Transfection Efficiency and Cytotoxicity of Nonviral Gene Transfer Reagents in Human Smooth Muscle and Endothelial Cells

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Purpose. Evaluation of a nonviral transfection reagent with respect to efficient gene transfer into primary human vascular cells.

Methods. Complexes consisting of seven commercially available transfection reagents (DAC-30, DC-30, Lipofectin, LipofectAMINE PLUS, Effectene, FuGene 6 and Superfect) and EGFP encoding plasmid DNA were studied. The *in vitro* transfection efficiency and cytotoxicity in human aorta smooth muscle cells (HASMCs) and endothelial cells (HAECs) and rat smooth muscle cells (A-10 SMCs) were assayed in the presence of serum using flow cytometric analysis and ATP-quantitation assay, respectively.

Results. Human primary cells were transfected less efficiently compared to the rat smooth muscle cell line. Transfection efficiency depended on the type of reagent, the reagent/DNA ratio, and, most importantly, on the cell type used. Determination of cytotoxicity showed that the effects of transfection on cell viability did not significantly differ from one another depending on the cell type. The exception to this was Superfect, which obviously reduced cell viability in all cell types.

Conclusions. Our experiments showed that DAC-30 is the preferred transfection reagent for HASMCs and HAECs, exhibiting an improved efficiency combined with an acceptable cytotoxicity. Therefore, it might offer a therapeutic option for the treatment of cardiovascular disease and prove suitable for further drug development.

KEY WORDS: cytotoxicity; human endothelial cells; nonviral gene transfer; smooth muscle cells; transfection efficiency.

INTRODUCTION

Cardiovascular diseases like arteriosclerosis are still some of the most common causes of morbidity and mortality. Invasive methods, such as angioplasty, are usually required as treatments (1). Limitation for this procedure is the development of restenosis (2). Gene transfer into the arterial wall has opened new therapeutic strategies in the treatment of vascular diseases (3–5). Endothelial and vascular smooth muscle cells are supposed to be the key factors with regard to the formation of these disorders (6). In order to improve the delivery efficiency of genetic material into these cells both *in vitro* and *in vivo*, the development of effective vectors for optimized gene transfer into target cells has become an important objective.

However, the current capability for transfection of these cell species is very low (7,8). Viral vectors have proven to deliver genetic material efficiently and to target a wide range of cells (9). However, several problems are associated with the use of viral vectors *in vivo* like adverse immune responses, toxic reactions, and the high risk of developing cancer, which diminish the possibility of repetitive treatments (10). Of further importance is the difficulty of purification of the viral vectors and their production on an industrial scale. For these reasons, synthetic delivery systems have been investigated during the last several years and have become more and more of an alternative to viral vectors. This is due in large part because they raise fewer safety issues, are easier to produce, and repeated administration is less of a problem (11–14). However, the lack of transfection efficiency of nonviral delivery systems *in vitro* and *in vivo*, especially in primary cells, is a major obstacle (8,15,16). Of relevance to this weak efficiency might be the presence of serum, reagent/DNA-charge ratios, DNA and reagent doses, size of transfection system, and cell cycle (17–21). Equally decisive for good transfection results are the cellular mechanisms involved in the transfection process like binding of the reagent/DNA-complexes to the cell surface, entry into the cells, dissociation of the complexes, and finally, their transport through the cytosol and the uptake of the DNA into the nucleus (22). Many of these factors are still poorly understood and require further investigation into the optimization of nonviral gene transfer.

Furthermore, with regard to subsequent federal approval of any gene transfer system and its eventual application in clinical trials, GMP-quality and GMP-conformity of all of the ingredients and processes has to be considered even at the early stages of drug development.

In this study, cytotoxicity and transfection efficiency of a variety of commercially available nonviral transfection reagents have been assessed. The transfection reagents have been chosen either according to their potential for transfecting human cells *in vitro* (as stated in the manufacturer's instructions) or to their possible applicability in humans *in vivo*. Our attention has been drawn to biological activity and compatibility in cell models. Characteristic influences on cultures of primary human aorta endothelial and smooth muscle cells, as well as on a rat smooth muscle cell line, have been investigated by flow cytometric analysis and toxicity testing. Our purpose was the evaluation of a transfection reagent suitable for further *in vivo* studies concerning cardiovascular problems and capability with respect to conforming to GMP standards during the scaling up process and production.

MATERIALS AND METHODS

Materials

3β-[*N*-(*N*,*N*′-dimethylaminoethane)-carbamoyl]-cholesterol (DAC-Chol)/dioleoylphosphatidylethanolamine (DOPE) 3:7 (w/w) (DAC-30) was obtained from G.O.T.

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ABBREVIATIONS: B, Buffer EC; DAC-Chol, 3β-[*N*-(*N*,*N*′-dimethylaminoethane)-carbamoyl]-cholesterol; DC-Chol, 3β-[(*N*′,*N*′-dimethylaminoethane)-carbamoyl]-cholesterol-HCl; DOPE, dioleoylphosphatidylethanolamine; EF, Effectene; FU, FuGene 6; GFP, green fluorescent protein; HASMC, human aorta smooth muscle cell; HAEC, human aorta endothelial cell; LF, Lipofectin; LP, LipofectAMINE PLUS; SF, Superfect; TM, transfection medium; TR, transfection reagent.

