CMLS, Cell. Mol. Life Sci. 57 (2000) 1326–1333
1420-682X/00/091326-08 $ 1.50 + 0.20/0

CMLS Cellular and Molecular Life Sciences

Research Article

Highly efficient cell-mediated gene transfer using non-viral vectors and FuGene™6: in vitro and in vivo studies

I. Hellgren[a,*], V. Drvota[a], R. Pieper[b], S. Enoksson[b], P. Blomberg[c], K. B. Islam[c] and C. Sylvén[a]

[a]Department of Cardiology, The Clinical Research Center, 5th floor, Novum, 141 86 Huddinge, Stockholm (Sweden), Fax + 46 8 58583810, e-mail: irina.hellgren@medhs.ki.se
[b]Department of Vascular Surgery, Huddinge University Hospital, Stockholm (Sweden)
[c]Gene Therapy Centre, Huddinge University Hospital, Stockholm (Sweden)

Received 22 May 2000; accepted 27 June 2000

Abstract. The present study was undertaken to develop an efficient non-viral gene delivery system for cardiovascular gene therapy. We investigated transfection efficiency and toxic properties of the new transfection reagent, FuGene™6, and compared it with two other transfection reagents, Tfx™-50 and LipoTaxi™. For in vivo experiments, the plasmid was delivered intramuscularly via transplantation of fibroblasts transfected with plasmid and FuGene™6. Conditions for efficient gene delivery were initially studied in vitro. Human and rabbit fibroblasts were isolated from skin, cultured and transfected with phVEGF165 or pCMVβgal plasmids, coding for vascular endothelial growth factor (VEGF) or β-galactosidase, respectively. The effect of the DNA amount and the DNA:transfection reagent ratio on plasmid uptake were studied. Of the transfection reagents tested, only FuGene™6 provided high-efficiency and dose-dependent plasmid transfer both for cell-localised (β-galactosidase) and secreted (VEGF) gene products. When analysed with an MTT assay, FuGene™6 showed no toxicity at low doses. Optimised conditions were applied for in vivo reporter gene delivery. Rabbits were injected intramuscularly with ex vivo-transfected fibroblasts. As in in vitro studies, ex vivo-transfected fibroblasts showed highly efficient gene expression in vivo. Tissue sections were analysed with macrophage-specific immunostaining. No signs of inflammation were seen in the region of fibroblast injection. This study demonstrates that FuGene™6 is a highly efficient transfection reagent that may be useful for in vitro non-viral transfection of primary human and rabbit fibroblasts and for in vivo therapeutic non-viral gene delivery.

Key words. Transfection; VEGF; FuGene™6; fibroblasts; gene therapy.

Gene therapy using vascular endothelial growth factor (VEGF) is an important alternative treatment for a number of cardiovascular diseases, including myocardial ischaemia [1–3] and peripheral artery disease [4].
However, to date, there are no ideal vectors for intracellular gene transfer. Viral systems deliver genes more efficiently than plasmids, but there are safety concerns regarding their immunogenicity, oncogenic properties, risk of recombination with wild-type viruses and unknown long-term effects [5, 6]. Another disadvantage of many viral vectors is their limited capacity for delivering large foreign genes. Moreover, viral vectors are difficult to prepare, they are expensive and they are potentially hazardous to work with.
Non-viral vectors do not have such safety concerns. They remain in a non-replicative, unintegrated form, have low immunogenicity and are easy and safe to prepare. Furthermore, plasmids may accommodate

* Corresponding author.

large fragments of DNA. In contrast to viral systems, plasmids provide temporary expression of the transfected gene. In certain cases, like the induction of angiogenesis with VEGF, such short-term expression is preferable to achieve a beneficial biological effect [7–9]. The main obstacle associated with non-viral vectors is their relatively low level of gene delivery.

Thus, the development of efficient gene delivery systems is one of the major goals of gene therapy. In the present study, we investigated the transfection efficiency of the new transfection reagent, FuGene™6, and tested cell-mediated intramuscular plasmid delivery via transplantation of fibroblasts transfected with plasmid and FuGene™6.

Materials and methods

Ethics. The investigation conforms with the principles outlined in the Declaration of Helsinki and with the Guiding Principles in the Care and Use of Animals.

Isolation of primary fibroblasts. Human skin biopsies were obtained from legs of patients 25–50 years old undergoing operation at the Surgery Department of Huddinge University Hospital, Sweden. Rabbit skin specimens were obtained from 4-month-old New Zealand White rabbits at the Animal Department of Huddinge University Hospital. Biopsies were minced to small pieces, placed in a tissue culture dish and incubated in an RPMI 160 cell culture medium containing 15% fetal calf serum, 2% L-glutamine and gentamicin at 37 °C. After 3–7 days, cells were detached with trypsin-EDTA and resuspended in the RPMI medium with 10% fetal calf serum, 2% L-glutamine and gentamicin.

