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In vivo production of human factor VIII in mice after intrasplenic implantation of primary fibroblasts transfected by receptor-mediated, adenovirus-augmented gene delivery

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ABSTRACT **Hemophilia A is caused by defects in the factor VIII gene. This results in life-threatening hemorrhages and severe arthropathies. Today, hemophiliacs are treated with human blood-derived factor VIII. In the future, it may be possible to use gene therapy to avoid long-term complications of conventional therapy and to improve the quality of life. However, initial gene therapy models using retroviral vectors and nonviral gene transfer techniques to introduce factor VIII gene constructs have been hampered by low expression levels of factor VIII. We show here that high expression levels of the B-domain-deleted human factor VIII in primary mouse fibroblasts and myoblasts are obtained by using receptor-mediated, adenovirus-augmented gene delivery (transferrinfection). We demonstrate that, presumably owing to the high molecular weight of factor VIII or its metabolic instability, secretion into the blood and attainment of therapeutic *in vivo* levels of factor VIII is achieved only if transfected autologous primary fibroblasts or myoblasts are delivered to the liver or spleen, but not if myoblasts are implanted into muscle, a strategy known to be successful for factor IX delivery.**

In hemophilia A there is a genetic defect of the coagulation factor VIII, a trace plasma glycoprotein, which acts as a cofactor for factor IX in the activation of factor X (reviewed in refs. 1 and 2). The factor VIII gene encodes a protein of 2351 amino acids containing six domains (3–5). The central, B domain of 980 amino acids, to which high-mannose oligosaccharides are added in the endoplasmic reticulum, is discarded during proteolytic activation of factor VIII (1, 3, 5). The B-domain-deleted form of the factor VIII gene has been chosen for use in gene transfer experiments with recombinant retrovirus because (*i*) the retroviral vectors do not readily accommodate the full-length cDNA (8.8 kb) and (*ii*) the B-domain-deleted protein is more easily processed in transduced cells than the full-length protein (6–9). However, the expression of factor VIII obtained with these retroviral vectors was too low for *in vivo* generation of detectable plasma levels of factor VIII (10). The insufficient synthesis rates after retrovirus-mediated transduction have been explained, at least in part, by the observation that sequences of the factor VIII gene interfere with its own transcription, resulting in low titers of the retroviral vectors and in low factor VIII production rates in the transduced cells (ref. 11 and R. C. Hoeben, personal communication). With "transferrinfection," neither the problem of interference of factor VIII sequences with vector production nor such size restriction for the delivery of DNA molecules exists, because the gene, highly condensed by polylysine-modified transferrin, is carried on the surface of the adenovirus (refs. 12–15 and unpublished results). Moreover, transferrinfection has been shown to introduce ≈20 copies of genes into cells, which correlates with high expression rates. To make our data directly comparable with published work, we have used the truncated version of the factor VIII gene in the present study.

Our aim is to test whether the specific technical features of transferrinfection permit increased factor VIII expression in various cell types. Moreover, it is necessary to define the *in vitro* expression level of factor VIII which is required for future gene therapy protocols for hemophilia A. We demonstrate here that the factor VIII expression rates of transferrinfected cells exceed the values which have been obtained with other gene transfer techniques by a factor of 10–100 and that production rates of 1000 milliunits per 10^6 cells in 24 hr are sufficient to generate therapeutic plasma levels in mice after implantation of *ex vivo* transfected cells. Comparison of various cell implantation strategies reveals that direct secretion of the factor VIII into the blood is a prerequisite for successful *in vivo* production.