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Therapeutics GmbH (Berlin, Germany). The lipids 3β-[(*N*′,*N*′-dimethylaminoethane)-carbamoyl]-cholesterol-HCl (DC-Chol) and DOPE were purchased from Avanti Polar Lipids (Birmingham, AL, USA). The binary lipid mixture composed of 30% (w/w) DC-Chol and 70% (w/w) DOPE (denoted as DC-30) was prepared according to standard protocols (see Avanti Polar Lipids homepage, www.avantilipids.com). Effectene (EF) and Superfect (SF) were obtained from Qiagen (Hilden, Germany). FuGene 6 (FU) was purchased from Roche Diagnostics (Mannheim, Germany). Lipofectin (LF) and LipofectAMINE PLUS (LP) were obtained from Invitrogen (Karlsruhe, Germany). Table I summarizes compositions of transfection reagents; chemical structures, as far as obtainable, are presented in Fig. 1. Medium 200 supplemented with low serum growth supplement and Medium 231 supplemented with smooth muscle growth supplement were obtained from Tebu (Frankfurt a.M., Germany). Dulbecco's Modified Eagle Medium, fetal calf serum, phosphate-buffered saline (PBS special), and other culture reagents were obtained from Biochrom (Berlin, Germany). The plasmid pAH7-EGFP (expressing enhanced green fluorescent protein) and the components of transfection medium 1 and 2 (TM1, a solution containing 250 mM sucrose and 25 mM sodium chloride, and TM2 containing 220 mM mannite, 29 mM sucrose, and 25 mM sodium chloride) were obtained from Boehringer Ingelheim Pharma (Biberach, Germany).

Preparation of the DNA Transfection Systems

For transfection experiments, the plasmid pAH7-EGFP (Boehringer Ingelheim Pharma) expressing enhanced green fluorescent protein was mixed with different transfection reagents in various transfection reagent (TR)/DNA ratios (w/w). Complexes were prepared according to the manufacturer's instructions. As a consequence of preparation techniques, the amounts of plasmid and reagent used were not alike but specific for each transfection reagent. Briefly, the single transfection reagents DC-30, DAC-30, Lipofectin, and LipofectAMINE PLUS and plasmid DNA were diluted separately in transfection medium (TM1 or TM2). Solutions were combined, gently mixed, and incubated for the appropriate time to allow formation of complexes. In the case of DC-30, 2 μg plasmid was mixed with 4, 8, 10, or 20 μg of the reagents (TR/DNA ratio (w/w) = 2:1, 4:1, 5:1, 10:1). DAC-30 was used with 2 μg plasmid in 1:1, 2:1, 2.5:1, 4:1, 5:1, 8:1, 10:1 and 12:1 ratios (w/w) and with 3 μg plasmid in the ratios 5:1 and 8:1 (w/w). For complexes containing Lipofectin, the ratios were 3:1, 6:1, and 12:1 (w/w) with both 1 and 2 μg plasmid per well. LipofectAMINE PLUS was used in 8:1 and 16:1 ratios, 1 or 2 μg plasmid was mixed with 0.5 or 1 μl PLUS-reagent before being mixed with the transfection reagent. For complexes containing Effectene, 0.4 μg or 1 μg plasmid was diluted in Buffer EC (B) and mixed with 3.2 μl or 8 μl Enhancer reagent prior to mixing with Effectene reagent. Superfect was used in the ratios 6:1, 15:1, 30:1, and 45:1 with 2 μg plasmid and 15:1 with 0.5 μg or 1 μg plasmid. The ratios of the complexes containing FuGene 6 were 3:1 and 6:1 (v/w) with 1 μg or 2 μg plasmid.

Plasmid, transfection reagents, and DNA condensation reagents were mixed in TM1, TM2, or in Buffer EC in the case of Effectene. Incubation times for the constitution of the complexes were chosen according to the manufacturer's instructions.

TR/DNA (+/–) charge ratio is evaluated assuming a molecular weight of plasmid DNA of 325 g/mol concerning one negative charge unit per DNA base. Positive charge units are calculated considering one positive charge per cationic lipid molecule and cationic lipid ratios of 3:7 (w/w) in DAC-30 and DC-30 and 1:1 (w/w) in Lipofectin (DOTMA/DOPE). Molecular weight (MW) of DAC-Chol and DC-Chol is 500 g/mol and MW (DOTMA) = 643.5 g/mol, respectively.

Cell Culture

All cells were cultivated at 37°C in a humidified atmosphere with 5% (v/v) CO_2. A-10 rat smooth muscle cells were obtained from DSMZ (Braunschweig, Germany) and cultivated in Dulbecco's Modified Eagle Medium supplemented with 20% (v/v) fetal calf serum. Human aorta smooth muscle cells (HASMCs) and human aorta endothelial cells (HAECs) were purchased from Tebu (Frankfurt/Main, Germany). HASMCs were maintained in Medium 231 with smooth muscle growth supplement, whereas HAECs were maintained in Medium 200 with low serum growth supplement.

In vitro Transfection Assay

Cells were plated in a 6-well cluster dish at a density of 7×10^4 cells and cultivated in the appropriate growth medium with serum. After 24 h in culture, the cells were washed with 2 ml PBS, and 1 ml fresh growth medium with serum was added to the cells. TR/DNA complexes were then added to each well and incubated with the cells for 3 or 5 h at 37°C [5% (v/v) CO_2]. The supernatants were then removed, and 2 ml of the appropriate growth medium was added to each well and cultured for 48 h after transfection at 37°C in 5% (v/v) CO_2.

