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The uptake and expression of the factor VIII and reporter genes by vascular cells

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The conditions and efficacy of transfection of vascular cells in primary culture using DEAE-dextran, calcium phosphate and lipofectin have been investigated using chloramphenicol acetyltransferase and luciferase as reporter genes. Subsequently factor VIII was expressed in endothelial and smooth muscle cells. Both reporter genes could be expressed after transfection of umbilical vein endothelial cells, umbilical artery smooth muscle cells and fibroblasts. The expression of both reporter genes in endothelial and smooth muscle cells was highest using lipofectin. After transfection of smooth muscle cells with both full-length and mutant factor VIII genes, factor VIII activity and antigen were secreted into the culture medium, the secretion remaining stable to serial cell passage. The secretion of factor VIII from transfected smooth muscle cells was confirmed by the immunoprecipitation of [^{35}S]methionine labelled protein. Endothelial cells also were successfully transfected with the mutant factor VIII gene.

Endothelial cell; Smooth muscle cell; Transfection; Factor VIII gene

1. INTRODUCTION

The continued flow of blood through the vasculature and delivery of nutrients to metabolizing tissues depends on an intact endothelium and the vascular tone. With ageing and atherosclerosis these properties of the arterial tree are impaired: blood flow may be restricted and limbs and organs put at risk. The clinical approach is to offer bypass surgery or endovascular intervention for critical ischemia. The success of these approaches is limited either by thrombosis or the response of the vessel wall to intervention and this has not been improved by pharmacological manipulation. The rapid advances in molecular and cellular biology have provided the basis for the suggestion that gene augmentation could underlay new advances in the treatment of cardiovascular disease [1,2]. Equally the vasculature could be used to augment deficiencies of plasma components by gene therapy. Several recent reports have demonstrated the feasibility of these approaches. Retroviral vectors and cationic liposomes have been used to introduce the β-galactosidase gene into arteries subject to balloon catheter angioplasty and genetically modified endothelial cells have been used to coat the surface of prosthetic grafts [3–5].

Most attention appears to have been given to the infectious methods for genetic modification of vascular cells. Here we have compared the use of three different non-infectious procedures for inserting and expressing 'foreign DNA' in primary cultures of endothelial, smooth muscle cells and fibroblasts using chloramphenicol acetyltransferase (CAT) and luciferase as reporter genes. Using the most successful conditions we have then attempted to insert and express the much larger gene of a protein with complex post-translational modifications, factor VIII, in endothelial and smooth muscle cells.

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2. MATERIALS AND METHODS

2.1. *Cell culture*

Endothelial cells were isolated from human umbilical veins, cultured and identified as previously described [6,7]. Smooth muscle cells were isolated from explants of human umbilical artery and cultured using the same conditions as for endothelial cells. These cells were identified with an antibody specific for smooth muscle cell actin. Fibroblasts were isolated from the adventitia of human umbilical arteries and subcultured using the same media and conditions as endothelial cells. Transfection experiments were conducted using endothelial and smooth muscle cells at second or third passage and fibroblasts at passage 5–8.

2.2. *Transfection studies*

All cells were transfected at 70–80% confluence. Cells were transfected using DEAE-dextran using 10 μg DNA for each 10 cm^2 dish [8]. After 2–2.5 h the cells were shocked by the addition of chloroquine to a final concentration of 80 μg/ml for smooth muscle cells and fibroblasts but a final concentration of 40 μg/ml for endothelial cells. Calcium phosphate transfections were conducted in Iscoves medium, supplemented with 20% pooled human serum, for 4 h using 10 μg DNA for each 10 cm^2 dish [9]. Chloroquine was added to the same final concentration, as for DEAE-dextran transfections, after 2 h. Lipofections were performed in RPMI-1640 supplemented with 2 mM

glutamine, penicillin (100 U/ml) and streptomycin (100 μg/ml), according to the manufacturers instructions (GIBW-BRL). On the day after transfection cells were washed twice with RPMI-1640 and replenished with fresh medium (2 ml/10 cm^2 dish). Cells were harvested with 0.25% trypsin 2–6 days after transfection. Trypsin was subsequently inhibited with soyabean trypsin inhibitor (10 mg/ml).