MATERIAL AND METHODS

Isolation of Primary Cells. Primary cells were isolated from the hindlimb muscles of 4-week-old C3H/He mice by repeated digestions of minced muscle tissue with 0.25% trypsin and 0.1% collagenase in phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (BSA), at 37°C. The enzymatically released cells were mixed with an equal volume of Dulbecco's modified Eagle's medium (DMEM) plus 20% fetal bovine serum, filtered, and centrifuged at 500 × *g* for 15 min. The cells were then resuspended in DMEM plus 20% fetal bovine serum and plated onto 10-cm culture dishes. After 60 min the nonadherent cells were found to be greatly enriched for myoblasts, whereas the adherent cells were predominantly fibroblasts; the myoblast-enriched, nonadherent fraction was seeded onto laminin-coated dishes.

Generation of the Factor VIII Gene Construct. The full-length factor VIII construct pF8CISa (16), a gift from Genentech, was digested with *Cel* II. The resultant 5648-bp *Cel* II fragment containing the B domain was replaced by a 2990-bp *Cel* II fragment isolated from the B-domain-deleted clone pUC/F8-dB2.6 (deletion from amino acid 746 to amino acid 1638), which was obtained from R. C. Hoeben (7). The 2990-bp fragment was ligated into pF8CISa so that the resulting expression vector pF8-dB2.6 was identical to the full-length F8 construct except for the B-domain deletion. The expression vector contained a cytomegalovirus promoter/enhancer followed by a synthetic intron and the B-domain-deleted factor VIII cDNA; 3′ of the cDNA there was a simian virus 40 poly(A)-addition sequence. The vector in-

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Abbreviation: BSA, bovine serum albumin.

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cluded also a β-lactamase gene, a prokaryotic origin of replication, and a dihydrofolate reductase gene.

Transfection of Cells. Cells (5×10^5) were transferrinfected with 2–12 × 10^9 particles of the biotinylated human E1A-defective adenovirus type 5 (dl312) which had been modified with 600 ng of streptavidin-polylysine and complexed to 6 μg of factor VIII expression plasmid. Then 6.8 μg of transferrin-polylysine was added (14). The resulting complexes were mixed with 1.5 ml of DMEM containing 2% fetal bovine serum, incubated with the cells for 4 hr, and then removed and replaced by 2 ml of DMEM containing 10% fetal bovine serum.

Immunofluorescence Microscopy. Transferrinfected C2C12 myoblasts were fixed with acetone:methanol (1:1) at −20°C for 10 min, washed with PBS, and incubated for 30 min at room temperature with an antibody directed against the human factor VIII:c antigen (ESH 4, American Diagnostica, Greenwich, CT) diluted 1:10 in PBS. After the cells were washed with PBS, a Texas Red-conjugated anti-mouse immunoglobulin antibody (EY Laboratories) diluted 1:20 in PBS was applied for 30 min to the cells. Thereafter the cells were washed three times with PBS, rinsed briefly in water and in 96% ethanol, and mounted with Moviol.

***In Vivo* Production of Factor VIII.** Primary mouse fibroblasts (from the first passage) and C2C12 myoblasts were transferrinfected with the B-domain-deleted factor VIII construct as described above. Six hours after transfection the cells were trypsinized, washed, and suspended at 1 × 10^6 cells per 100 μl of Earle's buffered salt solution (1 unit of heparin was added to the preparation for mice 5 and 6). After laparotomy, 1 × 10^6 cells were injected into the spleens of six female C3H/He mice (4 weeks old) that had been anesthetized with Avertin. The injection sites were then ligated and the abdominal wounds were closed. Alternatively, 1 × 10^6 C2C12 myoblasts were implanted into the musculus quadriceps femuris essentially as described (17). Blood was collected by cardiac puncture 24 hr later and factor VIII concentrations were determined with a sandwich ELISA specific for human factor VIII. For negative control, plasma from mice which had received implantations of primary fibroblasts transferrinfected with a luciferase reporter gene were analyzed in parallel.

