An Ex Vivo Keratinocyte Model for Gene Therapy of Hemophilia B

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We are investigating whether skin-targeted gene therapy may be used to treat hemophilia B by transplanting keratinocytes transduced by factor IX-expressing retroviral vectors. No pre-clinical animal model for keratinocyte-mediated gene therapy has shown long-term efficacy in vivo. It remains unclear whether this short-term expression is due to promoter shut-off or a reduced survival of grafted genetically modified cells. The purpose of this study was to determine the fate of primary human keratinocytes superficially grafted to nude mice in a silicone transplantation chamber. In addition, vectors containing keratinocyte-specific enhancers from the human papilloma virus-16 (HPV-16) and human keratin 5 and 14 genes were used upstream of the cytomegaloviral (CMV) immediate-early promoter/enhancer to control factor IX cDNA expression to avoid promoter shut-off. Factor IX was secreted by cultured keratinocytes after transduction by each of these chimeric promoter/enhancer vectors, although the levels varied according to the particular construct used. Keratinocytes transduced by the vector containing the HPV-16 enhancer were grafted into nude mice, and human factor IX was detected in plasma at 0.02-9 ng per ml for 4-5 wk for the duration of graft survival. The HPV-16 enhancer may be a useful addition to expression vectors for keratinocyte gene therapy. The transplantation chamber can be adapted to grafting retrovirally transduced keratinocytes for gene transfer studies. Key words: retroviral vector/epidermis/ factor IX/skin grafting. J Invest Dermatol 109:139-145, 1997

The keratinocyte, the major cell type of the epidermis, is an attractive target cell for the purpose of ex vivo gene therapy (reviewed by Greenhalgh et al., 1994). The epidermis can be noninvasively biopsied to provide a source of keratinocytes (Rheinwald, 1989) that can be serially cultivated (Rheinwald and Green, 1975), transduced by retroviruses, and expanded to large numbers in culture (Green et al., 1979). In addition, procedures for autografting keratinocytes onto burn victims are well established (Gallico et al., 1984). Furthermore, these grafts remain viable, enabling easy monitoring (e.g., for signs of malignancy) and removal if necessary.

As a result, the epidermis has become the focus of many gene therapy protocols, using not only an ex vivo retroviral approach (Morgan et al., 1987; Gerrard et al., 1993; Stockschlader et al., 1994; Choate et al., 1996) but also a direct in vivo approach with adenoviral vectors (Setoguchi et al., 1994; Bonnekoh et al., 1995), topically applied DNA-liposome complexes (Alexander and Akhurst, 1995; Li and Hoffman, 1995), or direct injection of naked DNA (Katsumi et al., 1994; Hengge et al., 1995).

Genetically modified epidermis can theoretically be used to treat a diverse range of diseases, either as a bio reactor to systemically deliver proteins such as clotting factors (Gerrard et al., 1993; Brownlee, 1995; Fenjves et al., 1996), growth hormone (Morgan et al., 1987; Jensen et al., 1994), and α1-anti-trypsin (Setoguchi et al., 1994) or as a metabolic sink to remove toxic products from the circulation, e.g., by expression of adenosine deaminase (Stockschlader et al., 1991). Alternatively, gene therapy may be applied to treat inherited (Choate et al., 1996) or acquired (Bonnekoh et al., 1995) disorders of the skin and might be useful in accelerating healing of epithelial autografted burn victims (Gallico et al., 1984).

