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Toward Gene Therapy for Cystic Fibrosis Using a Lentivirus Pseudotyped With Sendai Virus Envelopes

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Gene therapy for cystic fibrosis (CF) is making encouraging progress into clinical trials. However, further improvements in transduction efficiency are desired. To develop a novel gene transfer vector that is improved and truly effective for CF gene therapy, a simian immunodeficiency virus (SIV) was pseudotyped with envelope proteins from Sendai virus (SeV), which is known to efficiently transduce unconditioned airway epithelial cells from the apical side. This novel vector was evaluated in mice *in vivo* and *in vitro* directed toward CF gene therapy. Here, we show that (i) we can produce relevant titers of an SIV vector pseudotyped with SeV envelope proteins for *in vivo* use, (ii) this vector can transduce the respiratory epithelium of the murine nose *in vivo* at levels that may be relevant for clinical benefit in CF, (iii) this can be achieved in a single formulation, and without the need for preconditioning, (iv) expression can last for 15 months, (v) readministration is feasible, (vi) the vector can transduce human air–liquid interface (ALI) cultures, and (vii) functional CF transmembrane conductance regulator (CFTR) chloride channels can be generated *in vitro*. Our data suggest that this lentiviral vector may provide a step change in airway transduction efficiency relevant to a clinical programme of gene therapy for CF.

Received 26 February 2009; accepted 4 January 2010; advance online publication 23 March 2010. doi:10.1038/mt.2010.13

INTRODUCTION

Cystic fibrosis (CF) is a fatal genetic disorder caused by mutations in the CF transmembrane conductance regulator (*CFTR*) gene, one function of which is to act as a chloride channel in airway epithelial cells. CF is characterized by recurrent chest infections, increased airway secretions, and eventually respiratory failure.[1] Although symptomatic treatments have successfully increased median survival to ~36 years, definitive novel therapeutic approaches aimed at the basic defect are clearly needed.

Given the propensity of certain viruses for infection of the respiratory tract, *CFTR* gene transfer using these vectors has been extensively studied. However, to date no viral vector has met the requirements for clinical use.[2] Three major problems have been encountered. Gene transfer efficiency is generally poor, at least in part because the respective receptors for the viral vectors appear to be predominantly localized to the basolateral surface of the airway epithelium. Second, penetration of the augmented mucus layer typical of CF is generally poor. Finally, the ability to administer viral vectors repeatedly, mandatory for such transient expression systems in the treatment of a lifelong disease, is limited. With these limitations in mind, we describe a novel vector that is able to circumvent some of the difficulties described above.

We have previously described the use of Sendai virus (SeV) vectors for airway gene transfer.[3,4] SeV is a single-stranded RNA virus, belongs to the family of *Paramyxoviridae*, and is able to overcome the first two of the above-noted difficulties. First, gene transfer to the airway epithelium is highly efficient, because the sialic acid and cholesterol receptors needed for transduction are present on the apical surface of airway epithelial cells. Further, SeV uses a cytoplasmic expression system, thereby removing the limitations imposed by the nuclear membrane. Second, although mucus does act as a partial barrier to SeV-mediated gene transfer, the very high expression levels generated allow this limitation to be readily tolerated. However, despite our best efforts to date, we have been unable to overcome the third impediment to clinical translation, namely repeated application of SeV.

A different solution to this problem would theoretically be provided by transduction of progenitor or stem cells, normally used to replenish the airway epithelium. If this was feasible, "single-hit" gene therapy with a viral vector might overcome this remaining hurdle. However, a significant further issue is the identity and localization of these progenitor or stem cells. Existing data suggest the presence of specialized cells, whose identity and site varies with progression along the branching of the airways.[5]

In addition, the ciliated epithelium may be able to renew or repair itself; the lifespan of these epithelial cells has historically

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been estimated to be around 3 months (ref. 6), but has been extended to 6 months in trachea and 17 months in lung in a most recent publication.[7] Irrespective of the appropriate target cell, an integrating vector would clearly be needed to achieve the goal of "single-hit" gene therapy. Further, given our present lack of conclusive data regarding the identity of airway stem cells, a vector that could transduce cells irrespective of their level of turnover, would be clearly advantageous.

In contrast to retroviral vectors that can only transduce proliferating cells,[8] lentiviruses are able to produce gene expression in nondividing cells, including those of the airway epithelium. However, to allow for receptor-mediated cell entry these vectors require pseudotyping to allow display of appropriate ligands for their cognate receptors. The most common pseudotyping, with glycoproteins of the vesicular stomatitis virus (VSV-G), produces efficient transduction of a broad range of cells, and the virus can be readily concentrated and purified by high-speed centrifugation.[9,10] However, they are not able to transduce airway epithelial cells *in vivo* when delivered via the apical surface. This, in turn, relates both to the difficulties such vectors have in penetrating the overlying mucus, and the lack of apically localized receptors on the epithelial cells.[11–13] Thus, the use of detergents such as lysophosphatidylcholine or ethylene glycol bis(2-aminoethyl ether)-*N,N,N′N′*-tetraacetic acid, help to breach these barriers, thereby allowing the vector to penetrate to the basolateral surface where the appropriate receptors reside. However, the suitability of such an approach in the bacteria-laden airways of CF patients is debatable. Thus, several groups have examined the use of a number of different pseudotyped lentiviral vectors for their airway epithelial transduction efficiency, including envelope glycoproteins of paramyxoviruses,[14] filoviruses,[15–17] and orthomyxoviruses.[18] Although encouraging data have been generated, we are not aware of a vector that fulfills the three key requirements of (i) efficient transduction of airway epithelial cells without the need for chemical pretreatment, (ii) long-term transgene expression, and (iii) the ability to be produced to high vector titers suitable for clinical application.[19]

We describe here the development of a replication-defective lentiviral vector[20] derived from the simian immunodeficiency virus (SIV) of the African green monkey.[21] Using novel strategies, we have been able to pseudotype this vector with the key SeV envelope proteins, hemagglutinin-neuraminidase (HN) and fusion (F) protein.[14] The HN and F proteins function, respectively, to attach to sialic acids, the receptor of SeV, and mediate cell fusion for vector entry to target cells. We have optimized vector production and transgene expression level of this F/HN-pseudotyped SIV vector by introducing the central polypurine tract (cPPT)[22] and the Woodchuck hepatitis virus posttranscriptional regulatory elements (WPRE).[23,24] We show that this F/HN-pseudotyped SIV vector can efficiently transduce nasal epithelial cells from the apical surface *in vivo*, resulting in transgene expression sustained for periods far beyond the proposed lifespan of airway epithelial cells. Importantly, we show that readministration is feasible. Finally, we demonstrate that this vector can transduce a fully differentiated human airway epithelium and that functional CFTR chloride channels can be generated after transduction with F/HN-SIV carrying the human *CFTR* complementary DNA *in vitro*. This vector may, therefore, be able to produce a step change in airway transduction efficiency relevant to a clinical programme of CF gene therapy.

RESULTS

F/HN-pseudotyped SIV vectors can be generated and produced at high titers

To accomplish pseudotyping of SIV vector with SeV envelope proteins, we have previously described that modifications of the F and HN proteins were needed.[14] Briefly, the cytoplasmic domain of the F protein was truncated to four amino acids, and the HN protein was fused with the cytoplasmic tail of the SIV transmembrane envelope protein. These modifications enabled the incorporation of F and HN-derived proteins into vector virions, generating an F/HN-pseudotyped SIV vector.

We next constructed a series of gene transfer vectors in which the cPPT and/or WPRE were inserted into the parent gene transfer vector.[20] Their function was first evaluated using VSV-G pseudotyped SIV vectors encoding green fluorescent protein (GFP). As expected, insertion of cPPT and WPRE increased vector production, from 3.8×10^6 transduction units (TU)/ml (cPPT$^-$/WPRE$^-$) to 1.4×10^7 TU/ml (cPPT$^+$/WPRE$^-$) and 6.7×10^6 TU/ml (cPPT$^-$/WPRE$^+$), respectively. The insertion of both elements (cPPT$^+$/WPRE$^+$) led to the highest titer production (3.9×10^7 TU/ml). Because the number of particles produced in the supernatant (~2×10^8/ml) was similar in all cases, the introduction of these elements increased the quality of the vector by improving the TU to particle ratio from 1:100 to 1:10. Importantly, consistent with these data, insertion of these sequences into the SeV-F/HN-pseudotyped vectors encoding GFP increased virus production from 1×10^5 TU/ml (cPPT$^-$/WPRE$^-$) to 5×10^7 TU/ml (cPPT$^+$/WPRE$^+$), leading to an improvement in TU to particle ratio from 1:100 to 1:20. Thus, the cPPT/WPRE-containing vector was used in all subsequent experiments. The F/HN-pseudotyped SIV vector (**Supplementary Figure S1, Supplementary Materials and Methods**) could be further concentrated from $2.0 \pm 0.9 \times 10^7$ to $6.1 \pm 2.0 \times 10^9$ TU/ml ($n = 3$) through centrifugation, making high titer use *in vivo* feasible.

F/HN-SIV-GFP transduces the nasal epithelium of mice

We evaluated the *in vivo* gene transduction efficiency of the SeV-F/HN-pseudotyped SIV vector in the nasal airway epithelium of mice. This tissue was chosen because the characteristic pathophysiological abnormalities of CF are reproduced in the nasal, but not the lower airways of CF knockout mice. In addition, in contrast to the murine lung, cell composition in the murine nasal epithelium is more akin to the human lung and contains both submucosal glands, as well as mucous-secreting cells, thereby allowing the evaluation of whether the vector is able to penetrate a mucus-enriched airway surface fluid layer. As a part of this study, ~100 mice were treated (for details see individual figure legends). The overall survival over the course of the >15 months study was 100%.

To examine transduction efficiency of respiratory epithelial cells, we administered F/HN-pseudotyped SIV vector encoding GFP to the nose of mice at a dose of 4×10^8 TU/mouse. At 30 days after transfection, GFP-positive respiratory epithelial cells

1–4 mm from the tip of the nasal bone (**Figure 1**). Transduction efficiency along the nasal septum was 4.5 ± 0.7% (mean ± SEM, $n = 8$). This corresponds favorably with the ~5% of cells estimated to require transduction for generating clinical benefit.