In vitro transfection. 50×10^4 cells were seeded into the wells of a 24-well plate 1 day prior to transfection. Transfections were performed according to FuGene™6 (Boehringer Mannheim), Tfx™-50 (Promega) and LipoTaxi™ (Stratagene) transfection reagent instructions from the manufacturers with 0.25–4 μg of plasmid. Plasmids used were phVEGF165.sr coding for VEGF (generously provided by Prof. J. M. Isner, St Elisabeth's Medical Center, Boston, Mass.) and pCMVβ coding for β-galactosidase (Clontech, USA). Transfection reagents were used in amounts of 0.75, 1.5, 3 or 6 μl/well for FuGene™6; 2.5, 5 or 7.5 μl/well for LipoTaxi™; and Tfx™-50 in charge ratios to DNA of 2:1, 3:1 or 4:1. Transfection with Tfx™-50 was performed in a serum-free medium. After 1 h, cells were overlayed with the complete medium. To cells transfected with LipoTaxi™, medium with $\times 2$ serum was added 6 h after transfection.

Production of the VEGF protein was measured by ELISA over 7 days. Production of β-galactosidase was analysed by X-gal staining after 48 h.

X-gal assay for lacZ expression in fixed fibroblasts. Fibroblasts were fixed in 2% formaldehyde/phosphate-buffered saline (PBS; pH 7.4) for 15 min, rinsed twice in PBS, 0.1% bovine serum albumin, 0.25% Triton, 0.1% glycine for 15 min (the second time in the same buffer but without glycine) and then in X-gal buffer (5 mM K-ferricyanide, 5 mM K-ferrocyanide, 2 mM $MgCl_2$) for 5 min. After staining for 15 min at room temperature in X-gal staining solution (X-gal buffer with 1 mg/ml X-gal), cells were washed in PBS and analysed under the microscope. Blue X-gal-positive cells were counted and the transfection efficiency was expressed as the percentage of positive cells relative to the total number of cells.

VEGF ELISA analysis. The amount of VEGF secreted by transfected fibroblasts into the culture medium was analysed using the R&D Systems Europe Quantikine kit (UK) for human VEGF according to the manufacturer's instructions. Briefly, standard or samples were incubated in wells containing assay diluent for 2 h at room temperature, washed three times, incubated with conjugate for 2 h at room temperature, washed three times and incubated with substrate for 20 min at room temperature. The reaction was stopped by adding stop solution. The plate was analysed within 30 min at 450 nm with a wavelength correction 540/570 nm.

Toxicity assay. Primary human and rabbit fibroblasts were plated in 96-well plates at a density of 10,000 cells per well in RPMI 160 medium, 10% fetal calf serum, 5% L-glutamine and gentamicin. Cells were incubated for 24 h at 37 °C and transfected with 0.2 μg of pCMVβgal plasmid in combination with FuGene™6 according to the manufacturer's instructions. Mock-treated cells and cells treated with plasmid only were used as controls. Cells were incubated for 48 h. The medium was then replaced with 100 μl fresh medium. MTT solution [3-(4,5-dimethylthiazolyl-2)-2,5-diphenyl tetrazolium bromide, 10 μl from 5 mg/ml] was added and cells were incubated for 4 h. Sodium dodecyl sulphate (SDS)/dimethylformamide (DMF) buffer (20% SDS, 50% DMF, pH 4.7) was added and incubated for 18 h. Absorbance was measured at 570 nm. Survival percentage was calculated in a comparison to mock-treated cells (100% survival).

In vivo experiments: intramuscular injection of ex vivo-transfected rabbit skin fibroblasts and analysis of β-galactosidase expression. Fibroblasts were isolated from rabbit skin biopsies as described above. Cells were grown in 37-cm^2 bottles and were transfected after four passages with 1 μg pCMVβgal and 1.5 μl FuGene™6 per 50×10^4 cells. The day after transfection, fibroblasts were washed twice in PBS, placed in serum-free, antibiotic-free RPMI medium and used for in vivo experiments.

New Zealand White rabbits (female, mean weight 4000 g, n = 7) were anaesthetised with Hypnorm (0.5 ml/kg). The muscular surface of the quadriceps muscle was exposed and injected with 500 µl ex vivo-transfected fibroblasts (10 µg pCMVβgal, 50×10^4 cells). The injection site was identified with a suture.

The rabbits were allowed to recover from anaesthesia and returned to their cages. After 5 days, they were killed by standard intravenous barbiturate overdose.

Histological analysis and immunostaining. The injected segments of skeletal muscle as well as segments of non-treated muscle were removed, fixed in 4% formaldehyde/PBS (pH 7.4) for 4 h, rinsed in PBS, 0.1% bovine serum albumin, 0.25% Triton, 0.1% glycine for 4 h, rinsed a second time in the same buffer but without glycine for 30 min and then in X-gal buffer (5 mM K-ferricyanide, 5 mM K-ferrocyanide, 2 mM $MgCl_2$) for 30 min. After staining in X-gal staining solution (X-gal buffer with 1 mg/ml X-gal), blue-stained muscle regions were cut off and paraffin embedded. For microscopic analysis, 5-µm sections were cut and stained with eosin and haematoxylin. Macrophages were identified using RAM-11 antibodies (1:25 dilution; Dako).