Analysis of Factor VIII Production. In the *in vitro* experiments the factor VIII activity that was released into the medium within 24 hr was measured with a chromogenic substrate assay (Coatest; KabiVitrum, Mölndal, Sweden). For measurement of *in vivo* produced human factor VIII an ELISA which does not react with murine factor VIII has been established. The ELISA is based on two monoclonal antibodies directed against human factor VIII (ESH 4 and ESH 8; American Diagnostica). ESH 4 (25 μg/ml in 0.1 M $NaHCO_3$/0.5 M NaCl, pH 9.0) was coupled to the ELISA plates overnight at 4°C, washed with 0.1% Tween 20 in PBS, and blocked with 1% BSA in PBS. The samples were applied in 0.05 M Tris·HCl/1 M NaCl/2% BSA, pH 7.5, over 4 hr at room temperature, the plates were washed, and ESH 8 (2.5 μg/ml in 0.05 M Tris·HCl/1 M NaCl/2% BSA, pH 7.5) that had been biotinylated with *N*-hydroxysuccinimidobiotin (Pierce) was added for 2 hr at room temperature. The color reaction was performed with peroxidase-conjugated streptavidin (Boehringer Mannheim) and *o*-phenylenediamine dihydrochloride as substrate. For all factor VIII measurements, the international human factor VIII:c standard (from the National Institute for Biological Standards and Control, Hertfordshire, U.K.) and normal mouse plasma were used as references.

RESULTS

***In Vitro* Production of Factor VIII in Primary Cells and Cell Lines.** Primary myoblasts and primary fibroblasts transferrinfected with the B-domain-deleted factor VIII produced between 620 and 1250 milliunits of factor VIII within 24 hr per 10^6 cells (Table 1), which exceeded by a factor of 10–100 the values reached by retroviral transduction or nonviral gene transfer techniques (6–9, 11, 18, 19). Surprisingly, primary fibroblasts were even more active in producing the B-domain-deleted factor VIII than the 3T3 fibroblast cell line, although the cell line expressed a luciferase reporter gene driven by an identical cytomegalovirus promoter/enhancer, which served as an indicator for the transfection efficiency, at higher levels than the primary cells (Table 1). This suggests that primary cells are more suited for the production of active B-domain-deleted factor VIII than established cell lines, perhaps because they support transcription or the posttranslational maturation steps in a more efficient manner than corresponding cell lines.

Long-Term Factor VIII Production in Muscle Cells. Transferrinfected genes are expressed transiently in proliferating cells, but expression is protracted in growth-arrested cells almost to the lifespan of the cells *in vitro* (14, 15). To test whether factor VIII can be expressed for prolonged periods in muscle cells, C2C12 murine myoblasts were transferrinfected with the B-domain-deleted human factor VIII gene and were treated with horse serum to induce differentiation of myoblasts into nondividing myotubes. The initial factor VIII production per 24 hr was in the range of 4000 milliunits per 10^6 cells (Fig. 1). After the differentiation stimulus, which led to formation of myotubes from about two-thirds of the myoblasts, factor VIII production remained at high levels, in the range of 1000 milliunits per 10^6 cells per 24 hr for the first 3 weeks of culture and then after 35 days declined to ≈50 milliunits per 24 hr. The factor VIII values at the end of the observation period do not take into account that a considerable fraction of the cells (in transfected dishes as well as in nontransfected controls) had already died, whereas the calculation of the factor VIII activities was normalized to the cell number at the beginning of the experiment.

Factor VIII Production in Myoblasts and Myotubes. Immunofluorescence microscopy revealed a positive factor VIII reaction in up to 40% of the C2C12 myoblasts transferrinfected with the truncated factor VIII gene construct (Fig. 2*A*). However, there were fewer cells positive for factor VIII as compared to the number of β-galactosidase-positive cells seen in myoblasts transferrinfected in parallel with a β-galactosidase reporter gene as an independent indicator of the transfection efficiency (data not shown). After induction of differentiation, myotubes reactive for factor VIII (Fig. 2*B*) were observed, which indicates that neither the transferrin-

Table 1. Capacity of various primary cells and cell lines for production of B-domain-deleted human factor VIII