Flow Cytometric Assay

Flow cytometric analysis for GFP fluorescence was performed using a 4-color FACS-Calibur (Becton Dickinson,

Table I. Compositions of Transfection Reagents Used

Transfection reagent (TR)	Constituents
DC-30	DC-Chol/DOPE 3:7 (w/w), liposomal
DAC-30	DAC-Chol/DOPE 3:7 (w/w), liposomal
Superfect (SF)	Activated dendrimer
Effectene (EF)	Non-liposomal lipid with enhancer
Lipofectin (LF)	DOTMA/DOPE 1:1 (w/w), liposomal
LipofectAMINE PLUS (LP)	DOSPA/DOPE 3:1 (w/w), liposomal with DNA complexing PLUS agent
FuGene 6 (FU)	Blend of lipids and other components

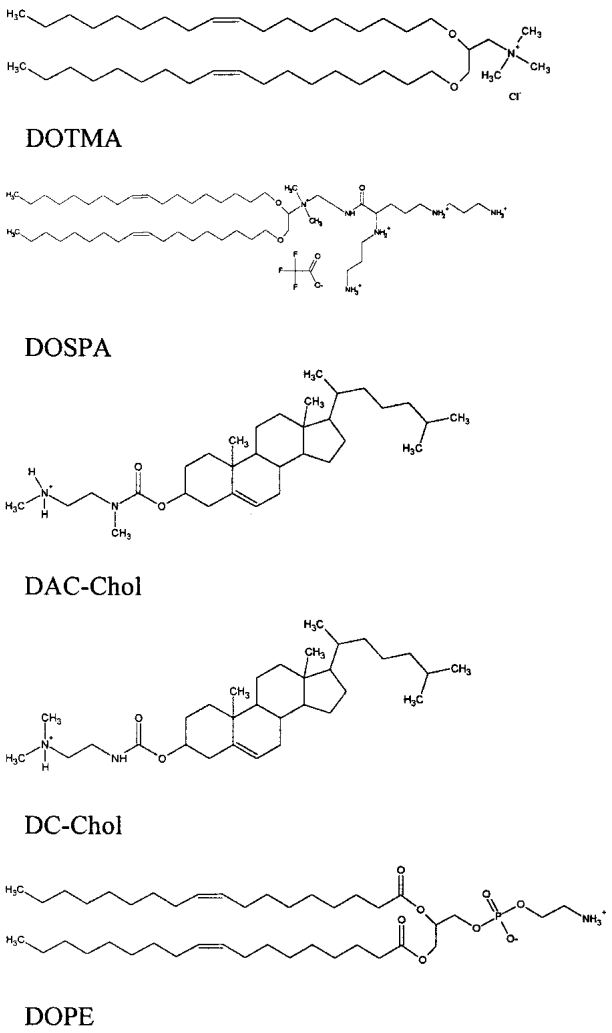


Fig. 1. Chemical structures of the ingredients of Lipofectin, LipofectAMINE PLUS, DAC-30, and DC-30.

Heidelberg, Germany) equipped with an argon laser exciting at a wavelength of 488 nm. For each sample, 10,000 events were collected by list-mode data that consisted of side scatter, forward scatter, and fluorescence emission centered at 530 nm (FL1). The GFP fluorescence was collected at a logarithmic scale with a 1024 channel resolution. Cell Quest Pro software (Becton Dickinson, Heidelberg, Germany) was applied for the analyses.

For sample preparation, 48 h after transfection the cells were washed once with 2 ml PBS, collected by trypsinisation, pelleted, washed with 1 ml PBS, and resuspended in 0.3 ml PBS. The cell suspension was analyzed within 30 min.

Toxicity Evaluation

Cell viability after transfection was determined by quantitative analysis of the amount of ATP present, produced by metabolically active cells. Therefore, CellTiter-Glo Luminescent Cell Viability Assay (Promega, Mannheim, Germany) was used. The medium was replaced with 500 μl Dulbecco's Modified Eagle Medium supplemented with 10% fetal calf serum and equivalent amounts of test reagent 48 h after transfection. The reagent induced cell lysis and generated a luminescent signal proportional to the amount of ATP present. After 2 min of mixing and 10 min of incubation at room temperature, the contents of the 6-well plates were transferred into 96-well plates; 3 values of 200 μl were obtained out of the 1000 μl content of a single well from the 6-well plate. The luminescent signal was quantified in a Luminometer (MicroLumat Plus LB 96 V, EG&G Berthold, Bad Wildbad, Germany). The percentage of cell viability was calculated by comparing the appropriate luminescent signal to the signal obtained with non-transfected control cells.

Complex Size Measurement

Complex size was measured by photon correlation spectroscopy using a Nicomp 380 (Nicomp, Santa Barbara, CA, USA). Sample size was analyzed by mono-modal Gaussian vesicle analysis with intensity weighting. Data represent the mean diameter and the calculated polydispersity of three measurements.

RESULTS

Transfection Efficiency

Transfection efficiencies of seven nonviral vector formulations in primary human aorta endothelial cells and primary human aorta smooth muscle cells were determined by flow cytometric analysis using the plasmid pAH7-EGFP. Optimal transfection conditions were evaluated by varying the amounts of plasmid DNA at different TR/DNA ratios, incubation times, and media in order to form the complexes (Fig. 2). With respect to planned scale-up processes, two different saccharide-containing media were assayed for protection of TR/DNA complexes during the required lyophilization step. Transfection experiments were also performed with a well-established rat smooth muscle cell line (A-10 SMC).