Results

Transfection efficiency in vitro. The efficiency of non-viral gene delivery was studied in vitro using FuGene™6, Tfx™-50 and LipoTaxi™ reagents. Transfections with naked DNA or with transfection reagents alone were used as controls.

Human and rabbit fibroblasts were isolated from skin, cultured in 24-well plates and transfected with VEGF- or β-galactosidase-coding plasmids. The amount of

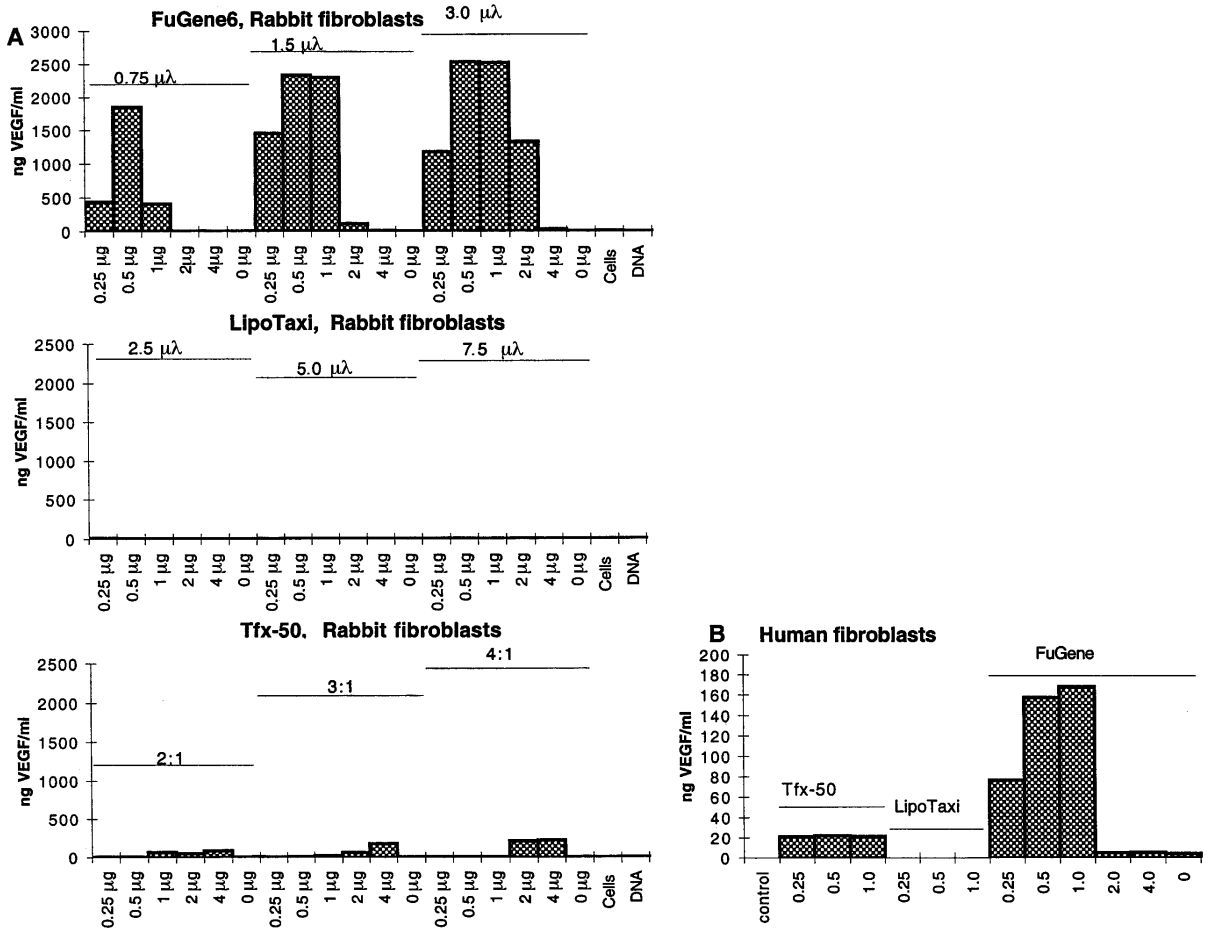

Figure 1. Transfection of primary rabbit (*A*) and human (*B*) fibroblasts with phVEGF165 plasmid complexed with FuGene™6, Tfx™-50 or LipoTaxi™ transfection reagents. Cells (50×10^4) were transfected in triplicate. The amount of VEGF secreted by cells into the medium (total volume 1 ml) is shown in ng/ml, the amount of applied plasmid in µg. VEGF expression in non-transfected cells (Cells) was taken as the background level (0 ng/ml in the figure). Transfection with naked DNA is also shown. Transfection reagents are shown as µl (FuGene™6 and LipoTaxi™) or as the ratio of transfection reagent to DNA (Tfx™-50).

Table 1. Transfection efficiency of pCMVβgal plasmid in combination with different transfection reagents in rabbit and human fibroblasts in vitro.