Cell type	Human factor VIII, milliunits per 10^6 cells in 24 hr		Luciferase activity, light units per 10^3 cells
	Exp. 1	Exp. 2	
pFB	800 ± 44	1247 ± 220	2,923 ± 255
3T3	448 ± 36	612 ± 36	18,364 ± 536
pMB	616 ± 79	1164 ± 36	3,860 ± 697
C2C12	735 ± 89	827 ± 42	9,545 ± 761

Primary mouse fibroblasts (pFB) and myoblasts (pMB) as well as the fibroblast cell line NIH 3T3 and the myoblast cell line C2C12 were transferrinfected in two independent series of experiments (Exp. 1 and Exp. 2) in triplicates. The indicated factor VIII activities represent mean values of each of the series with the standard deviations indicated. An international factor VIII:c standard served as reference. For negative control of the factor VIII assay, and as a parameter for the transfection efficiency, cells were transferrinfected in parallel to the Exp 2 series with a cytomegalovirus-driven luciferase reporter gene instead of the factor VIII construct.

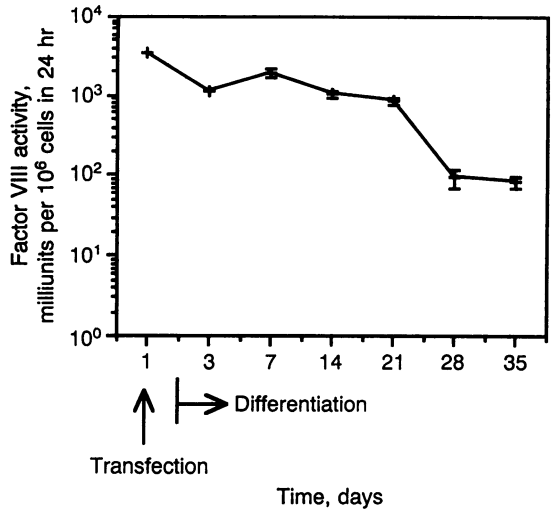

FIG. 1. Long-term expression of factor VIII. C2C12 myoblasts (5×10^5) were transferrinfected with the B-domain-deleted human factor VIII and induced to differentiate into myotubes 48 hr after transfection by incubation for 5 days in DMEM containing 2% horse serum. Factor VIII production per 24 hr was measured (Coatest; KabiVitrum) at the indicated time points after transfection. The factor VIII activities represent mean values of three parallel transfections with the standard deviations indicated by bars.

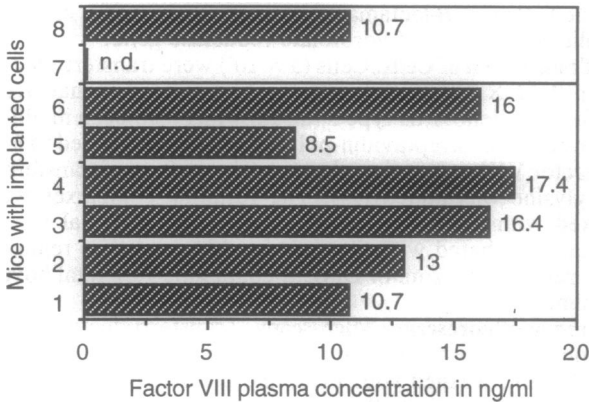

FIG. 3. *In vivo* production of factor VIII after implantation of transferrinfected cells into mice. Primary fibroblasts were implanted into the spleen of mice after transferrinfection with B-domain-deleted human factor VIII (mice 1–6). In parallel, transferrinfected C2C12 myoblasts were implanted into the muscle (mouse 7) or into the spleen (mouse 8) of mice. The generated plasma concentrations of human factor VIII were measured with an ELISA that is specific for human factor VIII (values represent ng of factor VIII per ml of plasma; n.d., not detectable).

fection nor the high-level production of factor VIII prevented myoblast differentiation.