F/HN-SIV transduces cells in both the respiratory and olfactory epithelium

Using high-power microscopic analysis, we were able directly to detect and identify GFP fluorescent cells 30 days after transfection. The murine nasal epithelium consists of squamous cells in the anterior part of the nose, changing to respiratory epithelium in the mid-portion and neuroepithelial olfactory epithelium in the dorsoposterior regions.[25] The majority (~69%) of GFP-positive cells were ciliated respiratory epithelial cells (**Figure 2a**) followed by neuronal cells (21%) in the olfactory epithelium (**Figure 2b**) and squamous cells (7%) (**Figure 2c**). The number of neuronal cells transduced was very few in the anterior part of the nose and increased in the posterior region as expected. The remainder (3%) were mainly non-neuronal cells in the olfactory epithelium, most likely sustentacular cells (**Figure 2d**), a major constituent of the olfactory epithelium. Interestingly, we did not detect GFP-expressing goblet cells, a cell type commonly transduced in VSGV-SIV perfused lysophosphatidylcholine-preconditioned nasal epithelium (data not shown).

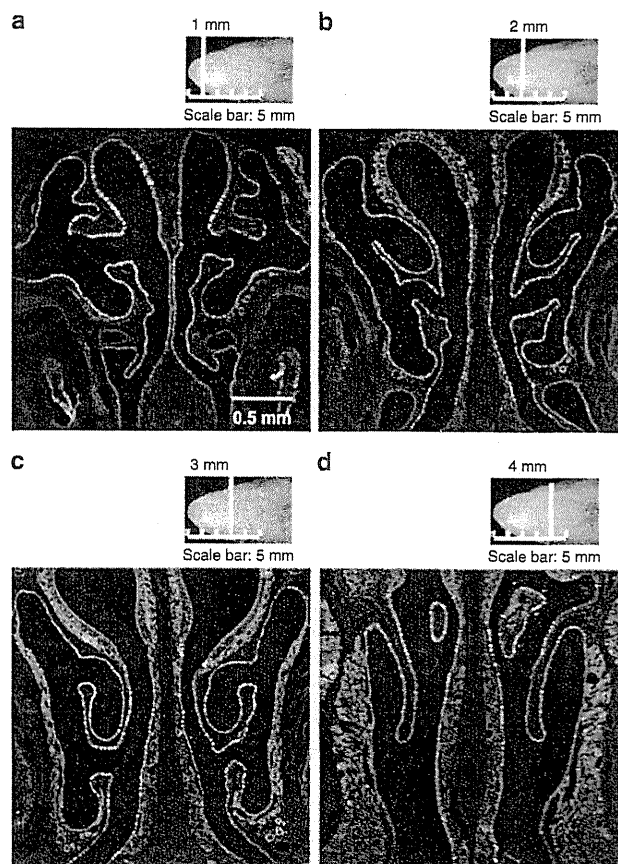

Figure 1 Transduction of mouse nasal epithelium with F/HN-SIV-GFP. The murine nose was perfused *in vivo* with F/HN-SIV-GFP (4 × 10^8 TU/mouse) vector and gene expression analyzed 30 days after transduction ($n = 8$). (**a,b**) *In situ* imaging of GFP expression in the nasal cavity. (**c,d**) Microscopic imaging of GFP in histological sections. The sections were collected (**a**) 1 mm, (**b**) 2 mm, (**c**) 3 mm, and (**d**) 4 mm into the nasal tissue (vertical white lines). GFP-positive cells appear as small white punctuate signals.

GFP expression persists for >1 year after F/HN-SIV-GFP transduction

To determine the duration of gene expression, mice were perfused with F/HN-SIV-GFP (4 × 10^8 TU/mouse) or phosphate-buffered saline (PBS) and harvested 3–449 days after transfection (**Figure 3a,b**). F/HN-SIV-mediated GFP expression was visible in the nasal cavity using *in situ* imaging as early as 10 days post-transduction and was still detectable in 7 out of 10 and 3 out of 10 mice at 360 and 449 days after a single administration of the vector, respectively. At this time point, GFP-positive cells appeared to be randomly distributed within the tissue similar to the distribution observed 30 days after transfection (**Figure 3c**). GFP was undetectable in lysophosphatidylcholine-preconditoned VSV-G-SIV-GFP-transduced tissue (data not shown). Histological sections were used to quantify the number of GFP-positive cells over time. As a whole, the number of GFP-positive cells gradually declined over time by ~70% (**Figure 3d**). In some mice, marked decline of GFP-positive cells were observed after 90 days post-transduction; however, in the other mice the number of transduced cells were maintained well even at 360 days post-transduction to the similar level of that at 30 days post-transduction. In addition, it is important to note that GFP-positive cells were still detectable in 16 out of 17 mice 12 months after transduction.

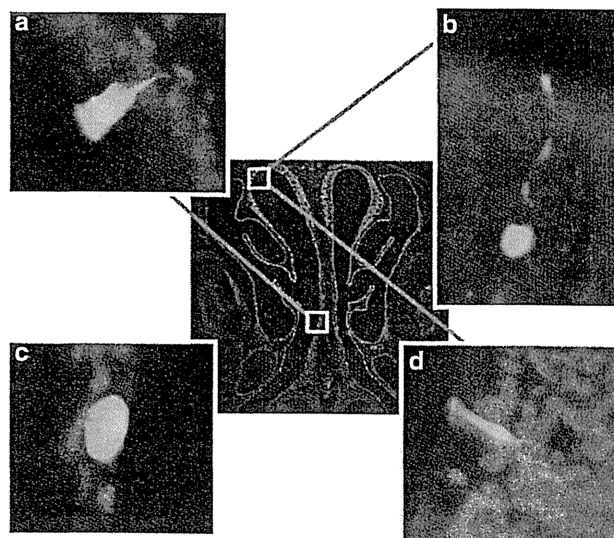

Figure 2 Determination of cell types transduced. The transduced GFP-positive cells were identified using fluorescent microscopy (original magnification ×63) 30 days after administration of F/HN-SIV-GFP (4 × 10^8 TU/mouse) vector to the mouse nose. (**a**) Ciliated respiratory epithelial cell, (**b**) neuronal cell in olfactory epithelium, (**c**) squamous epithelial cell, (**d**) non-neuronal cell in olfactory epithelium. The central image shows a cross-section through the mouse nose and white boxes indicate regions in mouse nasal epithelium where respective transduced cell types were found. Panels **a**, **b**, and **d** were rotated ~45°, 130°, and 180° counter clockwise, respectively, to improve clarity of the figure.

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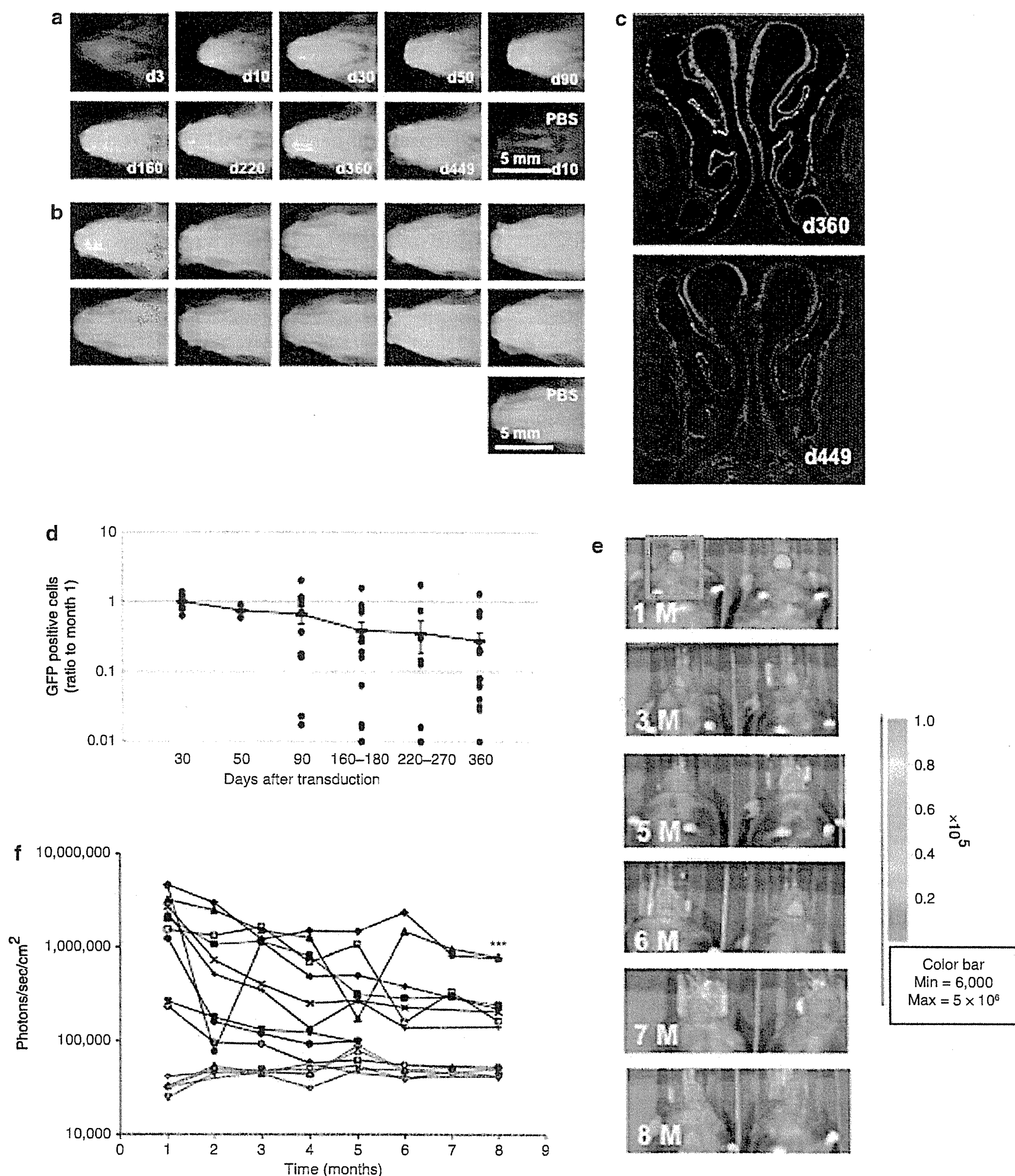

Figure 3 Duration of green fluorescent protein (GFP) expression after transduction with F/HN-SIV-GFP. Mouse nasal tissue was perfused with F/HN-SIV-GFP (4×10^8 TU/mouse or phosphate-buffered saline (PBS) and gene expression was analyzed at indicated time points after transduction. (**a**) Representative *in situ* images of GFP expression in the nasal cavity from mice analyzed 3–449 days after transduction. (**b**) *In situ* imaging of GFP expression in the nasal cavity from 10 mice analyzed 360 days after transduction. (**c**) Representative microscopic images of GFP expression in histological sections 360 and 449 days after transduction. GFP-positive cells appear as small white punctuate signals. (**d**) Quantification of transduced cells. GFP-positive cells were quantified on histological sections taken 2 mm into the nasal tissue of the nose. Data from 30 to 360 days after transduction are represented both by mean ± SEM and individual values (ratio to GFP cells positive on day 30). The number (*n*) per group are 13 (day 30), 3 (day 50), 12 (day 90), 14 (day 160–180), 10 (day 220–270), and 17 (day 360). (**e**) Bioluminescence *in vivo* imaging 1 (1 M) to 8 months (8 M) after transduction with F/HN-SIV-lux. Representative images of 2 out of 6 mice are shown. Red box indicates area chosen for quantification of photon emission. (**f**) Quantification of *in vivo* bioluminescence over time after transduction with F/HN-SIV-lux (black lines) or PBS (red lines). Each line represents photon emission over time in one animal. ***$P < 0.005$ compared to bioluminescence one month after gene transfer.