Previously we have shown that normal human keratinocytes, transduced by a retroviral vector in which the human clotting factor IX cDNA was under the transcriptional control of the Moloney murine leukemia 5' long terminal repeat (LTR) promoter/enhancer, were able to secrete ~600 ng of factor IX per 106 cells per 24 h in tissue culture. When these cells were grafted beneath a skin flap in athymic mice, however, the levels of human factor IX detected in the plasma (~3 ng per ml) were lower than expected. Moreover, factor IX rapidly declined and was not detected after 1 wk. The low levels and short duration of expression in vivo may have been a result of (i) a change in transcriptional activity of the promoter after transplantation; (ii) the "ectopic" environment of the graft affecting the growth, differentiation, and life-span of the transplanted keratinocytes; or (iii) inadequate transport of factor IX to the circulation via lymphatic drainage. This latter possibility is unlikely, however, because reasonably efficient transport of factor IX from a subcutaneous site to the bloodstream was demonstrated in model experiments (Gerrard et al., 1992), and factor IX expression directed to the naturally formed (i.e., nongrafted) epidermis in a transgenic mouse resulted in its transport to the blood (Alexander et al., 1995).

Similar in vivo observations of low and decreasing recombinant gene product under the transcriptional control of strong viral promoters [such as the cytomegalovirus (CMV) or retroviral LTR]
Figure 1. Retroviral vectors containing keratinocyte-specific enhancer elements. Vector nomenclature is derived from the individual components of the construct: L, LTR; N, neo (neomycin phosphotransferase selectable marker); C, CMV (immediate-early CMV promoter/enhancer); IX, human clotting factor IX cDNA; P, keratinocyte dependent enhancer from HPV-16; K5/K14, transcriptional sequence from the 5' noncoding region of hK5 and hK14 genes, respectively; K14R, reverse orientation. PA317 producer cell clones were isolated for each of these vectors with the exception of PA317-LNCIXc11, which had been previously isolated (Palmer et al., 1989). Footnotes to figure are as follows: “The number of PA317-producer clones secreting virus over the total number isolated for each construct; b The number of PA317-producer clones secreting virus and human factor IX over the total number isolated; c Best clone refers to the clone reference number with the highest viral titer; d The viral titer of the best clone.

Table 1

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Scale=1 kb HPV-16 hK14 hK14 (reverse) hK5

have been reported by others in keratinocytes (Fenjves et al., 1996), fibroblasts (Palmer et al., 1989), myoblasts (Dai et al., 1992), hepatocytes (Kay et al., 1992), and hematopoietic stem cells (Challita and Kohn, 1994). In some cases, persistence of vector DNA but declining levels of mRNA suggested that the decrease in recombinant protein may be a result of promoter down-regulation (Palmer et al., 1991; Scharffmann et al., 1991; Challita and Kohn, 1994; Dwarik et al., 1995). This problem of viral promoter shut-off has been partly overcome by the use of nonviral promoters such as the phosphoglycerate kinase (Moullier et al., 1993), the dihydrofolate reductase (Scharffmann et al., 1991), the metallothionein (Heartlein et al., 1994), the β-actin (Chowdhury et al., 1991), the polymerase II (Rettinger et al., 1994), or the human α-anti-trypsin (Hafenrichter et al., 1994) promoters. Despite the obvious success in achieving long-term expression in vivo, however, these “housekeeping” or tissue-specific promoters are generally much less active than viral promoters (Yao et al., 1991; Cheng et al., 1993; Rettinger et al., 1994). By using hybrid promoter/enhancer elements to combine the dual benefits of a strong promoter with the long-term effects of tissue-specific enhancers, Dai et al. (1992) and Okuyama et al. (1996) achieved sustained detectable levels of transgene expression in vivo. Not all combinations of promoter/enhancer hybrids, however, were successful (Dai et al., 1992; Wu et al., 1996).

Herein we sought to improve the duration and level of factor IX expression in vivo in a nude mouse model. We have investigated a superficial skin grafting technique as an alternative to grafting keratinocytes in an “ectopic” position beneath a skin flap, as we have previously reported (Gerrard et al., 1993). Keratinocytes were transplanted onto a superficial position of the skin of an athymic mouse within a silicone transplantation chamber. This chamber (Boukamp et al., 1985), used herein as part of a gene therapy protocol, protects the grafted keratinocytes from desiccation and mechanical irritation while enabling tissue architecture to form. Furthermore, the chamber defines the limits of the wound margin by preventing graft shrinkage and host-mediated re-epithelialization of the graft bed thus enabling the contribution of surviving bona fide transplanted cells to the re-formed epithelium to be determined without ambiguity. Retroviral vectors were constructed in which factor IX expression was controlled by the strong CMV immediate-early promoter/enhancer and keratinocyte-specific enhancer elements from either human papilloma virus 16 (HPV-16; Cripe et al., 1987) or just upstream of the human keratin 14 (hK14) (Jiang et al., 1990; Leask et al., 1990) or the human keratin 5 (hK5) (Ohtsuki et al., 1992; Byrne and Fuchs, 1993) genes. Factor IX produced from keratinocytes transduced by each of these vectors was assessed in vitro (tissue culture) and in vivo after superficial transplantation to nude mice.