Transfection reagent/ well	DNA (μg)	Serum	Rabbit fibroblasts (% cells transfected)	Human fibroblasts (% cells transfected)
FuGene™6				
0.75 μl	0.5	+	40	8
1.5 μl	0.5	+	50	10
1.5 μl	1	+	50	10
3 μl	0.5	+	50	10
3 μl	1	+	50	10
Tfx-50™ (charge ratio)				
2:1	2	−	2	<0.1
3:1	2	−	2	<0.1
4:1	2	−	5	<0.1
LipoTaxi™*				
2.5 μl	0.5	+/++	<0.01	<0.001
5 μl	0.5	+/++	<0.01	<0.001
7.5 μl	0.5	+/++	<0.01	<0.001

* After 4–6 h of transfection, LipoTaxi™ requires addition of cell culture medium at twice the normal serum concentration.

DNA applied varied between 0.25 and 4 μg. Transfection was performed three times in duplicate. The production of VEGF protein was analysed by ELISA over 7 days fig. 1). The production of β-galactosidase protein was visualised after 2 days by staining with X-gal (table 1, fig. 2).

The highest degree of gene transfer was achieved with the FuGene™6 transfection reagent as analysed both by VEGF ELISA assay and X-gal staining. Up to 2,300 ng VEGF/ml medium was secreted by rabbit fibroblasts (fig. 1A) and 170 ng/ml medium by human fibroblasts (fig. 1B). Expression of the β-galactosidase gene was detected in 50% of rabbit fibroblasts and in 10% of human fibroblasts transfected with pCMVβgal in the presence of FuGene™6 (fig. 2). VEGF and β-galactosidase expression were at background levels in fibroblasts transfected in the presence of LipoTaxi™ and were also low when Tfx™-50 was used (fig. 1, table 1). The amounts of pCMVβgal plasmid and transfection reagents for X-gal analysis were selected based on the best results obtained in VEGF experiments.

Transfection efficiency was shown to depend on the concentration of DNA, the ratio of transfection reagent to DNA and the origin of the fibroblasts. An initial increase in the amount of DNA (from 0.25 to 0.5–1 μg) led to an increase in the VEGF protein level, while a further increase in applied plasmid (to 2–4 μg) caused the VEGF protein level to decrease (fig. 1).

Human fibroblasts proved to be more difficult to transfect than rabbit fibroblasts (figs 1, 2, table 1). We detected neither expression of VEGF nor of β-galactosidase proteins when cells were transfected with naked DNA.

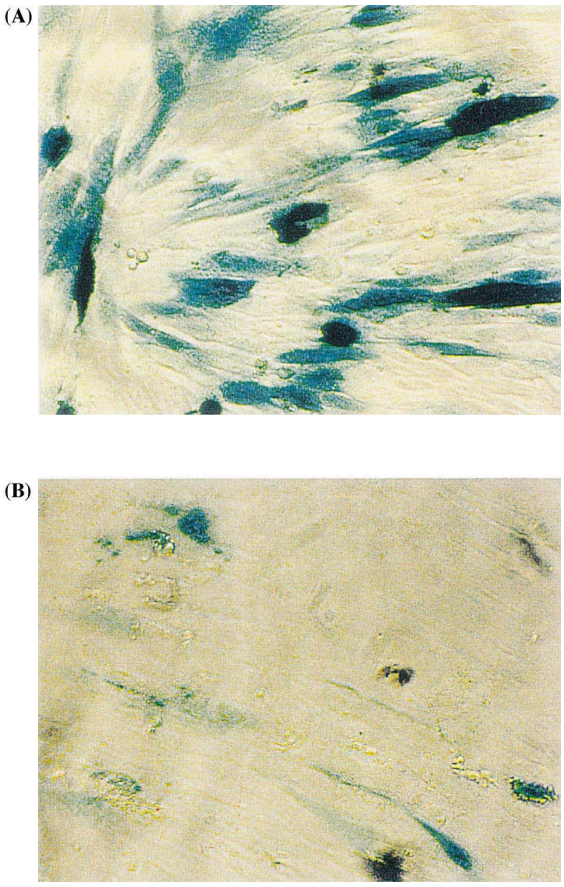

Figure 2. X-gal staining of primary rabbit (*A*) and human (*B*) fibroblasts transfected in vitro with the pCMVβgal-FuGene™6 complex. Representative micrographs are shown.

Taking into account the absence of toxicity of FuGene™6 at low doses, the optimal amount of applied DNA was found to be 0.5–1 μg per 1.5 μl FuGene™6.

Toxicity assay in vitro. The toxicity of the FuGene™6 transfection reagent was analysed by MTT assay. Fibroblast survival and the concentration of FuGene™6 were clearly correlated. The survival rate was very high, 98–100%, for cells transfected with low doses of FuGene™6 (0.75–1.5 μl FuGene™6/50×10^4 cells). However, cell survival decreased to 86% and 65% when FuGene™6 doses were raised to 3 μl and 6 μl, respectively.

In vivo experiments. On the basis of the in vitro results, FuGene™6 was selected for in vivo β-gal gene delivery via intramuscular injection of ex vivo-transfected rabbit skin fibroblasts. New Zealand White rabbits ($n = 7$) were injected intramuscularly in the upper back limb. The expression of β-galactosidase protein was analysed after 5 days by X-gal staining. A representative histology of skeletal muscles at the sites of injection is shown in figure 3.