We also used *in vivo* bioluminescence imaging after nasal perfusion with F/HN-SIV-lux to assess gene expression over time in the same animal (Figure 3e).

Figure 3f shows quantification of photon emission after intraperitoneal injection of luciferin substrate from 1 to 8 months after transduction. Although persistence of gene expression far exceeds the expected lifespan of airway epithelial cells of 100 days, possibly indicating progenitor cell integration, repeated bioluminescence *in vivo* imaging after F/HN-SIV-lux transduction shows a gradual decline in photon emission over an 8-month period (month 1: $2.2 \times 10^6 \pm 4.9 \times 10^5$, month 8: $2.7 \times 10^5 \pm 8.8 \times 10^4$, $n = 10$/group, $P < 0.005$), but was still significantly ($P < 0.01$) higher than the PBS control ($4.5 \times 10^4 \pm 2.0 \times 10^3$, $n = 5$) This result is consistent with the decline in GFP-positive cells described above. In addition, we analyzed all data using a repeat measure test to determine whether the decline in bioluminescence stabilizes during the 8-month study period. The analysis showed that gene expression significantly ($P < 0.05$) declined for the first 4 months but then stabilized with expression levels from 5 to 8 months not being different compared to the 4-month levels.

The SIV vector–transduced cells show clustering after induced regeneration of the epithelium

To ascertain further whether F/HN-SIV induced chromosomal integration into nasal respiratory progenitor or stem cells, we artificially induced cell division after SIV vector transduction by damaging the nasal tissue with the detergent (polidocanol),[6] which has previously been shown to strip the surface epithelium within a few hours, while retaining basal cells able to regenerate the epithelium within 7 days (Figures 4a–d). At 7 and 28 days after vector transduction (4×10^8 TU/mouse, $n = 3$) the nasal tissue was perfused with 2% polidocanol (10 µl/mouse) and gene expression analyzed 4 weeks after the last detergent treatment. Importantly, GFP-expressing cells now showed clustering after polidocanol treatment (Figure 4e and **Supplementary Figure S2**), possibly indicating origination from a common progenitor.

SIV-mediated gene transfer can be achieved after three applications of the vector

Although the above data are encouraging, gene therapy for CF will require lifelong treatment. We therefore, assessed the feasibility of

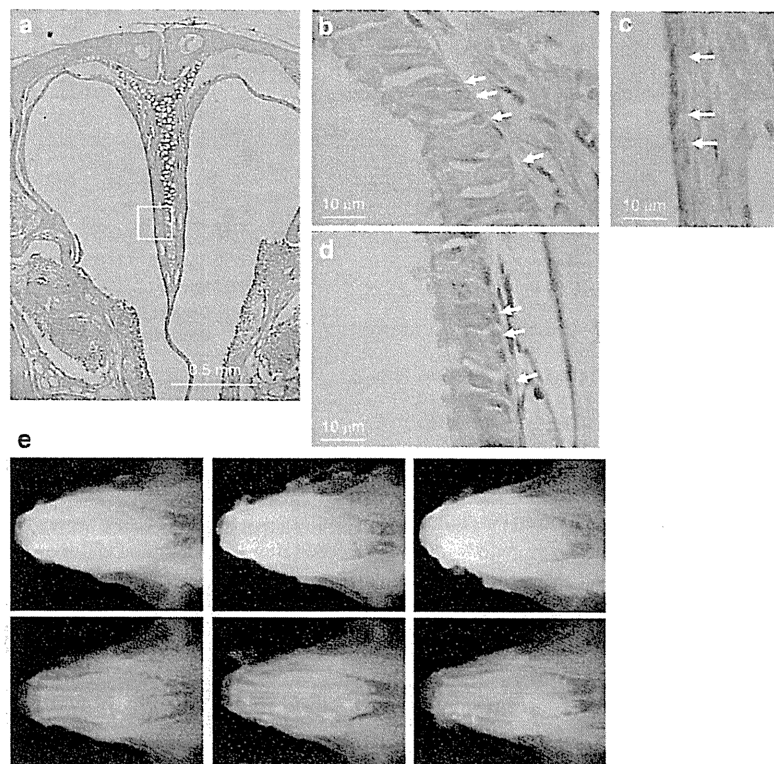

Figure 4 Clustering of transduced cells after the polidocanol-mediated stripping of epithelial cells followed by rapid regeneration. Mouse nasal tissue was perfused with 10 µl of 2% (vol/vol) polidocanol ($n = 3$). (**a**) Representative low-power view (original magnification ×50) of the nasal cavity 24 hours after perfusion. Respiratory epithelium, marked by a white box was further magnified (original magnification ×200). The respiratory epithelium before the treatment is shown in **b**. Arrow indicates basal cells. The respiratory epithelium was completely stripped 24 hours after polidocanol perfusion, whereas the basal cell layer was (**c**) retained and (**d**) regenerated 7 days after treatment. (**e**) This treatment was done after transduction with F/HN-SIV vector. Seven days after transduction of nasal epithelial cells with F/HN-SIV-GFP (4×10^8 transduction units/100 µl/mouse), the nasal epithelium was stripped via perfusion with 10 µl of 2% (vol/vol) polidocanol. Polidocanol treatment was repeated again 3 weeks later. Histological sections were analyzed 58 days after vector administration (30 days after the last polidocanol treatment). *In situ* imaging of GFP expression in the nasal cavity of untreated mice (top panel in **e**) or mice treated with polidocanol (bottom panel in **e**). Clusters of GFP-positive cells were seen in the polidocanol-treated mice. GFP, green fluorescent protein.

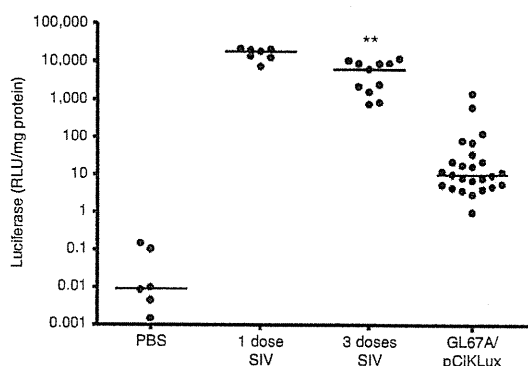

Figure 5 Repeat administration of F/HN-SIV to nasal epithelium. Mice were transduced with F/HN-SIV-lux (1 dose) or two doses of F/HN-SIV-GFP (day 0 and day 28) followed by F/HN-SIV-lux 4 weeks later (day 56 = 3 doses). Luciferase expression was measured 30 days after F/HN-SIV-lux transduction and compared to levels achieved with the non-viral gene transfer agent GL67A complexed to a luciferase reporter gene plasmid (pCIKLux). Each dot represents one mouse. Horizontal bars indicate the median per group (**$P < 0.01$) compared to mice receiving GL67A/plasmid DNA. PBS, phosphate-buffered saline; RLU, relative light units.

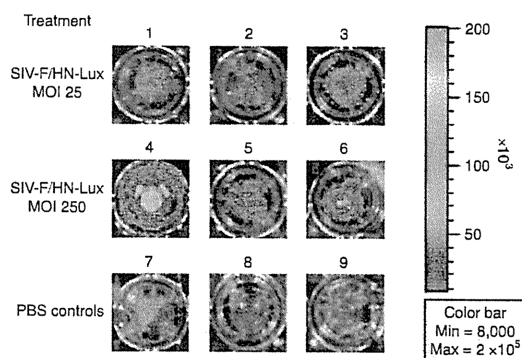

Figure 6 Transduction of human air–liquid interface (ALIs) cultures with F/HN-SIV-lux. ALIs were transduced with F/HN-SIV-lux at an approximate multiplicity of infection of 25 (1–3) and 250 (4–6) or treated with phosphate-buffered saline (PBS) (7–9). 5 days after transduction ALIs were treated with luciferin and bioluminescent imaging performed.

readministering this vector, and compared transduction efficiency to the current optimal nonviral formulation for airway gene transfer *in vivo*. **Figure 5** shows that following two administrations of F/HN-SIV-GFP separated by 1 month, a third administration of F/HN-SIV-lux (to prevent an immune response against the transgene) produced gene expression of ~40% of that seen following a single challenge with F/HN-SIV-lux. Further, these levels after three challenges with the SIV vector remained ~500-fold greater ($P < 0.01$) than seen with an optimal nonviral formulation, previously used in a CF clinical trial.

F/HN-SIV transduces differentiated human airway epithelium

Differentiated human airway epithelium is in general difficult to transduce. In preliminary experiments, we have shown that F/HN-pseudotyped lentivirus transduced human airway cells grown as air-liquid interphase (ALI) cultures. ALIs were transfected with F/HN-SIV carrying a luciferase reporter gene at

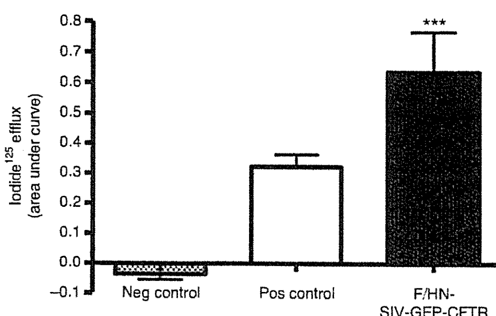

Figure 7 Functional confirmation of CF transmembrane conductance regulator (CFTR) production by F/HN-SIV-GFP-CFTR. HEK293T cells were transduced with F/HN-SIV-GFP-CFTR or a control virus carrying green fluorescent protein (GFP) (F/HN-SIV-GFP) at an multiplicity of infection of 500. The iodide efflux assay was performed 2 days after transduction. Cells transfected with an eukaryotic expression plasmid carrying the *CFTR* complementary DNA under the control of a cytomegalovirus promoter complexed to Lipofectamine 2000 were used as positive control. Data are presented as mean ± SEM. ***$P < 0.001$ compared to the control virus, $n = 6$/group. Neg, negative; pos, positive.