MATERIALS AND METHODS

Construction of Retroviral Vectors The human factor IX cDNA was excised from pKG5IX-2—a modified version of pKG5IX-1 (Rees et al., 1988), correcting a short nucleotide rearranged section present in earlier constructs (Anson et al., 1985; Gerrard et al., 1993)—with HindIII and XhoI, subcloned into pBluescript KS+ (Stratagene) to generate pBS-FIX, re-excised with BamHI, “filled-in” with the Klenow fragment of DNA polymerase I, and finally cloned into pHap-cut pLNCX (Miller and Ross, 1989) to generate pLNCIX. All other vectors were derived from this vector by amplifying keratinocyte enhancer elements by Pfu (Stratagene) polymerase chain reaction and cloning the BamHI-digested products into BamHI-digested pLNCIX (Fig 1): pLNCIX contained the enhancer region from positions 7500 to 7777 of the HPV-16 genome (Sedford et al., 1985) and was constructed by using the primers 5′-ACCGGATCCCTTCTATGTCAGCAACTATGG-3′ (forward) and 5′-ACCGGGATCCATTGGTGGCCCTTAGAAG-3′ (reverse); pL14CIX and pL14RCIX contained the proximal domain (positions −271 to −30) from upstream of the hK14 gene (Leask et al., 1990) in the forward and reverse orientation, respectively, and was constructed by using the primers 5′-ACCGGATCCCTATGTCAGCAACTATGG-3′ (forward) and 5′-ACCGGATCCCTATGTCAGCAACTATGG-3′ (reverse); pLNK5CIX contained the region from positions −102 to −29 upstream of the hK5 gene (Byrne and Fuchs, 1993) amplified with the primers 5′-CGGGGTACCGGATCCCTATGTCAGCAACTATGG-3′ (forward) and 5′-CGGGGTACCGGATCCCTATGTCAGCAACTATGG-3′ (reverse). Polymerase chain reaction fidelity and the orientation of cloned inserts was verified by automated DNA sequencing.

Cell Culture: Generation of Defective Retroviral Vector Producer Cell Lines and Transduction of Human Keratinocytes Defective retroviral vector producer cell lines were generated as previously described (Miller et al., 1993). Briefly, the packaging cell line 8-2 (Mann et al., 1983) was transiently transfected with vector plasmid and the resulting defective virus was used to transduce the packaging cell line PA317 (Miller and Buttimoore, 1986). Individual colonies of G418-resistant PA317 vector producer cells were isolated and the viral titer was determined on 3T3 tk−.
cells, essentially as described (Miller et al, 1993). The \( \Psi \)CRI/MFG-NB producer cell line secretes the retroviral vector MFG-NB, which contains the nuclear-localized \( \beta \)-galactosidase marker under the control of the retroviral LTR promoter/enhancer and lacks a neo selectable marker (Ferry et al, 1991). Low-passage (less than five passages) normal human foreskin keratinocytes strains kk, ka, or z were transduced by co-cultivation with lethally irradiated PA317 producer cells (Gerrard et al, 1993). Transduced keratinocytes were selected in 0.15 mg G418 per ml for 2 wk with the exception of cells transduced by \( \Psi \)CRI/MFG-NB, which were grafted without selection.