As in in vitro studies, fibroblasts transfected ex vivo with the pCMVβgal-FuGene™6 complex showed high efficiency gene expression in vivo when injected intramuscularly. No signs of inflammation were observed in the region of fibroblast injection. Using immunostaining with RAM-11 antibodies (fig. 4), macrophages were found only in a few areas as rare cells (less than 0.001% of the total cell population). Immunostaining was similar in all skeletal muscle sections.

Discussion

Recent studies have made serious efforts to develop efficient non-viral gene delivery systems that are competitive with viral vectors. Several reporter genes such as β-galactosidase, chloramphenicol transferase or luciferase have been used to test the in vitro and in vivo transfection efficiency of various transfection reagents, mostly cationic liposomes and polymers, in combination with plasmids. In the cardiovascular field, such complexes (lipofectin [10, 11], Tfx-50 [12], other cationic liposomes [13–15], polyethylenimines and fractured polyamidoamine dendrimers [16]) have been applied to facilitate intracellular gene delivery into the vascular wall. A gene transfer method involving Sendai virus (HVJ) liposomes improved gene transfer into vascular smooth muscle cell culture [17] and into the rabbit arterial wall in vivo [18]. Using the HVJ-liposome technology, both oligonucleotides and plasmid were introduced into the myocardium by coronary infusion during cardioplegic arrest of the rat heart [19].

The aim of our investigation was to develop an efficient non-viral gene transfer system that could be useful for intramuscular plasmid delivery. For this purpose, we tested a new transfection reagent with non-liposomal formulation, FuGene™6, and compared it with two liposome-based transfection reagents, Tfx™-50 and LipoTaxi™. All three, according to the manufacturers and some publications, have high in vitro transfection efficiency for a variety of mammalian cell lines and several primary cells. Transfection with these reagents, in contrast to most of the above-mentioned liposomes, lipopolyamines and dendrimers, can be performed in the presence of serum, which facilitates the ex vivo gene delivery procedure and is important for direct in vivo intramuscular injection of the transfection reagent-plasmid complex.

We are considering injection of ex vivo-transfected primary cells as an attractive method for intramuscular gene delivery. Skin fibroblasts are interesting candidates for this purpose since they are among the most studied primary cells and are relatively easy to isolate and grow in vitro. In previous earlier in vitro and in vivo studies, only viral-mediated gene transfer was successful in transfecting primary (non-immortalised) fibroblasts, with several exceptions, such as an electroporation method used for in vitro gene delivery into primary human fibroblasts [20] or ex vivo gene transfer into primary rabbit fibroblasts [21]. However, later it was shown that some transfection reagents, such as transferrin-polylysine alone or in combination with glycerol [22, 23], or a combination of polyethylenimine and plate centrifugation [24] could be efficient for in vitro non-viral gene delivery into primary fibroblasts. In other in vitro studies, techniques such as a gene gun [25], ultrasound cell culture treatment [14], ionising radiation [26] or transfection in the presence of lysosomal protease inhibitors [27] were applied to increase the transfection efficiency of primary fibroblasts.

Here, we demonstrated high-efficiency delivery of plasmid complexed with FuGene™6. In in vitro experiments, primary rabbit and human skin fibroblasts were used to test the transfection efficiency of phVEGF165 or pCMVβgal plasmids in combination with FuGene™6, Tfx™-50 or LipoTaxi™. Among the transfection reagents tested, only the use of FuGene™6 led to high levels of VEGF or β-gal gene expression as analysed by VEGF ELISA or X-gal staining, respectively. As expected, human skin fibroblasts proved to be more difficult to transfect than rabbit fibroblasts.

In our in vivo study, we tested intramuscular gene delivery via injection of rabbit skin fibroblasts, transfected ex vivo with a plasmid complexed with FuGene™6, and demonstrated high levels of reporter gene expression. This method of gene delivery may prove to be attractive for clinical gene therapy since, in contrast to direct intramuscular injection of naked DNA or DNA complexed with transfected reagents, it is highly

