Lee and others (Lee *et al.* 1998). NAC has been shown to inhibit the NF- κ B/Rel activity which is essential for bovine and human aortic SMC proliferation (Lee *et al.* 1998). On the other hand, NF- κ B/Rel has no effect on the viability or growth of aortic endothelial cells (Lee *et al.* 1998). In the experiment described here, porcine SMCs were used. It is probable that the NF- κ B/Rel activity cannot be stopped using NAC, as has been found for endothelial cells. In contrast, when FCS had been removed as well, there was not only a reduction in proliferation, but also a reduction in total cell numbers detectable. This appeared to be secondary to a toxic reaction caused by the withdrawal of serum. The use of NAC is not suitable for the induction of stationary cultures of porcine SMCs for transfection experiments because of its inefficiency in the presence of serum.

Using aphidicolin, there was complete inhibition almost immediately following the addition of antibiotics at concentrations of 0.5 μ g/ml and more. Aphidicolin binds DNA-polymerase α and inhibits DNA replication at different stages of the cell cycle. Cells with DNA not synthesized completely and no possibility to do so in a certain period of time often undergo apoptosis or necrosis (Cotter 1992). This may explain the high toxicity of aphidicolin, resulting in significant amounts of necrotic and apoptotic cells.

In contrast to the other methods used, there was almost complete inhibition of proliferation of porcine SMCs when heparin was added, with no or minimal toxic effects over a wide range of concentrations (100–500 µg/ml). At concentrations of 200–300 µg/ml, also in the presence of serum (10% FCS), SMCs remained quiescent without significant necrosis or apoptosis. The inhibitory effect of heparin is not mediated by a single cellular process. Heparin can bind to many growth factors such as basic fibroblast growth factor (bFGF) and transforming growth factor- β (TGF- β) or to their receptors (Skaletz-Rorowski *et al.* 1996), reducing the expression of certain proto-oncogenes (c-fos, c-myc) (Herbert *et al.* 1996). In addition, it may influence the inhibitory properties of certain matrix-degrading proteases and inhibit the mitogen-activated protein kinase (MAPK) and mitogen-activated protein kinase kinase (MAPKK) activity (Daum *et al.* 1997), as well as affecting the protein kinase C (PKC)-signal pathway.

Several possible methods of transferring genes into cells exist. Retroviral, adenoviral or non-viral gene transfer using cationic liposomes have commonly been used (Simoes *et al.* 1999; Yla-Herttuala & Martin 2000). The advantages of using liposomal rather than viral gene transfer include easy handling and low immune responses. Transfection using cationic lipids can result in transfection efficiencies as high as 90% in proliferating cell cultures, depending on the target cell type and liposomes used (Bebok *et al.* 1996; Egilmez *et al.* 1996; Zhang *et al.* 1997), whereas transfer rates achieved *in vivo* can be as low as 1% (Egilmez *et al.* 1996; Laitinen *et al.* 1997; Nikol *et al.* 1999). Transfer efficiency is particularly compromised by the cell proliferation status (Wilke *et al.* 1996).

To achieve high transfer efficiency using liposomes, optimization should be carried out *in vitro* for each targeted cell type (Armeanu *et al.* 2000). Optimization of liposomal gene transfer depends on DNA/liposome ratios, cell type, liposomes used and the state of cell proliferation. In this study, the *in vitro* experiments optimizing transfer conditions demonstrated that 50–100% higher amounts of liposomes are needed with the same amount of DNA in quiescent cultures of SMCs or ECs compared with replicating cells in culture. Thus, despite optimized transfer conditions, 10–40 times lower transfer efficiencies were possible in induced stationary cultures compared with proliferating cell cultures. The difference in transfer efficiencies between cells following growth inhibition with serum-free medium versus inhibition with heparin or aphidicolin (63–122 mU per 100 µg protein versus 31–42 mU per 100 µg protein) correlated to the very low proliferative status of SMCs in serum-free medium (0.5–1% versus 1.5–3%).