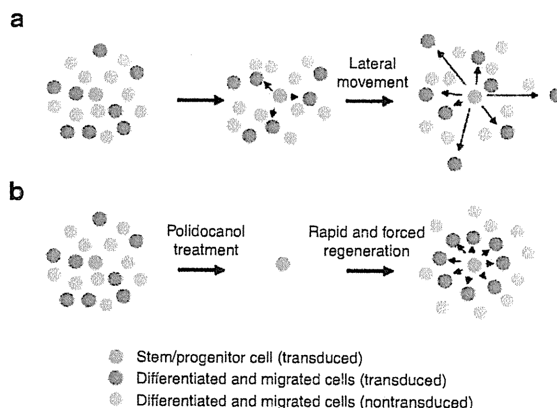

Figure 8 Schematic representation of epithelial cell migration in intact and damaged epithelium. (**a**) Scattered pattern with regeneration in normal condition. (**b**) Clustered formation after rapid and forced regeneration. We speculate that under normal physiological (undamaged) conditions, turnover may be comparatively slow and that newly generated epithelial cells may move laterally away from the stem or progenitor cell that they originated from **a**. In contrast, if rapid regeneration is forced (after tissue damage with polidocanol) stem or progenitor cells have to divide rapidly and newly generated epithelial cells may (transiently?) stay in closer proximity to the cell that they originated from **b**. This hypothesis may explain, why we observed clusters of GFP-positive cells in damaged, but not in undamaged epithelium.

an approximate multiplicity of infection of 100 and luciferase expression was detectable 10 and 26 days after gene transfer (day 10: 53.0 ± 6.3 relative light units/mg protein, day 26: 7.9 ± 3.4 relative light units/mg protein, untransfected: 0.01 relative light units/mg protein, $n = 3$/group) and **Figure 6**. Importantly, gene transfer occurred without the need for preconditioning.

Transduction with F/HN-SIV carrying the *CFTR* complementary DNA leads to expression of cAMP-dependent chloride channels

Iodide efflux is commonly used to demonstrate the presence of forskolin-activated chloride channels *in vitro*. In this assay, iodide

is used as a surrogate for chloride due to the shorter half-life of the radioactive material. We have constructed an F/HN-SIV carrying a GFP-*CFTR* fusion complementary DNA construct and transduced human embryonic kidney (HEK)293T cells to assess whether functional CFTR chloride channels are generated. Fusion of GFP to the N-terminus of CFTR has previously been shown not to effect CFTR function.[26,27] Figure 7 shows that cells transduced with F/HN-SIV-GFP-CFTR (multiplicity of infection 50) significantly ($P < 0.001$) increased cAMP-mediated efflux compared to cells treated with an F/HN-SIV-GFP control virus. We, therefore, conclude that transduction with F/HN-SIV-GFP-CFTR generates functional CFTR chloride channels *in vitro*.

DISCUSSION

Here, we show that (i) we can produce titers of a novel SIV vector pseudotyped with SeV envelope proteins appropriate for *in vivo* use, (ii) the vector can transduce the respiratory epithelium of the murine nose *in vivo* at levels that may be relevant for clinical benefit in CF, as previously suggested by *in vitro* mixing experiments,[28] (iii) this can be achieved in a single formulation, and without the need for preconditioning, (iv) expression can last for at least half the lifespan of a mouse, (v) the vector can produce levels of gene expression ~500-fold greater than the current optimal nonviral formulation after three repeated administrations, (vi) the vector is able to transduce a fully differentiated human airway epithelium, and (vii) can produce functional cAMP-dependent CFTR chloride channels *in vitro*.

We inserted cPPT and WPRE sequences into the SIV vector. Both elements have previously been reported to increase gene transduction efficiency possibly due to acceleration of the movement of the preintegration complex of the vector into the nucleus,[22,29,30] or other mechanisms.[23,31] Interestingly, a synergistic effect of these two elements has been observed for human immunodeficiency virus–based lentiviral vectors.[24] Our data are in keeping with these observations, with simultaneous insertion of cPPT and WPRE increasing productivity of both the VSV-G and SeV-F/HN-pseudotyped SIV vectors. Using these methods, we were able to reach titers of the SeV-F/HN-pseudotyped SIV vector of 5×10^7 TU/ml. Thus, this vector may be able to overcome one previously encountered important translational hurdle.

Lentiviral vectors pseudotyped with a variety of envelope proteins other than VSV-G have been described, including those from Ebola, Zaire,[15,16] influenza hemagglutinin from fowl plague virus,[18] and baculovirus GP64 envelopes.[17] Among these, arguably it is the transduction efficiency of the baculovirus GP64-pseudotyped vectors that is most impressive, when applied in a viscoelastic gel formulation (1% methylcellulose) as a vector solvent. However, the regulatory complexities of moving two new agents into the clinic simultaneously, underline the encouraging transduction efficiency and duration we report here without the need for additions to the formulation.

The likely target for CF gene therapy are the ciliated epithelial cells, and >70% of the cells transduced by the F/HN SIV vector were of this type. This is in keeping with transduction of these cells by the "parent" SeV vector, and overcomes a second hurdle in the translation of these vectors toward the clinic. The number of cells requiring transduction for clinical benefit is a vexed, and unresolved question. In part, this may depend on which of the many functions of CFTR requires correction. Thus, if the chloride channel function predominates, *in vitro* data suggest that as few as 5% of cells may be sufficient.[28] These values are in reach of the F/HN SIV vector described here.

Using a human cytomegalovirus promoter, we saw expression of GFP for >360 days in 16 out of 17 mice, although gene expression gradually decreased over time when quantified as number of cells expressing the GFP reporter gene or longitudinal assessment of bioluminescent imaging. The onset of transgene expression was typically delayed, with no GFP fluorescence detected at day 3, but clearly visible by day 10. This has also been reported for an Ebola virus Z protein–pseudotyped human immunodeficiency virus vector.[15] Possible explanations include the delayed movement of the preintegration complex to the nucleus, or the shutting off of promoter activity by concomitant inflammation consequent upon transduction,[32,33] with subsequent expression following the resolution of inflammation. Irrespective, transgene expression was still apparent up to 449 days after transduction the longest time point assessed. Because the lifespan of terminally differentiated airway epithelial cells has been estimated at around 90 days (ref. 6), both in mice and in man, we considered whether this vector may have transduced progenitor or stem cells within the airway epithelium. We would predict that in this case we would observe clonal expansion, with clustering of transgene positive cells, and this was seen following induced regeneration of epithelial cells after polidocanol treatment. Cells derived from progenitor or stem cells thought to reside near the basement membrane have previously been shown to move laterally during differentiation.[34] Thus, in the absence of epithelial damage, SIV vector–transduced progenitor or stem cell–derived cells should be observed in a scattered pattern without clustering (see schematic presentation in Figure 8a). In contrast, following epithelial stripping, a different pattern would be predicted to occur, with clusters of transduced cells becoming visible (see schematic presentation in Figure 8b). This hypothesis may explain, why we observed clusters of GFP-positive cells in damaged but not in undamaged epithelium. However, more extensive studies will be necessary to understand and more conclusively prove stem or progenitor cell transduction. Other explanations for the unexpectedly long duration of expression include (i) an alteration in the cell cycle of transduced respiratory epithelial cells, although to our knowledge this has not previously been reported, (ii) expression from -resident airway inflammatory or immune cells, for which we saw no evidence, or (iii) a >3 months half-life of respiratory epithelial cells, which has recently been suggested by Rawlins *et al.*[7]

The treatment of CF will require lifelong expression of the normal CFTR protein. Thus, despite these encouraging data showing long-lasting expression from a single administration, we assessed whether repeated application of this vector could sustain gene expression. We show that repeated mucosal administration of F/HN pseudotyped lentivirus, when given monthly over a 3-month period, is feasible and led to gene expression ~40% of that seen following a single administration. This dosing interval may be of subsequent clinical relevance. Sinn *et al.*[35] have recently shown that seven weekly administrations of a GP64-pseudotyped FIV, given in tandem with a ciliastatic agent, are able to produce repeatable expression. Further, each study was undertaken in a different inbred mouse strain. The significant differences between

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the studies, yet with similar outcome, provide a growing body of evidence that such vectors can be readministered.

Differentiated human airway epithelium is in general difficult to transduce. However, we have shown here that F/HN-SIV transduced fully differentiated human airway epithelium successfully, and that reporter gene expression could be detected for at least 26 days after transduction. Importantly, gene transfer occurred without the need for preconditioning with tight junction openers or cilia static agents that are often required with other viral vectors. This provides encouraging support for its use in human trial.

In addition to demonstrating that F/HN-SIV carrying the *CFTR* complementary DNA was able to generate cAMP-dependent chloride channels *in vitro*, we also attempted to correct nasal potential difference in CF knockout mice. However, we did not detect any changes in ion transport (data not shown). Importantly, the suitability of the CF mouse nasal epithelium as a model has been put into question by two recent publications showing that the nasal bioelectrics are dominated by the olfactory rather than the respiratory epithelium.[36,37] Our experience is in keeping with this observation. Transduction with SeV, which transduces respiratory and olfactory epithelium led to significant increases in chloride transport,[38] whereas lentivirus and nonviral gene transfer agents,[39] which predominantly transduce ciliated respiratory epithelial cells were unable to alter ion transport in the mouse nose. Until more appropriate animal models become widely available, analysis of *CFTR* function after gene transfer may, for certain gene transfer agents, be restricted to *in vitro* models.

Clearly, at least one remaining crucial hurdle is the risk–benefit ratio of these integrating vectors. The cases of leukemia in the severe combined immunodeficiency trial using a retroviral vector have been well documented, but lentiviral vectors are considered by many to be less susceptible to these problems. Further, the slowly dividing airway epithelium may represent a very different risk to the rapid turnover of bone marrow stem cells. Encouragingly, in our study of ~100 mice, over a 1-year period we saw no adverse events attributable to the vector. However, the encouraging increase in median survival of CF patients to the current ~36 years suggests that extensive toxicology studies will be needed before clinical trials can begin.