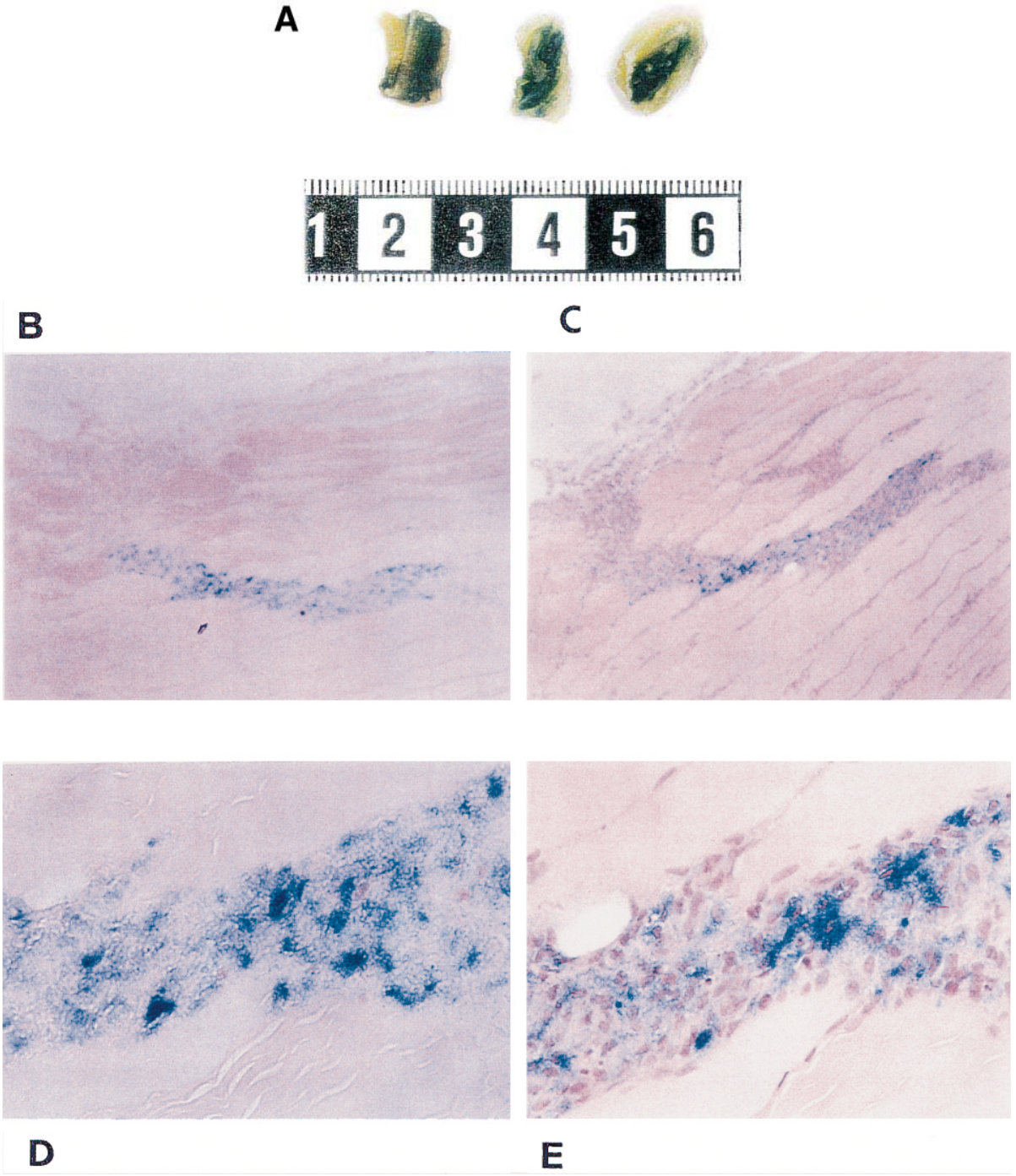

Figure 3. Histochemical analyses of rabbit skeletal muscles following injection of ex vivo-transfected rabbit skin fibroblasts. Fibroblasts were transfected with the pCMVβgal-FuGene™6 complex and injected into the rabbit upper back limb. Five days after transfection, skeletal muscles at the site of injection were isolated and analysed for β-galactosidase expression by staining with X-gal. (*A*) Representative pieces of skeletal muscles at the sites of injection (scale in cm). (*B–E*) Representative sections of muscles stained with eosin (*B*, *D*) or eosin-haematoxylin (*C*, *E*). Magnification: $\times$ 200 (*B*, *C*); $\times$ 400 (*D*, *E*).