In proliferating cells in culture, DNA/liposome ratios depended on the number of transfected cells. Using higher cell numbers per well, more liposomes were needed for the same DNA concentration to achieve optimal conditions. In contrast, stationary cells did not demonstrate any dependence of liposome concentration on transfected cell numbers. These results suggest that the optimal DNA/liposome ratio may depend upon the proliferating state of cells. The higher the cell number to be transfected, the lower the amount of proliferating cells as cell–cell contact inhibition occurs. Hence, the number of cells to be transfected needs to be constant during such experiments to optimize gene transfer conditions.

One major problem in gene therapy is the markedly lower transfer efficiency observed *in vivo* compared with results obtained from *in vitro* experiments. This problem concerns both liposomal and viral transfection. Additionally, most experiments using different transfection systems are conducted in proliferating cell cultures. The transfer efficiency achieved under these conditions may be as high as 100% transfected cells. However, the majority of cells in intact tissues such as vessels is quiescent and the percentage of proliferating cells in injured vessels is still markedly lower than in cultures of proliferating cells (Isner *et al.* 1994). Therefore, induced stationary cells appear to be a more suitable *in vitro* model for experiments investigating gene transfer conditions. These experiments might first permit rational investigation of the reasons for differences

in transfection efficiencies found *in vivo* and *in vitro*. Second, optimal conditions found for gene therapy in stationary cells may later be applied to *in vivo* experiments. Optimized delivery conditions may ultimately permit lower numbers of animals to be used. Porcine animal studies using optimized liposomal transfection via local application of genes have already confirmed results achieved in stationary cells (Armeanu *et al.* 2000; Nikol *et al.* 2000). Optimizing the gene transfer in various ways *in vitro* increased *in vivo* transfection rates from 0.1 to 15% of cells (Armeanu *et al.* 2000).

Further improvement in gene transfer rates may be possible by introducing agents facilitating transport into the nucleus via the nuclear membrane. It has been suggested that this has been a major obstacle for efficient plasmid DNA expression in stationary cell cultures and *in vivo* (Zabner *et al.* 1995; Brisson & Huang 1999; Simoes *et al.* 1999). There is no active cellular mechanism to transport DNA through the nuclear membrane. During mitosis, the nuclear membrane disintegrates and the transfected DNA can enter the nucleus. This may be one explanation why transfer efficiency is much higher in proliferating cells undergoing mitosis than in stationary cell cultures or tissues transfected *in vivo*, where proliferation is relatively low, as discussed above. A number of agents are suspected to improve gene transport into the nucleus. Ongoing investigations within our group emphasize this issue, and form part of the results presented in a forthcoming paper.

An alternative model to cell culture experiments for transfection techniques may be organ tissue investigated *in vitro*. However, it is very difficult to maintain the constant and reproducible transfection conditions that are needed for the successful optimization of lipofection techniques. In addition, in the immediate days following culture there is outgrowth of SMCs from the organ tissue, which are proliferating cells. These replicating cells may confuse data analysis.

Transfection of ECs *in vivo* was not detectable using local gene delivery under optimized gene transfer conditions (Nikol *et al.* 1999, 2000). This may be because the needle injection catheter delivers primarily to the adventitia. A modified balloon catheter for luminal delivery such as the porous or chamber balloon catheter (Hoeftling & Huehns 1995) may be more suitable for this type of experiment looking at endothelial delivery *in vivo*. Such investigations were not performed here, as the major goal of this paper was to investigate the induction of stationary cultures of SMCs, cells that might be valid targets for gene therapy after balloon injury, where there is endothelial removal.

In conclusion, we have shown that the addition of heparin to cell cultures is a simple physiological and low-priced method of halting cell growth, and can be used to induce stationary cultures of SMCs. Transfection experiments performed in non-proliferating cell cultures may better simulate *in vivo* conditions and lead to more directed and valuable experiments on animals.

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