Short-Term Generation of Therapeutic Factor VIII Plasma Levels in Mice. Primary fibroblasts were transferrinfected with the B-domain-deleted factor VIII gene construct and implanted into the spleens of syngeneic mice. For detection of the *in vivo* produced human factor VIII in normal mice, an ELISA specific for human factor VIII has been developed. All animals implanted with 1×10^6 cells had factor VIII plasma levels ranging from 8.5 to 17.4 ng/ml (mean value, 13.7 ng/ml) at 24 hr after cell transfer (Fig. 3), which clearly exceeded the 5-ng/ml limit anticipated as necessary for a therapeutic effect (normal factor VIII concentration in the plasma is between 50 and 200 ng/ml) (2). However, only negligible levels of factor VIII were found at 48 hr. The reason for this rapid turn-off is not known. As revealed by implantation of primary fibroblasts transferrinfected with a luciferase reporter gene into the spleen, the cells settled in the spleen and liver, but no luciferase activity was found in lung, kidney, ovary, or testis (data not shown). In an alternative approach, transferrinfected C2C12 myoblasts, which produced *in vitro* amounts of factor VIII similar to those produced by primary fibroblasts (see Table 1), were implanted into the musculus quadriceps femuris of mice. In this case, however, the animals did not generate detectable plasma levels of factor VIII. To demonstrate that the absence of human factor VIII in the circulation was not due to insufficient *in vivo* production of factor VIII by myoblasts, transferrinfected C2C12 myoblasts were implanted into the spleen of a control mouse. This animal revealed similar factor VIII plasma levels as had been obtained with transferrinfected fibroblasts.

DISCUSSION

The development of gene therapy for hemophilia A is dependent on a gene delivery system which guarantees high pri-

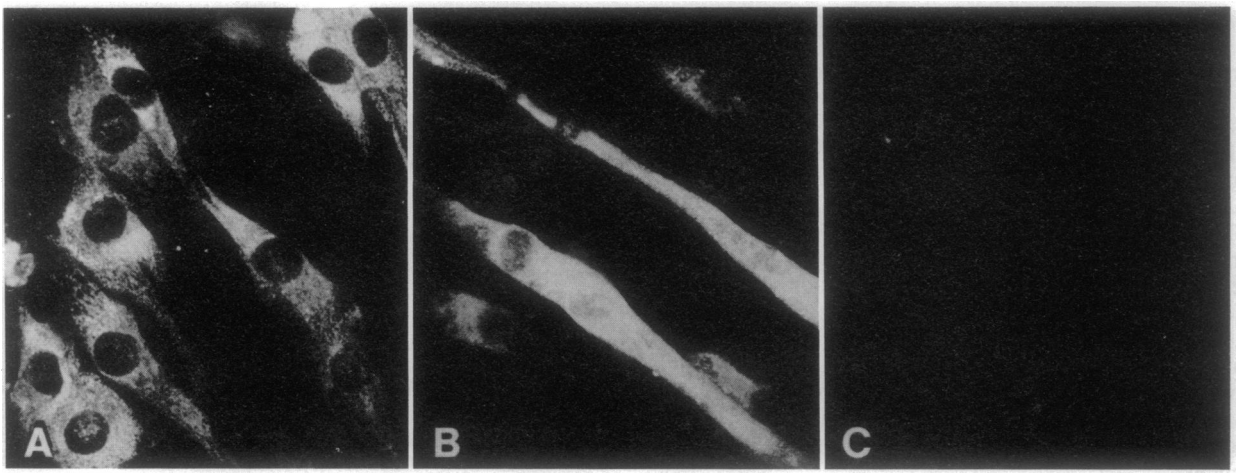

FIG. 2. Immunohistochemical detection of factor VIII in transferrinfected C2C12 myoblasts and myotubes. (*A*) C2C12 myoblasts transferrinfected with the B-domain-deleted human factor VIII. (*B*) Transferrinfected C2C12 myoblasts were induced to differentiate into myotubes after gene delivery. (*C*) Negative control with C2C12 myoblasts transferrinfected with a cytomegalovirus promoter/β-galactosidase reporter gene construct. The immunofluorescence reactions were performed on C2C12 myoblasts (*A* and *C*) 48 hr after transfection and after induction of differentiation into myotubes (*B*) at day 7 after transfection. (×250.)