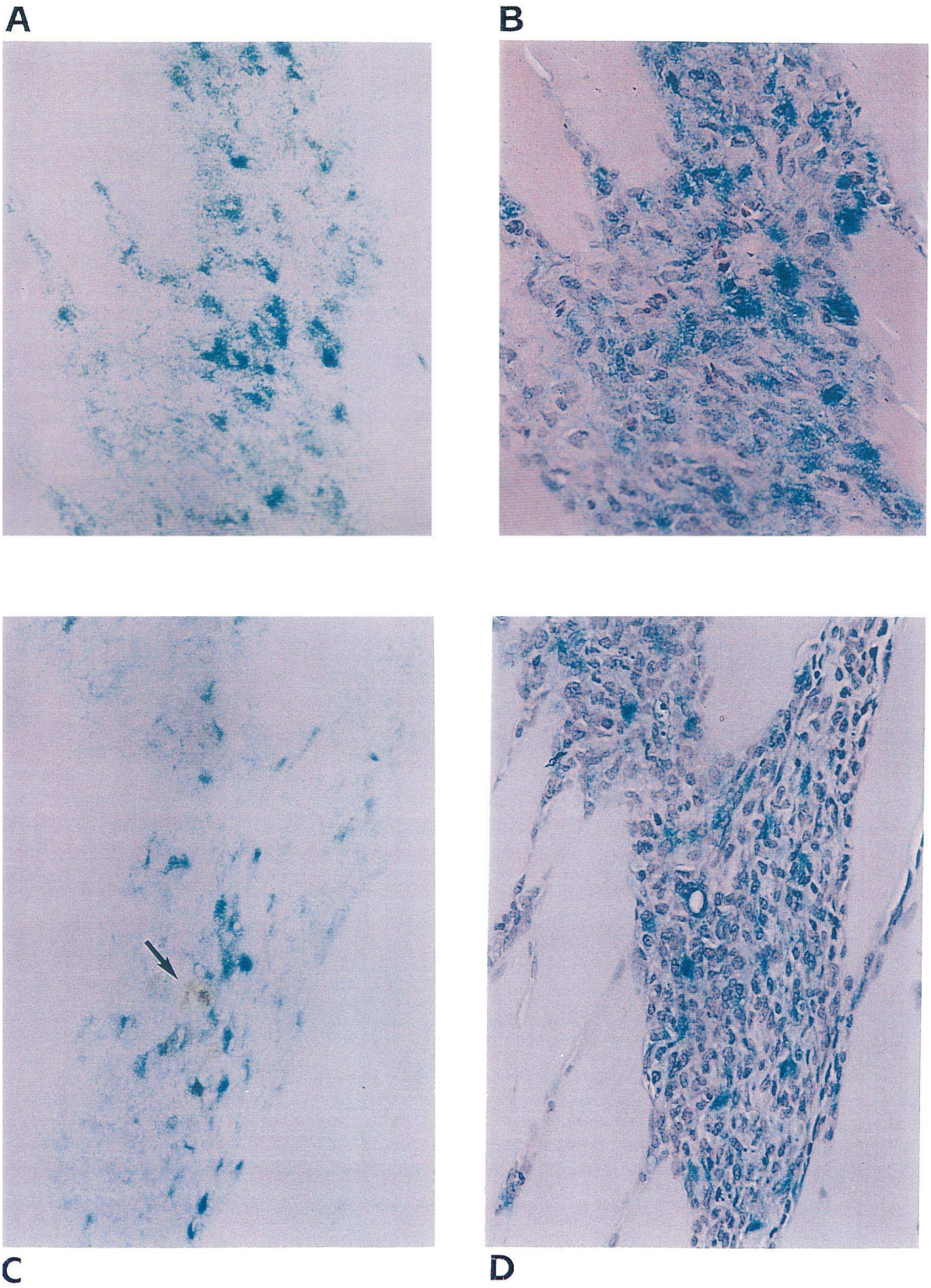

Figure 4. Histochemical analyses and immunostaining of rabbit skeletal muscles following injection of transfected ex vivo-transfected rabbit skin fibroblasts. Fibroblasts were transfected with the pCMVβgal-FuGene™6 complex and injected into rabbit upper back limb. Five days after transfection, skeletal muscles at the sites of injection were isolated and analysed for β-galactosidase expression by staining with X-gal. Macrophages were identified using RAM-11 antibodies (1:25 dilution; Dako). Magnification $\times 400$. (*A*, *C*) Representative sections stained with RAM-11 antibodies. (*B*, *D*) Corresponding sections stained with eosin-haematoxylin.

reproducible, requires small amounts of plasmid and avoids direct contact of tissues with DNA or the transfection reagent. Moreover, injection of naked DNA provides insufficient levels of gene expression. Furthermore, human fibroblasts do not immortalise spontaneously in cell culture.

In conclusion, FuGene™6 is a highly efficient transfection reagent that may be useful for therapeutic non-viral gene transfer via cell-mediated gene delivery.

Acknowledgements. We appreciate the assistance of Eva Wärdell and Anne Jämsä. This work was supported by the Swedish Medical Research Council, the Swedish Heart and Lung Foundation, The Swedish Society of Medicine Foundation, the Clas Groschinskys Minnesfond and the Karolinska Institute Research Foundation.