Human Aorta Endothelial Cells

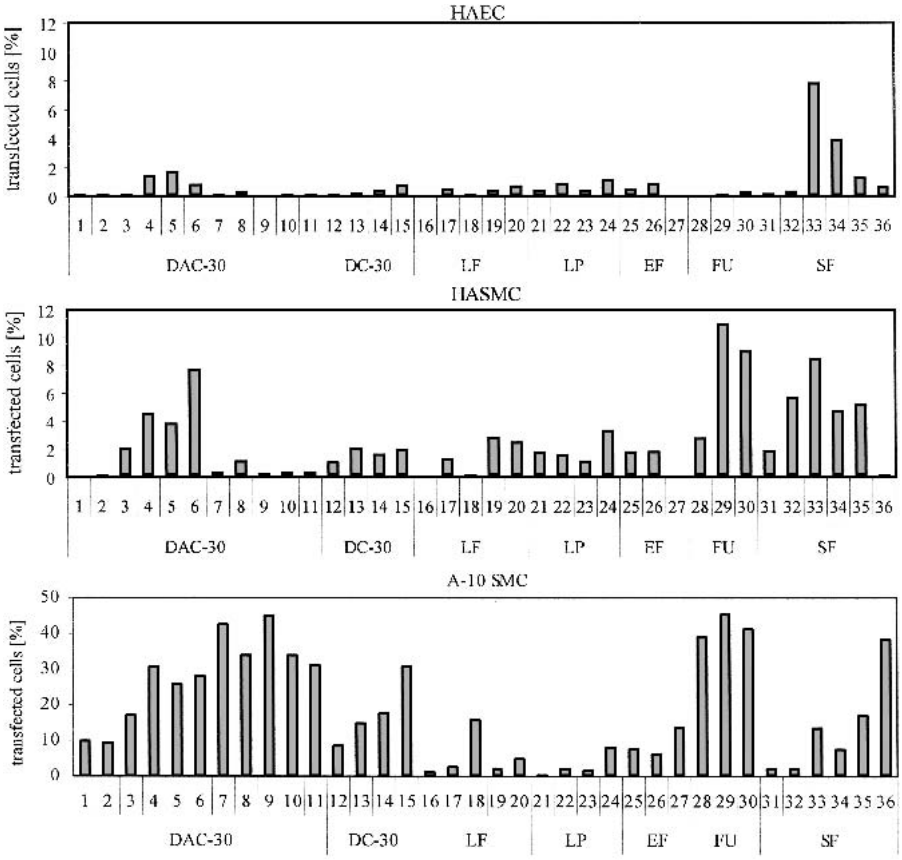
Results ranged from 0.1 to 8% transfection in HAECs. The best efficiency was achieved with Superfect, a cationic dendrimer. Up to 8% of cells were transfected with a formulation of Superfect and DNA in a ratio of 15:1 (w/w) and a plasmid DNA amount of 1.0 μg per well (Fig. 2). Liposomal formulations (DAC-30, DC-30, Lipofectin) ranged from 0.1 to 2% in HAECs. The best results for liposomal formulations were achieved with DAC-30, a combination of DOPE and DAC-Chol in a ratio of 7:3 (w/w). Two percent of HAECs were transfected with DAC-30 in a ratio of 5:1 (DAC-30 to plasmid DNA) and a plasmid amount of 2.0 μg per well (Fig. 2).

Detectable transfection efficiency in HAECs with LipofectAMINE PLUS and Effectene, two reagents consisting of a lipid and a DNA condensing component, was not more than 1%. Similarly, FuGene 6, a blend of lipids and other components, transfected HAECs only sporadically.

Smooth Muscle Cells

Human aorta smooth muscle cells and a rat smooth muscle cell line (A-10 SMC) were chosen for determination of transfection efficiency in smooth muscle cells.

Significant differences in amounts of transfected cells were measured depending on the transfection reagent, the



sample	conditions				sample	conditions			
	µg DNA	ratio	medium	incubation time		µg DNA	ratio	medium	incubation time
1	2.0	1:1	TM1	5h	19	1.0	12:1	TM1	5h
2	2.0	2:1	TM1	5h	20	2.0	12:1	TM1	5h
3	2.0	2.5:1	TM1	5h	21	1.0	8:1	TM1	3h
4	2.0	4:1	TM1	5h	22	2.0	8:1	TM1	3h
5	2.0	5:1	TM1	5h	23	1.0	16:1	TM1	3h
6	2.0	5:1	TM2	5h	24	2.0	16:1	TM1	3h
7	3.0	5:1	TM1	5h	25	0.4	10:1	B	3h
8	2.0	8:1	TM1	5h	26	0.4	25:1	B	3h
9	3.0	8:1	TM1	5h	27	1.0	25:1	B	3h
10	2.0	10:1	TM1	5h	28	1.0	3:1	TM1	3h
11	2.0	12:1	TM1	5h	29	2.0	3:1	TM1	3h
12	2.0	2:1	TM1	5h	30	1.0	6:1	TM1	3h
13	2.0	4:1	TM1	5h	31	2.0	6:1	TM2	5h
14	2.0	5:1	TM1	5h	32	0.5	15:1	TM2	3h
15	2.0	10:1	TM1	5h	33	1.0	15:1	TM2	5h
16	2.0	3:1	TM1	5h	34	2.0	15:1	TM2	5h
17	1.0	6:1	TM1	5h	35	2.0	30:1	TM2	5h
18	2.0	6:1	TM1	5h	36	2.0	45:1	TM2	5h

Fig. 2. *In vitro* transfection efficiency of transfection reagent/DNA complexes at different ratios (w/w) in HAECs, HASMCs, and A-10 SMCs (1–36, conditions as shown in table). Cells were transfected with TR/DNA complexes in the presence of serum with various amounts of plasmid and different incubation times. The complexes were formed in different media (TM1, TM2, B). Each value represents the mean of at least duplicates, deviation was not more than 5%.

TR/DNA ratio, and amount of plasmid per well. However, FuGene 6, the most effective transfection reagent for A-10 SMCs, also transfected HASMCs very well. HASMCs were transfected up to 11% and A-10 SMCs up to 50%. No remarkable differences were found between the obtained transfection results with varying ratios of FuGene 6 and plasmid DNA. Both the activated dendrimer Superfect and the liposomal formulation DAC-30 transfected HASMCs with an efficiency of about 8%. Yet in A-10 SMCs, DAC-30 showed significantly better efficiencies than Superfect (Fig. 2).