Quantitation of Human Factor IX by Enzyme-Linked Immunosorbent Assay (ELISA) Human factor IX in conditioned medium from transduced keratinocytes and PA317 producer cells was measured by ELISA (limit of sensitivity \( \sim 0.1 \) ng per ml) as previously described (Anson et al, 1985) using the monoclonal anti-human factor IX antibody 3A6 (Dako, Huko Wymouke, UK) followed by peroxidase-conjugated goat anti-rabbit IgG (Sigma, Poole, UK) as the primary and secondary detection antibodies, respectively. For quantitating human factor IX in mouse plasma, samples were diluted in phosphate-buffered saline containing 0.1% Triton X-100, 20 ng bovine serum albumin per ml, and 1 mM ethylenediamine tetraacetic acid, and the sensitivity of the ELISA was improved over that described by Anson et al (1985) to \( \sim 0.02 \) ng per ml by replacing the secondary antibody with biotinylated goat anti-rabbit IgG (Vector, Peterborough, UK) followed by horseradish peroxidase-avidin conjugate (Vector) before development (Anson et al, 1985).

Superficial Transplantation of Keratinocytes to Mouse Keratinocytes were grafted beneath a silicone transplantation chamber in a one-step procedure essentially as described (Boukamp et al, 1985). Briefly, the chamber (Renner, Dannestadt, Germany) was embedded beneath the full-thickness skin of 8- to 10-wk-old male MF1 Nu/Nu mice (Harlan Olac, Bicester, U.K.) and 100 keratinocytes in 200 \( \mu l \) of phosphate-buffered saline were carefully layered onto the surface of the mouse fascia by injection through the chamber. Blood was collected from the tail vein into one-ml centrifuge tubes containing 7.5% sodium citrate, and the plasma was separated by microcentrifugation. Animal husbandry and all procedures were performed according to British Home Office regulations. Mice were humanely sacrificed and the tissue beneath the chamber was processed for either routine hematoxylin and eosin or histochemical (Jensen et al, 1994) staining.

RESULTS

Generation of Defective Retroviral Producer Clones A series of retroviral vectors based upon the Moloney murine leukemia virus (Miller and Rosman, 1989) was constructed in which the factor IX cDNA was placed under the transcriptional control of either the CMV immediate-early promoter/enhancer alone (LN CIX) or a combination of the CMV promoter/enhancer and various keratinocyte-specific enhancer elements (Fig 1). The vector LNPCIIX contained the 777-bp (positions 7500–7777) keratinocyte-dependent viral-independent enhancer upstream of the P97 promoter of HPV-16 (Cripe et al, 1987); LNK14CIX and LNK14RCIX contained a 241-bp fragment of the proximal enhancer from the hK4 gene (liang et al, 1990; Leask et al, 1990, 1991) in the forward or reverse orientation, respectively, and LN5CIX contained 73 bp of sequence immediately 5’ of the TATA box from the hK5 gene (Ohkushi et al, 1992; Byrne and Fuchs, 1993) (Fig 1). The CMV promoter was selected because it is one of the strongest known viral promoters in human cells, including keratinocytes (Cheng et al, 1993), and because its activity is up-regulated by a heterologous enhancer (e.g., the muscle creatine kinase enhancer; Dai et al, 1992). The keratinocyte-specific enhancers were selected because they are among the best studied enhancers known to be active in normal keratinocytes.

Defective-retroviral producer clones were generated by standard techniques, and the individual clones of G418-resistant PA317 producer cells were screened for factor IX expression and virus in the conditioned medium. Clones were considered “functional” if they secreted factor IX and had a detectable viral titer (Fig 1). The inclusion of different keratinocyte-specific enhancer elements as part of the same parent vector backbone had variable effects on the ability to isolate functional virus-secreting producer clones (Fig 1). When the LNPCIIX construct was used, more than half of the clones isolated produced virus and a third also secreted factor IX, but when the LNK14CIX construct was used, only 4 of 27 produced virus and only one clone also secreted factor IX. High-titer producer clones (\(-10^{-6}\) colony-forming units per 10\(^{6}\) cells per 24 h) were isolated for the vectors LNK14CIX and LNK5CIX. Even the best PA317 producer clone isolated for vectors LNPCIIX and LNK14CIX, however, gave low viral titers (\(<10^{-6}\) colony-forming units per 10\(^{6}\) cells per 24 h).