1 Banai S., Jaklitsch M. T., Shou M., Lazarous D. F., Scheinowitz M., Biro S. et al. (1994) Angiogenic-induced enhancement of collateral blood flow to ischemic myocardium by vascular endothelial growth factor in dogs. Circulation **89:** 2183–2189
2 Pearlman J. D., Hibberd M. G., Chuang M. L., Harada K., Lopez J. J., Gladston S. R. et al. (1995) Magnetic resonance mapping demonstrates benefits of VEGF-induced myocardial angiogenesis. Nat. Med. **1:** 1085–1089
3 Losordo D. W., Vale P. R., Symes J. F., Dunnington C. H., Esakof D. D., Maysky M. et al. (1998) Gene therapy for myocardial angiogenesis: initial clinical results with direct myocardial injection of phVEGF165 as sole therapy for myocardial ischemia. Circulation **98:** 2800–2804
4 Baumgartner I., Pieczek A., Manor O., Blair R., Kearney M., Walsh K. et al. (1998) Constitutive expression of phVEGF165 after intramuscular gene transfer promotes collateral vessel development in patients with critical limb ischemia. Circulation **97:** 1114–1123
5 Miller A. D. (1992) Human gene therapy comes of age. Nature **357:** 455–460
6 Morgan R. A. and Anderson W. F. (1993) Human gene therapy. Annu. Rev. Biochem. **62:** 191–217
7 Finkel T. and Epstein S. E. (1995) Gene therapy for vascular disease. FASEB J. **9:** 843–851
8 Nabel E. G. and Nabel G. J. (1994) Complex models for the study of gene function in cardiovascular biology. Annu. Rev. Physiol. **56:** 741–761
9 Ylä-Herttuala S. (1997) Vascular gene transfer. Curr. Opin. Lipidol. **8:** 72–76
10 Nabel E. G., Plautz G. and Nabel G. J. (1990) Site-specific gene expression in vivo by direct gene transfer into the arterial wall. Science **249:** 1285–1288
11 Takeshita S., Gal D., Leclerc G., Pickering J. G., Riessen R., Weir L. et al. (1994) Increased gene expression after liposome-mediated arterial gene transfer associated with intimal smooth muscle cell proliferation: in vitro and in vivo findings in a rabbit model of vascular injury. J. Clin. Invest. **93:** 652–661
12 Keogh M.-C., Chen D., Lupu F., Shaper N., Schmitt J. F., Kakkar V. V. et al. (1997) High efficiency gene transfection of vascular tissue in vitro and in vivo using a cationic lipid-DNA complex. Gene Ther. **4:** 162–171
13 Chapman G. D., Lim C. S., Gammon R. S., Culp S. C., Desper J. S., Bauman R. P. et al. (1992) Gene transfer into coronary arteries of intact animals with a percutaneous balloon catheter. Circ. Res. **71:** 27–33
14 Kim H. J., Greenleaf J. F., Kinnick R. R., Bronk J. T. and Bolander M. E. (1996) Ultrasound-mediated transfection of mammalian cells. Hum. Gene Ther. **7:** 1339–1346
15 Leclerc G., Dal D., Takeshita S., Nikol S., Weir L. and Isner J. M. (1992) Percutaneous arterial gene transfer in a rabbit model: efficiency in normal and balloon-dilated atherosclerotic arteries. J. Clin. Invest. **90:** 936–944
16 Turunen M. P., Hiltunen M. O., Ruponen M., Virkamäki L., Szoka Jr F. C., Urtti A. et al. (1999) Efficient adventitial gene delivery to rabbit carotid artery with cationic polymer-plasmid complexes. Gene Ther. **6:** 6–11
17 Mann M. J., Morishita R., Gibbons G. H., Leyen H. E. von der and Dzau V. J. (1997) DNA transfer into vascular smooth muscle using fusigenic Sendai virus (HVJ)-liposomes. Mol. Cell. Biochem. **172:** 3–12
18 Yonemitsu Y., Kaneda Y., Morishita R., Nakagawa K., Nakashima Y. and Sueishi K. (1996) Characterization of in vivo gene transfer into the arterial wall mediated by the Sendai virus (hemagglutinating virus of Japan) liposomes: an effective tool for the in vivo study of arterial diseases. Lab. Invest. **75:** 313–323
19 Sawa Y., Kaneda Y., Bai H. Z., Suzuki K., Fujimoto J., Morishita R. et al. (1998) Efficient transfer of oligonucleotides and plasmid DNA into the whole heart through the coronary artery. Gene Ther. **5:** 1472–1480
20 Stankovics J. and Ledley F. D. (1993) Cloning and functional alpha propionyl CoA carboxylase and correction of enzyme deficiency in pccA fibroblasts. Am. J. Hum. Genet. **52:** 144–151
21 Heartlein M. W., Roman V. A., Jiang J. L., Sellers J. W., Zuliani A. M., Treco D. A. et al. (1994) Long-term production and delivery of human growth hormone in vivo. Proc. Natl. Acad. Sci. USA **91:** 10967–10971
22 Zauner W., Kichler A., Schmidt W., Sinski A. and Wagner E. (1996) Glycerol enhancement of ligand-polylysine/DNA transfection. Biotechniques **20:** 905–913
23 Zauner W., Brunner S., Buschle M., Ogris M. and Wagner E. (1999) Differential behaviour of lipid based and polycation based gene transfer systems in transfecting primary human fibroblasts: a potential role of polylysine in nuclear transport. Biochim. Biophys. Acta **1428:** 57–67
24 Boussif O., Zanta M. A. and Behr J. P. (1996) Optimized galenics improve in vitro gene transfer with cationic molecules up to 1000-fold. Gene Ther. **3:** 1074–1080
25 Turner J. G., Tan J., Crucian B. E., Sullivan D. M., Ballester O. F., Dalton W. S. et al. (1998) Broadened clinical utility of gene gun-mediated, granulocyte-macrophage colony-stimulating factor cDNA-based tumor cell vaccines as demonstrated with a mouse myeloma model. Hum. Gene Ther. **9:** 1121–1130
26 Stevens C. W., Zeng M. and Cerniglia G. J. (1996) Ionizing radiation greatly improves gene transfer efficiency in mammalian cells. Human Gene Ther. **7:** 1727–1734
27 Coonrod A., Li F. Q. and Horwitz M. (1997) On the mechanism of DNA transfection: efficient gene transfer without viruses. Gene Ther. **4:** 1313–1321