The other liposomal formulations (DC-30, Lipofectin) as well as the reagents consisting of lipid and DNA condensing components were less effective.

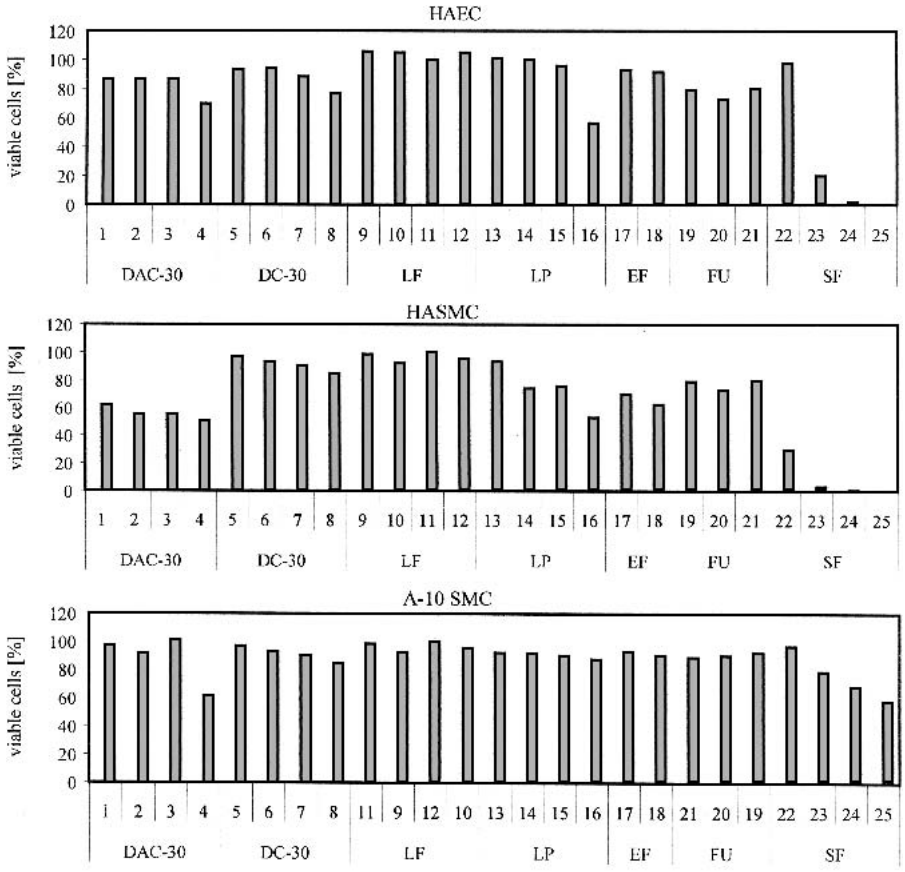
Effects of Transfection on Cell Viability

For the determination of cytotoxicity, composition and preparation of complexes as well as transfection conditions were the same as those used in the efficiency experiments.

Evaluation of the influence of transfection with naked DNA resulted in no decrease in cell viability (data not

shown). The lowest amount of viable cells was found after transfection with the activated dendrimer Superfect in A-10 SMCs, HASMCs, and HAECs. Almost no living HASMCs and HAECs could be detected after transfection with complexes containing 2 μg plasmid per well. Provoked by the toxic effects of transfection with Superfect, a strongly visible change in cell morphology occurred: cells disintegrated and finally were detached from the plate ground. None of the other transfection reagents had a similarly distinctive effect on cell viability as the dendrimer. A-10 SMCs were also more significantly affected by these complexes compared to the results obtained with the other transfection reagents (Fig. 3). This is particularly striking because for A-10 SMCs and HAECs most commonly, no apparent toxic effects were seen with the other transfection reagents. An exception was found for HAECs with Lipofect-AMINE PLUS in a ratio of 16:1 with 2 μg plasmid per well, which caused a more significant percentage of cell death. For A-10 SMCs, the DAC-30/DNA complex (ratio 10:1, w/w) was more toxic than the other ratios. Generally HASMCs were more affected by transfection than A-10 SMCs and HAECs.

In order to evaluate the effect of different incubation times, A-10 SMCs were exposed to complexes for both 3 and 5 h (Fig. 4). The evaluation of the toxicity test 48 h after transfection resulted in only minor differences in cell viability, which indicates that for the examined complexes, incubation time exhibits little influence on cytotoxicity.



sample	conditions			sample	conditions		
	μg DNA	ratio	incubation time		μg DNA	ratio	incubation time
1	1.0	6:1	5h	14	1.0	15:1	5h
2	2.0	6:1	5h	15	2.0	15:1	5h
3	1.0	12:1	5h	16	2.0	30:1	5h
4	2.0	12:1	5h	17	2.0	45:1	5h
5	1.0	8:1	3h	18	2.0	2:1	5h
6	2.0	8:1	3h	19	2.0	4:1	5h
7	1.0	16:1	3h	20	2.0	5:1	5h
8	2.0	16:1	3h	21	2.0	10:1	5h
9	0.4	25:1	3h	22	2.0	2:1	5h
10	0.4	10:1	3h	23	2.0	4:1	5h
11	1.0	3:1	3h	24	2.0	5:1	5h
12	2.0	3:1	3h	25	2.0	10:1	5h
13	1.0	6:1	3h				

Fig. 3. Cell viability of different samples (1–25, conditions as shown in table, for forming of complexes TM1 was used for all samples). Each value represents the mean of at least duplicates, deviation was not more than 5%. As a control (100% viability), non-transfected cells were used.