2.3. *Plasmid DNA*

The different cDNAs were all constructed in the pSVL series of plasmids and purified through two CsCl gradients [9]. The RSV promoter controlled luciferase and chloramphenicol acetyltransferase (CAT) plasmids were a gift from Dr. A.J. van Zonneveld [10]. Two factor VIII plasmids were used, both with the cDNA fused to the RSV promoter: a full-length 7,436 bp insert (FL) and a 5,351 bp insert, coding for factor VIII with deletion of amino acids 867–1,562 of the B domain, factor VIII-Δ (868–1562) [11].

2.4. *Enzyme assays*

Chloramphenicol acetyltransferase and luciferase were assayed in cell extracts as previously described [12,13]. Their specific activity was calculated after determination of the protein content of cell extracts using the dye binding assay described by Bradford [14]. Factor VIII activity was determined spectrophotometrically using a chromogenic substrate and purified bovine coagulation factors (Coatest Factor VIII, KabiVitrum, Stockholm, Sweden). Factor VIII activity is expressed in U/ml where 1 U/ml represents the concentration of factor VIII in pooled normal human plasma (about 100 ng/ml). Factor VIII antigen was measured by an enzyme-linked immunoabsorbant assay, as previously described [15].

2.5. *Metabolic labelling with [^{35}S]methionine and immunoprecipitation*

After transfection and medium change endothelial and smooth muscle cells were maintained for 72 h before washing with RPMI-1640 and transfer to serum-free RPMI medium lacking methionine for a period of 1.5 h. The methionine free medium was then removed and replaced with the same medium supplemented with [^{35}S]methionine (10 μCi/ml). Cells were maintained in the [^{35}S]methionine medium for 4 h before the cells were washed twice with RPMI-1640 and replenished with full medium. After 15 h the culture medium was assayed for factor VIII activity and if appropriately positive the metabolic labelling was terminated after 18 h. The labelled media were centrifuged for 10 min at 8,000 × *g* to remove cells and debris and concentrated lysis buffer added to achieve final concentrations of 1% Nonidet P40, 0.1 M NaCl, 20 mM Tris/Cl, 4 mM EDTA at pH 7.5. Cells were lysed by addition of this same buffer and freezing at −70°C. The lysis buffer was supplemented with a cocktail of protease inhibitors: 1 mM phenylmethylsulphonyl fluoride, 0.02 mg/ml soyabean trypsin inhibitor, 10 mM benzamidine, 5 mM *N*-ethylmaleimide. Pre-clearance was achieved by successive incubation with gelatin-Sepharose at 37°C for 2 h, passage through columns (0.5 ml) of protein A-Sepharose and mouse IgG-protein A-Sepharose, prior to specific immobilization of labelled factor VIII by incubation (16 h, 4°C) with a mixture of monoclonal antibodies (CLB-Cag 9, CLB-Cag 117 and CLB-CagA) coupled to protein A-Sepharose [15]. After extensive washing (3 times with lysis buffer, twice with 0.25 M $CaCl_2$ containing 25 mM Tris/Cl, pH 7.8 and 1% Nonidet NP40, once with 1 M NaCl containing 25 mM Tris/Cl pH 7.8 and the final wash with 0.1 M NaCl containing 25 mM Tris/Cl pH 7.8) immune complexes were removed from the Sepharose by boiling in 1% SDS for 5 min and analyzed by SDS/polyacrylamide gel electrophoresis under reducing conditions followed by autoradiography.

3. RESULTS

The efficiency of transfection varies greatly according to the conditions used, such conditions including the length of transfection, concentration of DNA, concentration of chloroquine used to shock cells, and cell density. Three methods of transfection, DEAE-dextran, calcium phosphate and lipofectin, were compared using cells at 70% confluence, 10 μg DNA with an RSV promoter/10 cm^2 well and a 4 h exposure to plasmid DNA. The expression of luciferase was highest in smooth muscle cells and least in fibroblasts, irrespective of the mode of transfection (Table I). The expression of chloramphenicol acetyl transferase was similar to luciferase (data not shown). After DEAE-dextran-mediated transfection, luciferase activity was maximal at 3 days and had reduced to background for all 3 cell types after 7 days. In contrast lipofectin-mediated luciferase activity remained stable between 3 and 14 days and was stable to at least one cell passage (1:2 split) in all 3 cell types.