In conclusion, we suggest that the SeV-F/HN-pseudotyped SIV vector reported here may represent a further step toward translating such integrating viral vectors into clinical use. Several key hurdles have been potentially overcome, pushing these vectors into the arena as candidates for clinical trials.

MATERIALS AND METHODS

Cell culture. HEK293T and 293T/17 cells (CRL-11268; ATCC, Manassas, VA) were maintained in Dulbecco's minimal Eagle's medium (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum and supplemented with penicillin (100 U/ml) and streptomycin (100 µg/ml).

Plasmid construction. pCAGGS-Fct4 and pCAGGS-SIVct+HN were constructed as previously described.[14] The cPPT and WPRE sequences[40] were inserted in the SIV-derived gene transfer plasmid.[21]

Production of SIV vector. Replication-defective self-inactivating SIV vector was constructed as previously described[20] with minor modifications. Briefly, the SeV-F/HN-pseudotyped SIV vector was produced by transfecting 293T/17 cells (15 cm diameter culture dishes) with four plasmids complexed to Lipofectamine/Plus reagents (Invitrogen) according to the manufacturer's recommendations [Plasmid-1: 10 µg SIV-derived transfer plasmid carrying a GFP, a luciferase (lux) reporter gene, or a GFP-*CFTR* fusion construct,[26] Plasmid-2: 3 µg packaging plasmid, Plasmid-3: 2 µg pCAGGS-Fct4, Plasmid 4: 2 µg pCAGGS-SIVct+HN]. The VSV-G pseudotyped SIV vector was produced using a similar protocol, but a pVSV-G plasmid (2 µg; Clontech, Mountain View, CA) was used instead of pCAGGS-Fct4 and pCAGGS-SIVct+HN. At 12 hours after transfection the culture medium was replaced with 30 ml serum-free Dulbecco's modified Eagle medium containing 5 mmol/l sodium butyrate. Sodium butyrate stimulates the vector production to inhibit histone deacetylase.[41,42] The culture supernatant containing the SIV vector was harvested 48 hours after transfection, filtered through a 0.45 µm filter membrane, and further concentrated by high-speed centrifugation (20,000 *g* for 4 hours at 4 °C, Avanti JA18 rotor; Beckman Coulter, Brea, CA). The vector pellets were suspended in PBS (Invitrogen) to 100- to 200-fold concentration and stored at −80 °C.

Vector titration. The particle titer was determined using real-time reverese transcriptase-PCR. Virus RNA was purified using a QIAamp viral RNA mini-kit (QIAGEN, Strasse, Germany), and reverse transcribed using Superscript II (Invitrogen). The QuantiTect probe PCR system (QIAGEN) and primers for amplifying 131 nucleotides (bp) spanning the WPRE sequence (forward primer: 5′-ggatacgctgctttaatgcc-3′, reverse primer: 5′-acgccacgttgcctgacaac-3′) were used according to the manufacturer's protocol in an ABI PRISM 7700 Sequence Detector System (PE Applied Biosystems, Foster City, CA). SIV gene transfer plasmid DNA (3×10^4 to 2×10^6 molecules) was used as standard.

Transduction units (TU/ml) were determined by transducing 293T cells with serial dilutions of vector stock and quantification of transduced cells by GFP fluorescence (for F/HN-SIV-GFP and VSV-G-SIV-GFP) or staining with anti-luciferase antibody (for F/HN-SIV-lux). To work with a consistent virus concentration throughout the study, virus stocks were all adjusted to a final volume of 4×10^9 TU/ml. A titer of 4×10^9 TU/ml was used for all animal studies.

In vivo *administration to the mouse nose.* All animal studies had been approved by the DNAVEC Animal Care Committee and the Imperial College Animal Ethics Committee and were carried out according to Home Office regulations. C57BL/6N mice (female, 6–8 weeks) were used. Mice were anesthetized, placed horizontally on their backs onto a heated board, and a thin catheter (<0.5 mm outer diameter) was inserted ~2.5 mm from the tip of nose into the left nostril. Using a syringe pump (Cole-Parmer, Vernon Hills, IL), vector (100 µl) was then slowly perfused onto the nasal epithelium (1.3 µl/min) for 75 minutes. Despite perfusion of virus into the left nostril, we routinely observe transfection in both left and right nostrils, which is due to dispersion of the solutions throughout the entire nasal cavity. PBS and VSV-G-SIV transduced mice preconditioned with 1% lysophosphatidylcholine as described by Limberis *et al.* were used as controls.[11] At indicated time points (3–360 days after transduction), mice were culled to visualize GFP expression.

In the repeat administration experiments groups of mice were transduced with either one dose of F/HN-SIV-lux (single-dose group), or two doses of F/HN-SIV-GFP (day 0, day 28), followed by F/HN-SIV-lux on day 56 (repeat-dose group). Importantly, mice receiving F/HN-SIV-lux (single-dose group) and F/HN-SIV-lux on day 56 (repeat-dose group) were of similar age and were transduced at the same time. Gene expression was analyzed 30 days after F/HN-SIV-lux administration. For comparison, mice were transfected with the cationic lipid GL67A complexed to a luciferase reporter gene as previously described[43] and luciferase expression was measured 2 days after transfection.

Induced regeneration of nasal epithelial cells by polidocanol treatment. Nasal epithelial cells were stripped by polidocanol treatment according to

the method described by Borthwick *et al.*[6] with some modification. In brief, mice were anesthetized and 10 µl polidocanol (2%) were administered to the nose as a bolus by "nasal sniffing". To confirm the stripping and regeneration of nasal epithelial cells, nasal tissue was perfused with 10 µl of 2% (vol/vol in PBS) polidocanol (nonaethylene glycol mono-dodecyl ether; SIGMA, St Louis, MO) and histological analysis undertaken 24 hours and 7 days after treatment (n = 3/group). To analyze transduction of possible progenitor or stem cells, we first administered F/HN-SIV-GFP (4×10^8 TU/mouse) vector to mouse nasal epithelium. Seven days after transduction, nasal tissue was perfused with 10 µl of 2% (vol/vol in PBS) polidocanol, and this treatment was repeated again 3 weeks later. Histological sections were analyzed 58 days after vector administration (30 days after the last polidocanol treatment).

Bioluminescent imaging. Mice were injected intraperitoneally with 150 mg/kg of D-luciferin (Xenogen, Alameda, CA) 10 minutes before imaging and were anesthetized with isoflurane. Bioluminescence (photons/s/cm²/sr) from living mice was measured using an IVIS50 system (Xenogen) at a binning of 4 for 10 minutes, using the software programme Living Image (Xenogen). For anatomical localization a pseudocolor image representing light intensity (blue: least intense, red: most intense) was generated using Living Image software and superimposed over the grayscale reference image. To quantify bioluminescence in the nose, photon emission in a defined area (red box) was measured by marking a standardized area for quantification. The size of the red box was kept constant and was placed over the heads of the animals as indicated in the figure. Importantly, the areas were marked using the grayscale reference image to avoid bias.

Tissue preparation for histological assessment of GFP expression. Mice were culled and the skin was removed. The head was cut at eye level and skin, jaw, tongue, and the soft tip of the nose were carefully removed. For *in situ* imaging of GFP expression in the nasal cavity, GFP fluorescence was detected using fluorescence stereoscopic microscopy (Leica, Ernst Leitz Optische Werke, Germany). Subsequently, the tissue was fixed in 4% paraformaldehyde (pH 7.4) overnight at room temperature and was then submerged in 20% EDTA (pH 7.5 for 5 days) for decalcification. The EDTA solution was changed at least every second day. After decalcification, the tissue was incubated in 15% sucrose overnight at room temperature and was then embedded in Tissue Mount (Chiba Medical, Soka, Japan). Ten micrometer sections were cut at six different positions in each mouse head (~0–6 mm from the tip of nasal bone). GFP expression was observed using a fluorescent microscope (Leica). Quantification and identification of cell types were carried out on six levels per mouse using a ×40 or ×63 objective. Prolonged image exposure was necessary to capture the structure of the nasal epithelium using fluorescent microscopy. This led to pixel saturation of GFP-positive cells and caused GFP-positive cells to appear almost white rather than the common green appearance that we, and others, observe under higher magnification.

Transduction of ALI cultures. Fully differentiated airway epithelial cells grown as ALI cultures were purchased from Epithelix (Geneva, Switzerland). ALIs were transfected with F/HN-SIV-lux at a multiplicity of infection ranging from ~25 to ~300. The virus was dissolved in 50 µl PBS and applied to the apical surface. After 6 hours, the virus was removed and ALIs were incubated for 10–26 days. The basolateral medium was changed every 48 hours during this incubation period. At specified time points, the ALIs were lysed in 100 µl reporter lysis buffer and luciferase expression was quantified using the Luciferase Assay System (Promega, Southampton, UK) according to the manufacturer's instructions. The total protein content of the cultures was quantified using the BioRad protein assay kit (BioRad, Hemel Hempstead, UK). Each sample was assayed in duplicate. Luciferase expression was then presented as relative light units/mg total protein. For bioluminescence imaging 100 µg luciferin in PBS were added to the apical membrane.

Iodide efflux assay. HEK293T cells were transfected with F/HN-SIV-GFP-CFTR or an F/HN-SIV-GFP control virus at a multiplicity of infection of 500 and cultured for 2 days. CFTR chloride channel activity was assayed by measuring the rate of ^{125}iodide efflux as previously described.[44] The ^{125}iodide efflux rates were normalized to the time of forskolin/IBMX addition (time 0). Curves were constructed by plotting rates of ^{125}iodide efflux against ime. To reflect the cumulative levels of ^{125}iodide efflux following agonist-stimulation, all comparisons are based on areas under the time-^{125}iodide efflux curves. The area under the curve was calculated by the trapezium rule. Experiments were carried out in duplicate (n = 6 wells/group/experiment).

Statistical analysis. Normal distribution was assessed for all data and parametric or nonparametric statistical analysis was performed as appropriate. Data in **Figure 3f** were analyzed using the Mann–Whitney U-test to compare bioluminescence at month 1 and 8, as appropriate for nonparametric data. In 2 out 10 mice, bioluminescence had returned to baseline levels before month 8 and these mice did not undergo additional bioluminescence *in vivo* imaging. For the final quantification of gene expression at 8 months the mean bioluminescence of the PBS control cohort was used for these two mice. In addition, data in **Figure 3f** were analyzed using a Friedman repeat measure test followed by Dunn's multiple comparison *post hoc* test, as appropriate for nonparametric data.