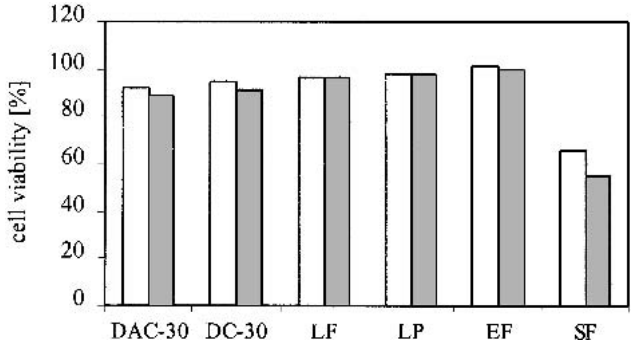


Fig. 4. Evaluation of the influence of incubation time on the viability of A-10 SMCs. Different commercially available transfection reagents in varying TR/DNA ratios (w/w) were selected for cytotoxicity determination, DAC-30 (5:1), DC-30 (4:1), Lipofectin (6:1), Lipofect-AMINE PLUS (8:1), Effectene (25:1), and Superfect (15:1). Bright and dark bars indicate the cell viability after 3 or 5 h incubation time, respectively (n = 4, deviation was not more than 5%).

Characteristics of Complexes

Table II summarizes the mean diameters and homogeneity, expressed as polydispersity, obtained with the complexes composed of different transfection reagents. Measuring the size of the complexes obtained with different transfection reagents led to a significant discrepancy in mean diameters. Polydispersities ranged from low (0.18) to high values (1.06) indicating that the complexes are not homogeneous. No correlation could be observed between polydispersity values and experimental variations. Sizes of complexes composed of DAC-30, DC-30, or LipofectAMINE PLUS ranged between 400 and 1000 nm. Significant variations in size were observed measuring complexes formed with Lipofectin and Effectene: mean diameter size went up to 7000 nm. The mean diameters of complexes composed of FuGene 6 and Superfect seemed to show no dependence with regards to the TR/DNA ratio. These results suggest that the size of complexes composed of LipofectAMINE PLUS, Effectene, FuGene 6, and Superfect seems not to be significantly influenced by varying TR/DNA ratios.

Table II. Size and Polydispersity of Transfection Reagent/DNA Complexes

Transfection reagent (TR)	Sample	Ratio TR/DNA (w/w)	DNA amount (μg)	Medium	Size (nm)	Polydispersity
DAC-30	1	1:1	2.0	TM1	735	0.46
	2	2:1	2.0	TM1	610	0.31
	3	2.5:1	2.0	TM1	580	0.60
	4	4:1	2.0	TM1	775	0.44
	5	5:1	2.0	TM1	605	0.46
	6	5:1	2.0	TM2	1040	0.81
	7	5:1	3.0	TM1	420	0.26
	8	8:1	2.0	TM1	690	0.25
	9	8:1	3.0	TM1	1015	0.22
	10	10:1	2.0	TM1	800	0.23
	11	12:1	2.0	TM1	770	0.24
DC-30	12	2:1	2.0	TM1	640	0.62
	13	4:1	2.0	TM1	760	0.45
	14	5:1	2.0	TM1	565	0.38
	15	10:1	2.0	TM1	1075	0.54
Lipofectin	16	3:1	2.0	TM1	200	0.23
	17	6:1	1.0	TM1	3440	0.67
	18	6:1	2.0	TM1	7250	0.85
	19	12:1	1.0	TM1	250	0.18
	20	12:1	2.0	TM1	115	0.19
Lipofect AMINE PLUS	21	8:1	1.0	TM1	510	0.25
	22	8:1	2.0	TM1	490	0.25
	23	16:1	1.0	TM1	465	0.27
	24	16:1	2.0	TM1	830	0.44
Effectene	25	10:1	0.4	B	2085	0.29
	26	25:1	0.4	B	455	0.31
	27	25:1	1.0	B	220	0.52
FuGene 6	28	3:1	1.0	TM1	1320	0.38
	29	3:1	2.0	TM1	1190	0.51
	30	6:1	1.0	TM1	1835	1.06
Superfect	31	6:1	2.0	TM2	950	0.32
	32	15:1	0.5	TM2	500	0.21
	33	15:1	1.0	TM2	820	0.16
	34	15:1	2.0	TM2	1215	0.38
	35	30:1	2.0	TM2	1150	0.21
	36	45:1	2.0	TM2	1665	0.61

Considering the (+/–) charge ratio of complexes composed of DAC-30, DC-30, and Lipofectin, there was a correlation between charge ratio and mean diameter. Figure 5 illustrates the size of complexes of plasmid DNA and DAC-30, DC-30, or Lipofectin at different lipid/DNA (+/–) charge ratios. Complexes of DAC-30 or DC-30, with charge ratios of 1:1 and 1.6:1 (+/–), exhibited a mean diameter of 700 and 500 nm, respectively, while lipoplexes with charge ratios of 2:1 and 3:1 (+/–) exhibited larger diameters (about 900 nm) (Figs. 5A and 5B). More significant is the change in the size of complexes with different (+/–) charge ratios obtained with Lipofectin as lipid component. Complexes with charge ratios of 0.8:1 or 3:1 (+/–) exhibited mean diameters of about 200 nm, whereas complexes of Lipofectin/DNA of a (+/–) charge ratio of 1.6:1 had a size of 4500 nm (Fig. 5C).

DISCUSSION

In this study, a comparative analysis of transfection efficiency and cytotoxicity of seven nonviral transfection reagents was performed. Gene transfer in human aorta smooth muscle cells (HASMCs) and human aorta endothelial cells (HAECs) was carried out in the presence of serum. The results of the experiments showed that both the efficiency and the cytotoxicity of gene transfer depended on the transfection reagent used as well as on experimental details such as TR/DNA ratio and amount of plasmid.