After transfection of smooth muscle cells with factor VIII genes with lipofectin, factor VIII activity and factor VIII antigen could be detected in the culture medium (Table II). Three days after transfection with factor VIII Δ(865–1562) construct the mean activity of the culture medium was 3.5 ± 1.8 mU/ml (n = 8) with a concentration of 2.5 ± 1.2 mU/ml being detected by ELISA. After transfection with full-length factor VIII cDNA the mean activity of the culture medium was only 1.0 ± 0.8 mU/ml 72 h after transfection but rose to 1.8 ± 1.2 mU/ml 6 days after transfection (n = 9); antigen was not detectable 3 days after transfection but was detected 6 days after transfection (1.8 ± 1.1 mU/ml). The amount of factor VIII secreted into the culture medium could be increased by process of differential adhesion. Brief trypsinization (2 min, 37°C) removes a population of cells which, after replating and growth, secreted very little factor VIII activity into the culture medium. After growth of the residual cells to confluence the factor VIII-Δ(868–1562) activity in the medium increased 2–3-fold to 8.5 ± 2.8 mU/ml or antigen concentration to 5.5 ± 1.7 mU/ml. The stability of factor VIII (Δ695) gene expression by transfected smooth muscle cells was investigated by serial passage of cells after lipofection. All the cells were detached by trypsinization (7 min, 37°C), subcultured at half density and grown up to

Table I

Comparison of non-infectious methods of transfection in primary vascular cells

Cell	Luciferase (ng/mg protein) after transfection with		
	DEAE dextran	Calcium phosphate	Lipofectin
Endothelial	8.9 ± 1.0	28.0 ± 1.7	86.7 ± 16.7
Smooth muscle	8.2 ± 0.3	18.9 ± 7.7	65.3 ± 11.4
Fibroblast	1.4 ± 0.5	11.8 ± 3.0	5.6 ± 0.5

The cells were exposed to 10 μg RSV luciferase DNA for 4 h using DEAE-dextran, calcium phosphate or lipofectin as the carrier. Cells were harvested 72 h following the termination of transfection, lysed by freeze-thaw cycles and luciferase, and protein was determined in the cell extracts. The results are given as the mean ± S.D. of 6 experiments.

Table II

Lipofectin-mediated transfection of smooth muscle cells with factor VIII expression plasmids

Cell passage	Factor VIII cDNA	Time after transfection (days)	Activity mU/ml ± S.D.	Antigen mU/ml ± S.D.	n
3	full length	3	1.0 ± 0.8	nd	6
3	full length	6	1.8 ± 1.2	1.8 ± 1.1	5
3	Δ(868–1562)	3	3.5 ± 1.8	2.5 ± 1.2	8
3	Δ(868–1562)	6	4.0 ± 0.9	3.0 ± 0.7	4
3	Δ(868–1562)	7	8.5 ± 2.0	7.5 ± 2.0	2*
4	Δ(868–1562)	9	11.2 ± 3.6	15.5 ± 5.2	(4 flasks)
5	Δ(868–1562)	11	8.2 ± 1.8	14.1 ± 3.3	(8 flasks)
6	Δ(868–1562)	15	6.7 ± 1.1	13.0 ± 2.2	(8 flasks)
7	Δ(868–1562)	18	7.8 ± 0.8	13.2 ± 2.3	(8 flasks)
8	Δ(868–1562)	22	8.1 ± 0.9	9.1 ± 2.4	(8 flasks)

*The experiment was repeated twice, the smooth muscle cells being derived from two separate transfections of smooth muscle cells in their third passage. A maximum of four flasks (80 cm^2) were retained after each successive passage, each flask contained ~5×10^5 cells. nd, not detectable.

confluence. Secretion of factor VIII activity and antigen was stable through 5 passages (Table II). Immunofluorescent microscopy demonstrated intracellular localization, probably associated with Golgi membranes, of factor VIII protein in a few of these cells (Fig. 1).

The expression of factor VIII and factor VIII-Δ(868–1562) by transfected smooth muscle cells was confirmed by metabolic labelling of the proteins with [^{35}S]methionine. From smooth muscle cells transfected with the mutant factor VIII-Δ(868–1562) plasmid-lipofectin micelles a strong, sharp band of about 190 kDa was visible after resolution on 6% acrylamide gels and autoradiography (Fig. 2). For smooth muscle cells transfected with full-length factor VIII plasmid–lipofectin micelles only a weak, broad band was observed at high molecular weight, 320 kDa (Fig. 2). No specific bands were observed after immunoprecipitation of the culture medium of smooth muscle cells similarly transfected with chloramphenicol acetyltransferase plasmid.