Data in **Figure 5** were analyzed by Kruskal–Wallis followed by Dunn's Multiple Comparison test, as appropriate for nonparametric data. Data in **Figure 6** were analyzed by ANOVA followed by Bonferroni's Multiple Comparison *post hoc* test, as appropriate for normal distributed data. The null hypothesis was rejected at $P < 0.05$.

SUPPLEMENTARY MATERIAL

Figure S1. SeV F and HN envelope proteins are incorporated into SIV vector particles and form infectious SIV pseudotyped vectors.
Figure S2. GFP-positive cluster in F/HN-SIV-GFP transduced and polidocanol-treated mouse nasal epithelium.
Materials and Methods.

ACKNOWLEDGMENTS

We acknowledge Hitoshi Iwasaki and Akihiro Iida for help in drafting the manuscript, and Kentaro Washizawa, and Satoshi Fujikawa for their excellent technical assistance. We thank Genzyme Corporation for supplying GL67. This work was in part funded by the Cystic Fibrosis Trust and the Dr Benjamin Angel Senior Lectureship (U.G.).

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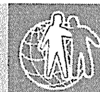

Haemophilia (2010), 16, 740–746

DOI: 10.1111/j.1365-2516.2010.02244.x

ORIGINAL ARTICLE *Clinical haemophilia*

Continuous infusion during total joint arthroplasty in Japanese haemophilia A patients: comparison study among two recombinants and one plasma-derived factor VIII

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Summary. As for the available factor VIII (FVIII) concentrates in Japan, there are two recombinant FVIII concentrates (Kogenate-FS and Advate) and one highly purified plasma-derived FVIII concentrate (Cross-Eight M). To evaluate the inter-product variability, the differences in the continuous infusion rates and total consumption of the above three concentrates were compared when continuous infusion was used as the administration mode to control bleeding during 28 total joint arthroplasties (TJAs) for 17 patients. There were no significant differences among the FVIII plasma levels during surgery, except day 0. Advate needed to be given at a significantly higher infusion rate (4.2–2.1 IU kg^{-1} h^{-1}) than the other two concentrates (Kogenate-FS: 1.0–2.9 IU kg^{-1} h^{-1}, $P < 0.01$ and $P < 0.05$; Cross-Eight M: 3.2–1.8 IU kg^{-1} h^{-1}, $P < 0.01$); however, their infusion rates were within the rates which were previously reported. The total consumption of Advate (652.1 IU kg^{-1}) was also significantly greater than either of the other concentrates (Kogenate-FS: 395.1 IU kg^{-1}, $P < 0.01$; Cross-Eight M: 519.1 IU kg^{-1}, $P < 0.05$). The results of this study showed that the continuous infusion of three FVIII concentrates is effective and safe during TJA, and also showed the differences in the continuous infusion rates and total consumption among concentrates when continuous infusion was used to control bleeding during surgery. These two results suggested that the continuous infusion of FVIII concentrate is a good administration mode, but there is still room for further investigation to use it as a more cost-effective and safer administration mode.

Keywords: continuous infusion, haemophilia A, inter-product variability, total joint surgery

Introduction

There are many *in vitro* and *in vivo* studies on the complications of continuous infusion of factor VIII (FVIII) such as, stability after reconstitution, biological sterility, thrombophlebitis and inhibitor formation [1–8], and there is still room for further investigation [9]. However, continuous infusion is known worldwide as a cost-effective and safe administration mode, because this mode can be used to avoid unnecessary high-peak factor levels and unexpected low-trough levels.

Also, many clinical success results were reported using continuous infusion [1–5]. In those reports, the continuous infusion speed was from 2.0 to 6.0 IU kg^{-1} h^{-1} when the desired factor level was aimed at 1.0 IU mL^{-1}. However, no consideration was given to the different states in which bleeding occurs, such as trauma, major surgery and minor surgery, when attempting to achieve haemostasis, nor were the differences in individual concentrate efficacy considered during the administration of continuous infusion. In other words, there are no reports focused on inter-product variability, or inter-individual variability during continuous infusion.

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Accepted after revision 9 February 2010

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As for the available concentrates in Japan, there are two recombinant FVIII concentrates: Kogenate-FS® (Bayer Healthcare, Berkeley, CA, USA) and Advate® (Baxter Healthcare Corporation, Westlake Village, CA, USA), and one highly purified plasma-derived FVIII: Cross-Eight M® (Japanese Red Cross Society, Chitose, Hokkaido, Japan). In this study, to evaluate the inter-product variability, the differences in the above three concentrates were compared when continuous infusion was used as the administration mode to control bleeding during total joint arthroplasty (TJA).

Patients and methods

In total 66 orthopaedic surgeries for 43 haemophilic patients were performed between April 2006 and August 2009 at one centre: 46 surgeries for 29 haemophilia A patients, nine surgeries for seven haemophilia B patients and 11 surgeries for seven haemophilia patients with inhibitors. Among the surgeries for haemophilia A, 28 TJAs for 17 severe haemophilia A Japanese patients without inhibitors were performed using three kinds of concentrates to control bleeding during surgery. Those concentrates are the available concentrates in Japan; Kogenate-FS® (Bayer Healthcare), Advate® (Baxter Healthcare Corporation) and Cross-Eight M® (Japanese Red Cross Society). All concentrates were labelled at 1000 IU per vial. The type of concentrate used during surgery was chosen based on the type previously used by the patient, and never exchanged with another concentrate.

A simple pharmacokinetics study was done for all patients before the first operation, and the FVIII *in vivo* recovery (IVR) and half-life were calculated. The FVIII activity (FVIII:C) was measured in our hospital by a one-stage activated thromboplastin time assay using the CA-1500 analyzer (Sysmex, Hyogo, Japan) before and at 30 min, 6 and 24 h after a 1000 IU concentrate was injected. The FVIII IVR (IU dL^{-1}) per (IU kg^{-1}) was defined as the ratio of FVIII:C increment to the units per kilogram infused of FVIII, and the half-life (h) is the length of time it takes for a 50% drop in plasma drug concentration as one compartment model in this study.

A bolus infusion of FVIII concentrate at a dose calculated to raise the plasma level to 80–100% was administered 30–60 min before the start of the surgery. The plasma FVIII level was confirmed at 10–20 min after bolus injection. At the same time, continuous infusion was initiated at a rate calculated to maintain a steady concentration of 80–100%. The bolus infusion dose of FVIII concentrate and initial concentrate infusion rate were determined according to the results of the simple pharmacokinetics study. Continuous infusion was maintained until day 6, and the daily continuous infusion dosage was adjusted throughout surgical periods to maintain a FVIII:C level of 80% for the first 2 days, 60–80% until day 4 and 40–60% until day 6 at surgery. As a rule, the plasma FVIII level was checked at 30 min, 3 h after surgery, day 1 or 2, day 3 or 4, day 5 or 6 and day 7 at surgery. Based on the FVIII levels at three points at day 0, the actual continuous infusion rate was determined to maintain a steady concentration of 80–100%. Also, the daily continuous infusion rate was determined based on the individual continuous infusion rate at day 0. The factor levels after day 0 were measured to confirm that the individual continuous infusion rate maintained the target FVIII level of the haemostatic protocol, and minor adjustments were done periodically.

Reconstituted FVIII concentrate in a 50 mL polypropylene syringe pump (Terumo, Tokyo, Japan) and a polyvinyl chloride extension tube that has a volume of 0.5 m: (Terumo) were connected parallel to a lactic ringer fluid drip system (drip speed: 500 mL per 24 h). It was infused continuously and exchanged within 12 h to maintain the stability and sterility of reconstituted FVIII at room temperature. The three concentrates were used without heparin, and none were diluted except for Kogenate-FS. Kogenate-FS was diluted (×4) with distilled water to avoid thrombophlebitis [10]. After all data were confirmed to be normally distributed by the Shapiro–Wilk test, they were analysed by the Bonferroni test (multiple comparisons) as parametrical data. SPSS (version 15.0, 2006, Troy, NY, USA) was used for all statistical analysis.

Results

Patient parameters

Patient parameters are shown in Table 1. All patients were adult severe haemophilia A Japanese patients, and there were no significant differences in other parameters among the three concentrates, except platelet counts. None of the patients in this study had inhibitors for FVIII before and after surgery.

Pharmacokinetic analysis and product factor activity

The average FVIII IVR was 1.86 ± 0.4 (IU dL^{-1}) per (IU kg^{-1}), and there were no significant differences among the three concentrates (Table 2). Also, the

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Table 1. Patient and surgical parameters.

	Unit	n	Kogenate-FS	n	Advate	n	Cross-Eight M	n	Total	Statistical analysis
Age	Year	7	39.7 ± 12.9	9	44.3 ± 7.5	12	42.2 ± 8.8	28	42.3 ± 9.4	*ns*
Body weight	kg	7	54 ± 4.0	9	58.7 ± 11.1	12	61.7 ± 6.1	28	58.8 ± 8.0	*ns*
Platelet	10^4 μL	7	23.7 ± 6.1	9	17.9 ± 4.2	12	15.4 ± 7.9	28	18.3 ± 7.1	*
Blood loss	mL	7	1120.4 ± 470.2	9	868.4 ± 361.1	12	933.2 ± 326.7	28	959.2 ± 375.5	*ns*
Transfusion volume	mL	7	620.0 ± 293.3	9	482.2 ± 310.9	12	530 ± 344.0	28	537.1 ± 314.3	*ns*
TKA (re-TKA)	Case		4		8		9 (1)		21 (1)	
THA (re-THA)	Case		3				2 (1)		5 (1)	
TEA	Case				1				1	
BHA	Case						1		1	
Hepatitis C virus positive	Case	7	5	9	9	12	12	28	26	
HIV positive	Case	7	5	9	6	12	3	28	14	
HAART	Case	5	2	6	2	3	3	14	7	

ns, no significant differences among the three concentrates; TKA, total knee arthroplasty; THA, total hip arthroplasty; TEA, total elbow arthroplasty; BHA, bipolar hip arthroplasty; Re-TKA, revision TKA; Re-THA, revision THA; HAART, highly active retroviral therapy.
*$P < 0.05$ comparison between Kogenate-FS and Cross-Eight M.

Table 2. Pharmacokinetic analysis and product factor activity.