Human aorta endothelial cells have previously been shown to be very difficult to transfect (23). However, in our study, complexes composed of Superfect and DAC-30 reached transfection efficiencies of 2% or 8%, respectively. Other transfection reagents (DC-30, Lipofectin, LipofectAMINE PLUS, Effectene) were less efficient. Published results described transfection using DC-30 as more efficient than using Lipofectin in minipig primary endothelial cells (12). In contrast, our studies showed that transfection with lipoplexes containing DC-30 did not result in higher efficiencies than lipoplexes composed of Lipofectin. FuGene 6, which according to the literature is an efficient gene delivery reagent in serum-free medium, such as Opti-MEM I (GIBCO) for human endothelial cells like HUVEC (24), proved to result in no protein expression in our experiments with HAEC in the presence of serum. However, it turned out to be most efficient for transfection of both human and rat smooth muscle cells in serum containing medium as shown in Fig. 2. More than 11% and 50%, respectively, showed expression of the gene product. Despite these satisfying transfection results in HASMCs, we considered FuGene 6 as nonoptimal because of its lack of efficiency in endothelial cells, its undefined composition, and its sensitivity to plastic surfaces, which would cause problems when the production process is scaled up.

FuGene 6, Superfect, and DAC-30 turned out to be most effective in human aorta smooth muscle cells. These three transfection reagents representing different vector systems achieved better results than the widely used reagents Lipofectin and Effectene (7,25).

Low transfection efficiencies similar to those seen with Lipofectin or Effectene were obtained with complexes composed of LipofectAMINE PLUS or the frequently used cationic lipid DC-30 (12).

Our results differed from literature data considering multivalent lipids to be more effective than monovalent lipid-

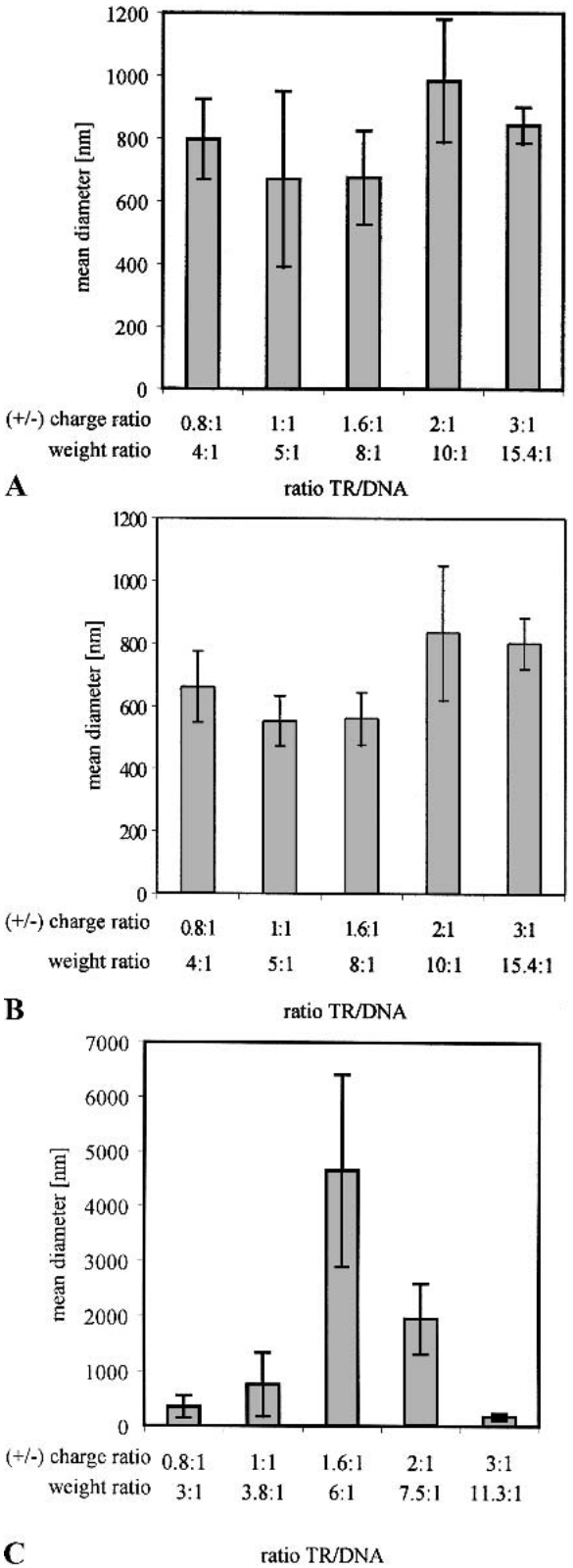


Fig. 5. (A) Correlation between size and (+/–) charge ratio of lipoplexes composed of DAC-30 and plasmid DNA. (B) Correlation between size and (+/–) charge ratio of lipoplexes composed of DC-30 and plasmid DNA. (C) Correlation between size and (+/–) charge ratio of lipoplexes composed of Lipofectin and plasmid DNA.

containing liposomes (26). DAC-30, a single charged liposomal formulation of DAC-Chol and DOPE (3:7 w/w), showed better results than LipofectAMINE PLUS, a multivalent cationic lipid with five potentially charged amino groups.

LipofectAMINE PLUS and Effectene used in conjunction with DNA compacting agents are reported to be effective for enhancing gene transfer in the presence of serum (27,28). In our experiments, however, LipofectAMINE PLUS and Effectene led to lower efficiencies in all cell types. Better results were reached with cationic liposomes (DAC-30) or the highly branched polymer Superfect.