Extensive modification of the transfection conditions was necessary before any secretion of factor VIII activity and antigen could be detected. Endothelial cells, in 25 cm^2 flasks, were exposed to micelles of DNA and lipofectin (10 μg DNA, 35 μg lipofectin in 100μl of water) in serum free medium for 2.5 h before the addition of Ultroser G to a final concentration of 1%. After 15 h, the cells were washed and fed with complete me-

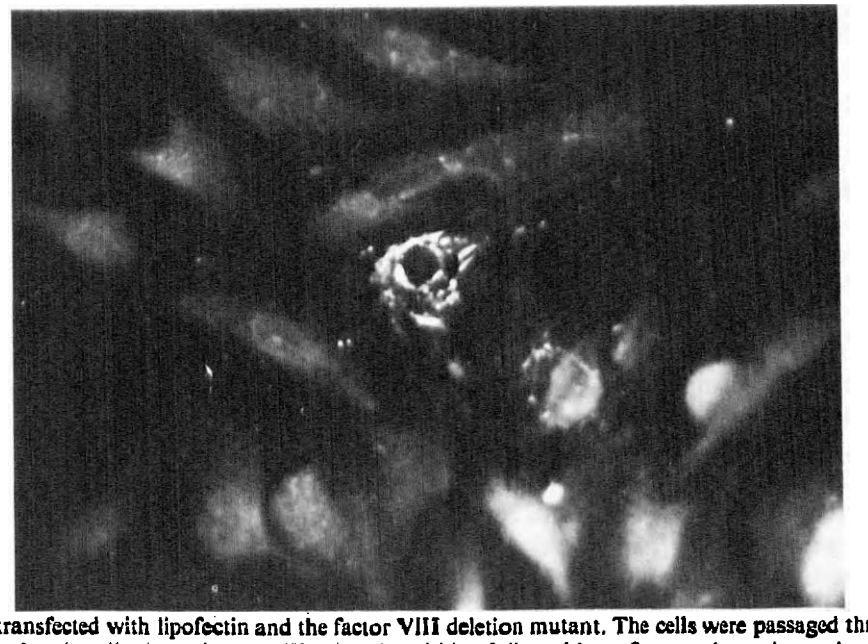

Fig. 1. Smooth muscle cells transfected with lipofectin and the factor VIII deletion mutant. The cells were passaged three times after transfection, fixed and reacted with a polyclonal antibody to factor VIII raised in rabbits, followed by a fluorescein conjugated second antibody. A few cells show bright staining of intracellular organelles, possibly Golgi membranes; final magnification ×315. Cells transfected with chloramphenicol acetyltransferase do not show these structures when similarly stained.

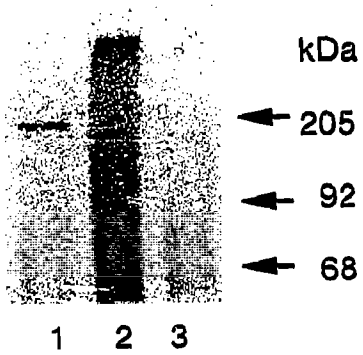

Fig. 2. Immunoprecipitation and resolution of metabolically labelled factor VIII secreted by transfected smooth muscle cells. Smooth muscle cells were transfected with chloramphenicol acetyltransferase, the factor VIII deletion mutant, factor VIII-Δ(868–1562) and full-length factor VIII using lipofectin. The cells were labelled with [^{35}S]methionine, factor VIII immunoprecipitated from the culture medium amd separated on polyacrylamide gels, as described in the text. The autoradiograph shows the results from the factor VIII deletion mutant transfected cells in lane 1, from cells transfected with full-length factor VIII in lane 2 and from chloramphenicol acetyltransferase transfected cells in lane 3. The molecular weight calibration is shown on the right hand side.

dium: about 10–15% of cells remained. After 7 days the cells had again reached confluence and factor VIII activity (1–2 mU/ml) could be detected in the medium of cells transfected with the Δ(868–1562) plasmid. After detaching and discarding about half the cells by brief trypsinization (2 min, 22°C) the remaining cells were allowed to again grow to confluence and the accumulation of factor VIII in the medium increased to 4–5 mU/ml and antigen could be detected in the medium (3 mU/ml). Factor VIII secretion could not be detected from endothelial cells similarly transfected with CAT or full-length factor VIII plasmids.