	Unit	Kogenate-FS	Advate	Cross-Eight M	Total	Statistic
Sample	Counts	11	6	11	28	
IVR	(IU dL^{-1}) per (IU kg^{-1})	1.91 ± 0.4	1.86 ± 0.5	1.83 ± 0.2	1.86 ± 0.4	*ns*
Half-life	h	13.7 ± 3.2	11.7 ± 4.0	13.2 ± 3.5	13.1 ± 3.5	*ns*
Batches	Counts	15	14	21		
Activity	IU per vial	1147 ± 47.4	1041 ± 34.7	1135.2 ± 39.1	1112 ± 60.3	*,†

ns, no significant differences among the three concentrates; IVR, *in vivo* recovery.
*$P < 0.01$ comparison between Kogenate-FS and Advate.
†$P < 0.01$ comparison between Advate and Cross-Eight M.

average half-life was 13.1 ± 3.5 (h), and there were no significant differences among the three concentrates. Several different batches of concentrates were used for surgery; 15 batches of Kogenate-FS® (Bayer Healthcare), 14 batches of Advate® (Baxter Healthcare Corporation) and 21 batches of Cross-Eight M® (Japanese Red Cross Society). The batches differed according to the time the operation was performed. Those product FVIII activity data were obtained from the product companies. The average activity of Advate was significantly lower than the average activity of Kogenate-FS and Cross-Eight M. Compared with the labelled activity on the vial, the average activity of Advate was as labelled, but the average activity of Kogenate-FS and Cross-Eight M was approximately 1.1 times as labelled.

Factor VIII:C (%) and concentrate doses

The actual FVIII plasma level at peri-operative periods was close to the target FVIII level according to the above haemostatic plan. The FVIII plasma levels of the Kogenate group were significantly higher than that of the others at just the first two measurement points, and there were no significant differences among the three concentrates later (Fig. 1).

To reduce the differences in product variability among the three concentrates, the original bolus infusion dose and continuous infusion rate of Kogenate-FS and Cross-Eight M were adjusted according to the differences in product activity. Even after adjustments were made to the bolus infusion dose and continuous infusion rate, there were significant differences among the three concentrates. There were no significant differences among the bolus infusion doses of the three FVIII concentrates (Kogenate-FS: 61.2 ± 11.8; Advate: 65.1 ± 10.1; Cross-Eight M: 62.5 ± 7.6) calculated to raise the plasma level to 80–100%. However, the continuous infusion rate of Advate (2.1–4.2 IU kg^{-1} h^{-1}: average range during postoperative 7 days) was significantly higher than that of Kogenate-FS (1.0–2.9 IU kg^{-1} h^{-1}, $P < 0.01$ and $P < 0.05$) and Cross-Eight M (1.8–3.2 IU kg^{-1} h^{-1}, $P < 0.01$) to maintain the target

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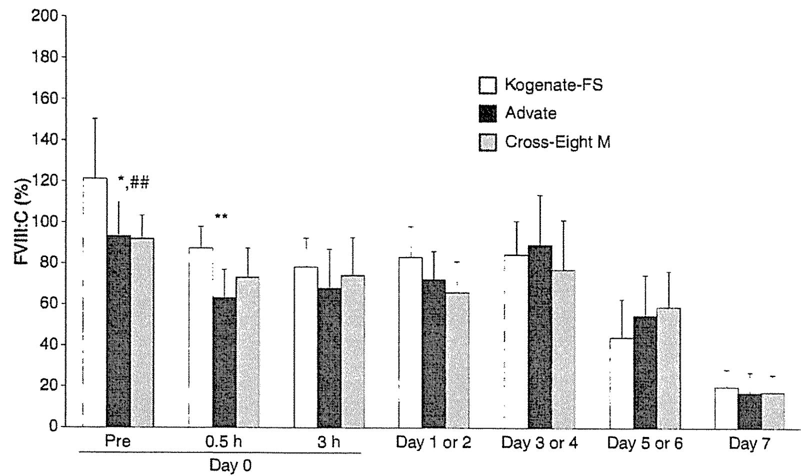

Fig. 1. Factor VIII (FVIII) levels during peri-operative periods. FVIII levels of the Kogenate group at preoperation and 0.5 h after operation were siginificantly higher than that of other concentrate cases ($^{*}P < 0.05$; $^{**}P < 0.01$ compared with Advate; $^{\#\#}P < 0.01$ compared with Cross-Eight M).

plasma level (Fig. 2). Also, the total consumption of Advate (652.1 ± 183.0 IU kg^{-1}: average) was significantly greater than either of the other concentrates (Kogenate-FS: 395.1 ± 65.0 IU kg^{-1}, $P < 0.01$; Cross-Eight M: 519.1 ± 68.0 IU kg^{-1}, $P < 0.05$).

Surgical parameters

Various types of TJAs were performed for patients, but there were no significant differences in blood loss from the period during surgery until day 2 among the three concentrates (Table 1). Between day 0 and day 2, unexpected bleedings and drops in FVIII levels occurred during eight surgeries. As a result, one injection of concentrate was required to control bleeding and raise the plasma level. For Kogenate-FS, one total knee arthroplasty (TKA) required one extra bolus injection. One TKA, one total hip arthroplasty (THA), one revision TKA, and one revision THA for Cross-Eight M, and three TKAs for Advate also required one extra bolus injection.

For all patients, 400 g of whole blood was pooled several days before surgery, and then returned to the patient at day 0 or day 1 as an autologous blood transfusion. Drainage blood from the wound was also returned to the patient by using the ConstaVac CBC II Blood Conservation System (Stryker, Mahwah, NJ, USA). Homologous blood was transfused for two cases; one THA using Kogenate-FS, and one TKA using Cross-Eight M. There were no differences in the total transfusion volumes among the three concentrates.

Other optional treatments

A tourniquet was not routinely used for TKA, instead, strict protocols were followed to coagulate bleeding during surgery to avoid complications, such

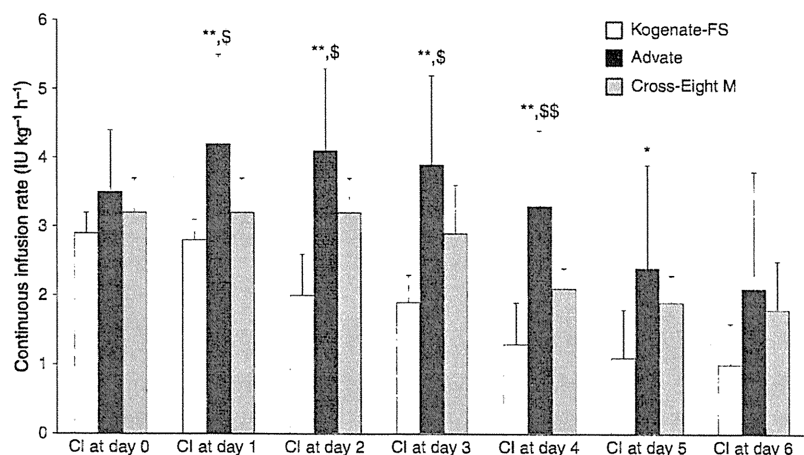

Fig. 2. Continuous infusion rate. The continuous infusion rate of Advate is significantly greater than that of other concentrates ($^{*}P < 0.05$; $^{**}P < 0.01$ compared with Kogenate-FS; $^{\$}P < 0.05$; $^{\$\$}P < 0.01$ compared with Cross-Eight M).

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as deep vein thrombosis, after surgery. Tranexamic acids were not used for any of the patients.

Other adverse effects

Based on appearance, the reconstituted concentrate in the infusion system was clear, and there were no signs of bacterial contamination. Also, none of the patients showed any signs of inhibitors, thrombosis or thrombophlebitis.

Discussion

Continuous infusion of FVIII was described already in 1954 [11] and proposed in 1970 as an effective alternative to the traditional bolus infusion to provide a safe, steady-state haemostatic factor level [12]. Many pharmacokinetics and biological studies, *in vivo* and *in vitro*, confirmed the efficacy, stability and microbiological safety of reconstituted FVIII at room temperature for at least 24 h. Neidhardt *et al.* [6] reported the stability of B-domain deleted recombinant FVIII within 72 h after reconstitution *in vitro*. Parti *et al.* [7] also reported the stability of plasma/albumin-free recombinant human FVIII at room temperature within 24 h after reconstitution *in vitro*. Stachnik reviewed clinical reports about continuous infusion of factor products for haemophilia and concluded that factor products were stable and sterile for periods longer than several hours [8]. Martinowitz *et al.* [4] reported that continuous infusion of sucrose-formulated recombinant FVIII is stable, effective and safe during major surgery within 24 h. Lee *et al.* [3] described that continuous infusion was a cost-effective delivery system with the portable minipumps, as approximately 30% of the total consumption of the concentrate can be saved, when compared with intermittent bolus infusion. Also, many comparison clinical studies between bolus infusion and continuous infusion [1,2,5] made clear some additional advantages of continuous infusion; avoidance of unnecessary high-factor peak levels and unexpected low-trough levels, and reduction of factor consumption at haemostasis. In this study, continuous infusion was also a safe and effective administration mode, because there were no adverse effects, such as biological infection, or an unexpected drop in FVIII activity.

However, it was difficult to judge the continuous infusion rate and clearance. The initial infusion rate in previous studies [2,5,13–15] was from 1.8 to 6.0 IU kg^{-1} h^{-1}, and the average clearance of FVIII in previous studies [1,4,16] was from 2.65 to 3.89 mL kg^{-1} h^{-1}. In the above results, there are only three reports that described the clearance and infusion rate for a single product during some kinds of surgeries. Stieltjes *et al.* [15] described that the initial infusion rate of ReFacto® (Wyeth Pharmaceuticals Inc., Philadelphia, PA, USA) was from 1.8 to 6.0 IU kg^{-1} h^{-1} during 20 surgical procedures. Negrier *et al.* [5] also described that the initial continuous infusion rate of Advate was from 3.2 to 3.7 IU kg^{-1} h^{-1} during 18 surgical procedures for major and minor orthopaedic surgeries, and non-orthopaedic surgeries. Martinowitz *et al.* [4] described that the clearance of Kogenate-FS was 3.02 mL kg^{-1} h^{-1} (1.6–4.6 mL kg^{-1} h^{-1}) according to a pharmacokinetics study. In this study, the average for the initial continuous infusion rate of Kogenate-FS, Advate, and Cross-Eight M was 2.9 ± 0.3, 3.5 ± 0.9, and 3.2 ± 0.5 IU kg^{-1} h^{-1}, respectively, during TJA. This result showed the efficacy and safety of continuous infusion of these three concentrates, because their initial infusion rates were in the range of the above previous results.

As for the causes of influence on continuous infusion rates, many factors are nominated such as, the adsorption in the infusion system, the dilution of concentrate, and inter-product, inter-individual, and inter-surgical procedure variations.