The DNA protective effect of cationic liposomes and the resistance to serum of cationic liposome/DNA complexes depend on their lipid/DNA (+/−) charge ratio. Complexes composed of DOTAP/Chol at a charge ratio of 2:1 (+/−) lipid/DNA ratio were reported to be the most active ratio in Faneca *et al.* (17). On the other hand, it has been reported that lipoplexes reach maximal cell association (which includes cell binding, membrane fusion, and endocytosis) when prepared at a 1:1 (+/−) charge ratio (18). Considering that the transfection efficiency is dependent on the (+/−) charge ratio of DAC-30/DNA complexes in our experiments, the best (+/−) charge ratio in both HAECs and HASMCs seems to be 1:1, which corresponds to a weight ratio of 5:1. The observation that DC-30 lipoplexes with increasing (+/−) charge ratios resulted in higher transfection efficiencies in HAECs but not in HASMCs cannot currently be explained. Lipoplexes composed of Lipofectin showed best transfection results at a (+/−) charge ratio of 3:1.

Evaluation of TR/DNA (+/−) charge ratios was only possible with DAC-30, DC-30, and Lipofectin, because no detailed information concerning electrostatic charges for the other commercial reagents is available.

Considering the sizes (mean diameters) of the complexes composed of cationic cholesterol derivatives in combination with DOPE and plasmid DNA, a moderate size from 0.4 to 1.4 μm was found to be the most efficient size for endocytosis-mediated uptake of complexes (20). Neither smaller vesicles (<400 nm) nor larger vesicles (>1.4 μm) led to adequate gene transfer results. We suggest that size dependency cannot be generalized but is specific for each transfection reagent. In agreement with Kawaura *et al.* (20), lipoplexes containing DAC-30 exhibited sizes in the range of 400 to 1000 nm for achievement of best transfection results in all cell types, whereas FuGene 6 generated complexes with mean diameters from 1200 to 1800 nm, which led to best transfection results in HASMCs and A-10 SMCs, but showed no detectable transfection in HAECs.

These results lead to the assumption that tendencies of optimal size ranges are only apparent within the cell types and transfection reagents but cannot be generalized overall.

Estimation of cytotoxicity is as important as efficiency in the evaluation of effective transfection reagents, especially for *in vivo* applications. In this study cell viability after transfection was determined by quantification of the ATP amount produced by metabolically active cells.

Despite the interexperimental variability in the performed transfection experiments, the effects of transfection on cell viability differed only slightly within each trial depending on the cell type. The exception in the range of studied TRs was represented by the polymer-based reagent Superfect. Recently it was shown to be slightly toxic to cells if added in a pure state (23). In our study, however, Superfect turned out to be the most toxic transfection reagent in combination with plasmid DNA for the targeted cells. Cell viability decreased particularly in HASMCs and HAECs after transfection with 2 μg plasmid per well independently of TR/DNA ratio of the complexes. This observation is in contrast to former studies that claim cationic lipids to be more toxic than polymers, even with lower doses of DNA (29).

In contrast to efficiency, where the most effective complexes varied depending on the cells, toxicity was more uniform. Superfect, which produced the most efficient protein expression in HAECs, was also the only explicitly toxic reagent for these cells. FuGene 6 was very efficient in HASMCs and A-10 SMCs and showed a cytotoxicity similar to those of the other reagents. In particular, DAC-30 emerged to be the transfection reagent with the most suitable correlation between transfection efficiency and toxicity. Other transfection reagents like Lipofectin, LipofectAMINE PLUS, and Effectene were not significantly toxic for the cells in the applied ratios, but at the same time gene expression did not occur at an effective level.

Similar to transfection efficiency, toxic effects due to the applied transfection reagents also depend on the TR used as well as on the conditions of the transfected cells (23,30,31). Significant differences in cell viability were seen after transfection of human and rat smooth muscle cells. Regardless of the reagent used, viability of HASMCs was lower than viability of A-10 SMCs. HAECs were also affected to a lesser extent than the primary muscle cells. Furthermore, it could be observed that toxic effects in HAECs seemed to be associated with positive transfection results. One reason for this behavior might be the physiological function of different cell types. Endothelial cells outline the entire vessel system. Their function is the uptake of substances from the blood and their transcytosis into the tissue behind. We suppose it is possible that complexes remain within the endothelial cells for only a brief period of time, which is too short for dissociation and protein expression. This could be the reason for low cytotoxicity as well as low efficiency occurrence in HAECs.

Other factors thought to influence cytotoxicity as well as transfection efficiency are the presence or absence of serum and the charge ratios of TR/DNA complexes, incubation time and reagent doses (7,29,30,32,33). However, we could not determine significant changes in toxicity caused by increasing the incubation time or altering charge ratios or reagent doses.

The variation in size and surface charge observed for complexes prepared at different TR/DNA ratios and even for those prepared at the same ratio are experimental evidence of the heterogeneous and dynamic nature of the complexes. We suppose this heterogeneity, combined with the different characteristics of the cell types used, such as differences in endocytosis and intracellular transport of the complexes, to be the reason for the variations seen in the results of the transfection experiments.

In this study, a suitable gene transfer system has been evaluated and optimized for *in vitro* conditions. One has to be aware that these data are preliminary with regards to use *in vivo*. However, as a first step in the development of a gene transfer system, the *in vitro* experiments as presented in this study are essential and the only way to define the systems thoroughly. Here, transfection efficiency and cytotoxicity

have been analyzed according to selected parameters such as cells of interest, ratio of transfection reagent to DNA, transfection medium, and incubation time.

For our purpose, DAC-30 was comparatively the best TR in reaching the requirements necessary to achieving transfection of HAECs and HASMCs combined with acceptable cytotoxicity and good practicability in terms of large-scale production.

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