4. DISCUSSION

Chloramphenicol acetyltransferase and luciferase are small intracellular proteins whose genes have been used widely in transfection experiments to report both the efficiency of transfection and to study the activity of promoters fused to these reporter genes: usually, for such studies established cell lines are used. Here experiments with reporter genes clearly demonstrated that cultured primary cells of the normal vasculature, including endothelial, smooth muscle cells and fibroblasts, also could take up plasmid DNA and transiently express luciferase and/or chloramphenicol acetyltransferase. In additon, the present study demonstrates that the factor VIII gene, a gene that both in a transient manner and in established cell lines is difficult to express, is readily expressed in smooth muscle cells, particularly when lipofectin was the carrier of DNA into the cells [16,17].

The procedures for lipofection appear to alter the interactions of cultured cells with their subtratum. Irrespective of the expression plasmid used, endothelial, smooth muscle cells and fibroblasts resisted complete detachment under the normal conditions of trypsin detachment. After lipofection, endothelial cells also became resistant to detachment with EGTA. We have no evidence concerning the mechanism of this altered cell–substratum adhesion. The transfection techniques evaluated here provided a rationale to examine the expression of genes that may modulate the haemostatic potential of the vasculature in a transient manner, a potential application of the present study. Lipofection became the method of choice to attempt to introduce the factor VIII gene, and a mutant thereof, in endothelial and smooth muscle cells.

Factor VIII, a protein that is deficient in haemophilia A and von Willebrand's disease, is a large molecule, the cDNA encodes a single chain of 2,332 amino acids which can be divided into six domains [18,19]. The largest of these, the B-domain occupies over 900 amino acids in the middle of the polypeptide chain and contains most of the potential glycosylation sites [16,19,20]. The construction and expression of deletion mutants has indicated that this domain is not necessary to the function of factor VIII [21]. The reduction in length of the cDNA encoding such deletion mutants appears to be advantageous for the expression of factor VIII activity after transfection of mammalian cell lines [16,17,21]. For these reasons we attempted the transfection of vascular cells with both expression plasmids containing the full-length factor VIII cDNA and a mutant with 695 amino acids of the B-domain deleted, factor VIII-Δ(868–1562) [11]. We too observed increased expression of factor VIII activity and/or antigen after transfection of muscle cells with this deletion mutant as compared with full-length factor VIII plasmids. It has become accepted that factor VIII is difficult to express efficiently in mammalian cell lines [16]. Again we made similar observations after lipofection of smooth muscle cells, these cells expressing the reporter genes 1–2 orders of magnitude more efficiently than the deletion mutant: maximum expression of factor VIII-Δ(868–1562) protein about 0.5 ng/10 cm^2 well compared with luciferase, about 20 ng/10 cm^2 well.

Lipofectin permits the stable integration and expression of foreign genes in mammalian cells and may explain why the factor VIII secretion from successfully transfected smooth muscle cells is stable through several cell passages (Table I). Factor VIII protein could be immunolocalized to cytoplasmic organelles, probably the Golgi, in a few cells (Fig. 1). This would be appropriate since the Golgi is the site of sulphation and terminal glycosylation of secreted proteins; the deletion mutant factor VIII-Δ(868–1562) contains both sulphation and glycosylation sites [16,17,19]. This mutant and probably full-length factor VIII appear to be secreted as single chain proteins, apparent molecular weights 190

kDa and 320 kDa respectively (Fig. 2). A molecular weight of 330 kDa has been reported previously for the mature, glycosylated full-length factor VIII [22]: the considerably smaller size of the mutant protein probably results from the deletion of most of the glycosylation sites. The expression of both these forms of factor VIII as single chains might appear surprising in view of the complex proteolytic processing observed in factor VIII preparations from human plasma, nevertheless previous expression studies in COS cells have also demonstrated the secretion of factor VIII as a single chain [17].

A potential application of the present study is the transfection of genes which modulate haemostasis at the blood–vessel interface. It is evident that primary vascular cells readily can be transfected by small reporter genes, without using retroviral vectors or other infectious techniques. Significant expression of such reporter genes by endothelial and smooth muscle cells can be obtained after even brief lipofection and provides for stable expression of augmented genes. The potential of this technique to gene augmentation in cardiovascular disease may be limited by the difficulties of augmenting cells with the larger genes of more complex proteins or the specific biology of each particular vascular cell. Much remains to be learnt before such technologies find application in vascular medicine.

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