Henze *et al.* described that the activity of Kogenate-FS is reduced in polyvinyl bags within the first few hours, as a result of the adsorption of recombinant FVIII protein to the inner surface of the tubing and bags. However, the results suggested that the minimal binding of protein to the infusion tubing at the start of continuous infusion would probably not alter the plasma FVIII concentration in a clinical situation [17]. McLeod *et al.* [18] also pointed out that the amount of FVIII activity significantly decreased during storage in the polyvinyl chloride bags for Kogenate-FS, diluted from 146–2 IU mL^{-1}. In this study, Kogenate-FS maintained good factor activity even if it was diluted (×4) with distilled water and infused continuously by using a polyvinyl chloride delivery system. Fernandez *et al.* [19] researched the stability of Advate during simulated continuous infusion based on three delivery systems, factor potency (high or mid potency) and dilution, and concluded that Advate is stable under conditions typically encountered during continuous infusion and suggested that Advate should be safe and effective when used for FVIII replacement by continuous infusion. The volume (0.5 mL) of the polyvinyl chloride extension tube in this study seemed to be too small to influence the adsorption of a concentrate. However, in this study, the adsorption in this infusion system might have

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influenced the continuous infusion rate, because the IVR and half-life of Advate were the same as those of the other concentrates according to the simple pharmacokinetic study, but Advate required a higher infusion speed when infused continuously.

As for the inter-product variability, Hermans *et al.* [16] described that the clearance of recombinant FVIII (2.65 ± 0.8 mL kg^{-1} h^{-1}) was significantly lower than that of plasma derived FVIII (3.97 ± 1.38 mL kg^{-1} h^{-1}). On the other hand, Bidlingmaier *et al.* [2] described that the product type, such as plasma derived or recombinant factor concentrates, did not influence the infusion rate, and the median infusion rate was 4.4 IU kg^{-1} h^{-1} (range: 2.8–9.5 IU kg^{-1} h^{-1}). This study showed that the continuous infusion rate of Advate is significantly higher than that of Kogenate-FS and Cross-Eight M, and the total consumption of Advate is significantly larger than that of Kogenate-FS and Cross-Eight M. These results suggested that the clearance of Advate was more easily influenced by the surgical condition such as a massive vascular injury or exsanguinations, or the administration method such as continuous infusion, compared with the other concentrates.

All the previous studies discussed the continuous infusion rates without consideration given to the variability of inter-surgical procedures; however, in relation to continuous infusion, it is an important factor for discussion, as well as, inter-individual variability. Batorova and Martinowitz described that the possible inter-individual variations of clearance, according to age, bodyweight, laboratory assay employed and type of concentrate used, should be considered. And to prevent an unexpected drop in the factor level in a particular patient, because of higher than expected clearance, it is advised to check the factor level a few hours later and to increase the rate if necessary [16].

In this study, the variability of inter-individuality, inter-product activity and inter-surgical procedure was made as small as possible; however, there were significant differences in the consumption of concentrates. Schulman described that there is still room for further investigation concerning the complications of continuous infusion of FVIII concentrates, and that future studies should address the question of minimum steady-state levels required for haemostasis [9]. Further studies are needed to conclude what factors influence the infusion speed of FVIII concentrates when infused continuously.

This study showed that the continuous infusion of two recombinant factors and highly purified plasma-derived FVIII are effective and safe during TJA, because the continuous infusion rates of those three concentrates are in the usual infusion rate range. Importantly, Advate needed to be given at a significantly higher infusion rate than the other two concentrates. The total consumption of Advate was also significantly greater than either of the other concentrates. These two results suggested that the continuous infusion of FVIII concentrate is a good administration mode, but there is still room for further investigation to use it as a more cost-effective and safer administration mode.

Disclosures

The author stated that he had no interests which might be perceived as posing a conflict or bias.

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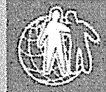

Haemophilia (2010), **16**, 290–295 DOI: 10.1111/j.1365-2516.2009.02149.x

ORIGINAL ARTICLE *Paediatrics*

Major orthopaedic surgeries for haemophilia with inhibitors using rFVIIa

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Summary. Between 2000 and 2008, 11 major orthopaedic surgeries for 7 congenital haemophilia patients with inhibitors were performed by the first author as the primary doctor using recombinant activated factor VII (rFVIIa). Orthopaedic surgical treatments were performed for six surgeries for four high-responder haemophilia A patients, three surgeries for two high-responder haemophilia B patients and two surgeries for one low-responder haemophilia B patient. This low-responder patient is allergic to factor IX products, so he usually uses rFVIIa as a haemostatic agent. All of the surgeries were major, such as joint arthroplasty, arthroscopic synovectomy, and a combination of both, and excellent surgical results were achieved. Seven cases were controlled by bolus infusion of rFVIIa, and the other four cases were controlled by combined bolus and continuous infusion of rFVIIa. An anti-fibrolytic agent was used for all cases. There were no thrombogenic adverse effects, only two bleeding episodes. As for haemostatic control, nine surgeries were excellent, one was good and one was fair. This report is the largest clinical report on major orthopaedic surgeries at a single institute. We have concluded that the combination of bolus and continuous infusion of rFVIIa is safe and effective, and more convenient to administer than simple bolus infusion therapy to achieve haemostasis at peri-operative periods. In addition, our data also concurs with the data of several previous reports which showed that orthopaedic surgery for haemophilia patients with inhibitors by means of rFVIIa is safe and effective.

Keywords: inhibitor, orthopaedic surgery, rFVIIa

Introduction

For haemophilia patients without inhibitors, orthopaedic surgery is becoming popular. However, for haemophilia patients with antibodies for the deficient factor VIII or IX (inhibitor), this surgery is an elective treatment [1,2], because there is still no guideline, based on surgical case studies, which specifies a proper monitoring marker to monitor the coagulation factor plasma levels and how to effectively use concentrates to control bleeding during surgery. However, many haemophilia patients with inhibitors also complain about joint dysfunction and seek out orthopaedic surgical treatments. Surgical results were reported for some surgeries that were performed using bypassing agents, such as activated prothrombin complex concentrate (aPCC) and activated recombinant factor VII (rFVIIa). Most of these results were of minor surgeries such as radioactive synovectomy or tooth extraction. In some multicentre studies [1,3–6], the results of more than 10 major orthopaedic surgeries were reported, but there is no large report from a single treatment centre. This report includes the results of 11 major surgeries that were performed for 7 haemophilia patients by one orthopaedic surgeon as the primary doctor who is

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Accepted after revision 19 October 2009

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sufficiently knowledgeable in matters related to haemostasis and an experienced and active surgeon at two hospitals.

Patients

Between 2000 and 2008, the first author as the primary doctor performed 16 orthopaedic surgeries at 2 hospitals for 10 haemophilia patients with inhibitors. During 11 orthopaedic surgeries and postoperative periods for 7 patients, rFVIIa was used to control bleeding. Seven surgeries were performed at the first hospital between 2000 and 2006, and four surgeries were performed at the second hospital between 2007 and 2008. All surgeries were performed by one primary doctor, knowledgeable in haemostasis and an active surgeon, thus, making this the largest clinical report based on major orthopaedic surgeries at a single institute. For Case 1, there was a pseudotumour (20 × 10 cm) in the left femoral region with two skin ulcers. For Case 2, the abduction of both shoulders was less than 80° and flexion was less than 60° owing to osteophytic impingement. Total knee arthroplasty and bipolar hip arthroplasty (Cases 3 and 7) were indicated for end-stage arthropathy with severe pain and recurrent bleeding. Arthroscopic synovectomy (Cases 4, 5 and 6) was indicated for early or progressive arthropathy with recurrent bleeding.

Results

Six orthopaedic surgical treatments were performed for four high-responder haemophilia A patients, three surgeries for two high-responder haemophilia B patients and two surgeries for one low-responder haemophilia B patient. This low-responder patient is allergic to FIX products, so he usually uses rFVIIa as a haemostatic agent (Table 1). The average age at operation was 26 years (11–52 years old) and the average body weight was 56 kg (43–80 kg). The preoperative inhibitor titres were 1–54.3 Bethesda Units (BU) mL^{-1} and the plasma-deficient factor levels in all cases were less than 1%, except one (1.7%). All of the surgeries were major, such as joint arthroplasties, arthroscopic synovectomy and a combination of both (Table 2). Four arthroscopic knee synovectomies and two total knee arthroplasties were performed using a tourniquet. There was very little bleeding during the surgeries except for the one that was combined with septoplasty. The blood loss volumes for five orthopaedic surgeries performed for haemophilia patients with inhibitors, without the use of a tourniquet, were comparatively the same with the blood loss volumes for orthopaedic surgeries for haemophilia patients without inhibitors. The average operation period was 125.3 min (69–193 min).

The average of one bolus infusion dose per body weight was 113.6 mcg kg^{-1} (range 95.2–39.5 mcg kg^{-1}) (Table 3). For the first case (Case 1), which was our first experience, we administered the first bolus infusion before general anaesthesia, and then the second bolus infusion after bleeding occurred following the first incision. For cases that followed, we administered the first bolus infusion before general anaesthesia, and then the second bolus infusion just before the first incision. The average total period for peri-operative bleeding control using rFVIIa was 11.5 days (range 6–23 days). This period, compared with the first two cases (Cases 1 and 2-1), was longer than that of the other cases and the total dose per body weight was also gradually decreased. Bleeding complications during seven surgeries were controlled by bolus infusions of rFVIIa (bolus group) and the other four surgeries were controlled by combined bolus and continuous infusions of rFVIIa (combined group). The total period for peri-operative bleeding control of the bolus group

Table 1. Patient demographics and clinical characteristics: 11 major orthopaedic surgeries were performed for 7 congenital haemophilic patients with inhibitors.

Case	Age (years)	Body weight (kg)	Deficient factor	Deficient factor level (%)	Inhibitor titre (BU mL^{-1})	Anamnestic response
1	52	58	VIII	1.70	17.5	Yes
2-1	29	51	VIII	<1	2.3	Yes
3-1	38	60	VIII	<1	6.5	Yes
4-1	11	43	IX	<1	28.8	Yes
5	27	48	VIII	<1	1	Yes
3-2	39	60	VIII	<1	5.8	
6	13	45	IX	<1	54.3	Yes
4-2	13	47	IX	<1	29	
2-2	33	80	VIII	<1	13	
7-1	16	63	IX	<1	1	No
7-2	17	60	IX	<1	1	No

BU, Bethesda Unit.

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