mary transfection efficiencies and does not require selection for transfected cells, since for this therapy primary patient cells are the target for genetic modification. Therefore the strategies (i.e., selection for stable expression and amplification of the inserted gene) which have been previously used to increase factor VIII expression in transfected cell lines for *in vitro* production of recombinant factor VIII are not applicable for gene therapy (20). Transferrinfection is characterized by its high transfection efficiency, with detectable gene expression in 30% to almost 100% of the transfected cells. Moreover, transferrinfection introduces multiple copies of the gene into the target cells, which leads to very high expression rates. Israel and Kaufman (19) have demonstrated in stably transfected cells that there is a correlation between the number of amplified copies of the factor VIII gene construct and the amount of secreted factor VIII.

The cell types which normally secrete factor VIII have not been definitely identified, but liver transplantation rectifies hemophilia A and factor VIII mRNA is detectable in the liver and in isolated hepatocytes, which suggests that liver cells are a primary site for factor VIII synthesis (21–24). For gene therapy of hemophilia A, it may be possible to implant genetically modified cells other than hepatocytes. Primary fibroblasts and myoblasts are interesting target cells for ectopic expression of factor VIII, since these cells can be easily isolated from patients and allow high transfection efficiencies *in vitro*. There are many groups which have demonstrated secretion of proteins into the blood from *ex vivo* transfected cells after implantation into various sites (reviewed in ref. 25). For instance, transfected myoblasts can be implanted into the muscle of mice, which leads to cell fusion between the myoblasts and preexisting muscle fibers. This is a very promising approach, since upon fusion of the myoblasts with the muscle fibers the genetic information is transferred into the muscle fiber, thus generating long-lived genetically modified cells. An alternative technique for *in vivo* production of recombinant proteins is to implant transfected fibroblasts into the subcutis or into the peritoneum. However, Hoeben *et al.* (10) reported that subcutaneous implantation of 5×10^6 fibroblasts that were retrovirally transduced with a B-domain-deleted factor VIII and encapsulated in a collagen matrix generated no detectable plasma levels of factor VIII. In contrast to this finding with factor VIII, it was reported that factor IX was released into the circulation from myoblasts that had been implanted into the muscle (17, 26, 27). We found that intramuscular implantation of myoblasts producing high amounts of factor VIII *in vitro* resulted in no detectable blood levels (Fig. 3, animal 7), whereas implantation of myoblasts or fibroblasts into the spleen and liver generated plasma levels which clearly exceeded the limit required for therapy. One possible explanation for these discrepancies is that factor VIII is a much larger molecule than factor IX, and, therefore, diffusion from the muscle or subcutaneous implantation site into blood or lymph capillaries is restricted. Moreover, rapid association of newly synthesized factor VIII with von Willebrand factor in the blood is required for stabilization of factor VIII (1). The implantation of factor VIII-producing cells at a site where direct secretion of factor VIII into the blood is possible, as is the case in the liver or spleen, appeared to be essential.

These data demonstrate the high capacity of transferrinfection for the delivery of truncated factor VIII gene constructs into primary cells. The expression, which was obtained without selection for transfected cells and/or amplification of the introduced factor VIII gene construct, was sufficient for the generation of therapeutic blood levels in mice. For application of this gene transfer technique in gene therapy of hemophilia A, further developments in the field of cell transplantation technology are required so that long-term survival of *ex vivo* modified cells can be achieved. Moreover, improvements in the factor VIII vector design are expected to further increase factor VIII production in transfected cells (11).

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