Transduced Keratinocytes Secrete Factor IX into Conditioned Medium Normal human foreskin keratinocytes were transduced by each of the five factor IX retroviral vectors (Fig 1) by co-cultivation with the corresponding lethally irradiated PA317 producer clone, and the conditioned medium from G418-selected keratinocytes was assayed for factor IX by ELISA (Fig 2). Transduction was reasonably efficient with the high-titer producer clones PA317-LN5CIXc23 and -LNK14CIXc12 but was inefficient with the lower-titer clones PA317-LNClIXc11, -LNPCIIXc23, and -LNK14CIXc13 (data not shown). Keratinocytes transduced by the parent vector PA317-LNClIXc11 secreted 255 ± 51 ng of factor IX per 10\(^{6}\) cells per 24 h in culture and by PA317-LNPCIIXc23 secreted marginally higher levels of 387 ± 66 ng of factor IX per 10\(^{6}\) cells per 24 h (Fig 2), although this difference is not statistically significant in a Student’s t test (\( p > 0.1 \)). These values are also similar to the levels reported for keratinocytes in which the factor IX was under the control of the Moloney murine leukemia retrovirus LTR promoter/enhancer (Gerrard et al, 1993; Fenjves et al, 1996). By contrast, keratinocytes transduced by PA317-LN5CIXc22 and -LNK14CIXc13 secreted \(-10^{-5}\) fold lower levels of factor IX at 19.5 ± 2.3 and 52.5 ± 15.5 ng per 10\(^{6}\) cells per 24 h, respectively, and transduction with PA317-LNK14CIXc13 resulted in even lower factor IX levels of 1.6 ± 0.13 ng of factor IX per 10\(^{6}\) cells per 24 h.

Human Factor IX Is Detected in Plasma of Mice Grafted with Transduced Keratinocytes Because the HPV-16 enhancer did not inhibit the level of factor IX secreted by keratinocytes in vitro (Fig 2), keratinocytes transduced by this vector were...
grafted onto nude mice. A silicone transplantation chamber was embedded beneath the full-thickness mouse skin and the vector-transduced keratinocytes were injected as a cell suspension directly onto the muscle. Transplanted keratinocytes were injected as a cell suspension directly onto the muscle.

After transplantation, plasma samples were periodically taken from and assayed for human factor IX. After transplantation (Fig 5A), human keratinocytes, as identified by the occasional blue histochemical staining, were found along the surface of the mouse fascia graft bed. By 6 d (Fig 5B), the keratinocytes had differentiated to form a mature epidermis complete with the four layers of stratified tissue characteristic of normal skin architecture. 

β-Galactosidase-positive cells were detected in all layers (Fig 5C), and the mouse mesenchyme tissue had contracted to form a thick neo-dermis containing densely packed fibroblasts. After 2 wk, the keratinocytes on the surface began to be engulfed by the dense mouse mesenchyme tissue (Fig 5D) until they became fully encapsulated to form subcutaneous keratinocyte-containing cysts (data not shown). By 4 wk, the dermis consisted of a very thickened layer of densely packed fibroblasts: keratinocytes, however, were not detected, either on the surface or buried in the mesenchyme (data not shown).

To investigate the fate of transplanted keratinocytes, a set of nude mice was grafted with lacZ-marked keratinocytes and grafts were retrieved at regular intervals for histochemical analysis. Unlike the factor IX-expressing vectors (Fig 1), the MFG-NB vector does not carry a neo selectable marker (Ferry et al, 1991); keratinocytes were transduced by co-cultivation as before but were then directly grafted without G418 selection. Two days after transplantation (Fig 5A), human keratinocytes, as identified by the occasional blue histochemical staining, were found along the surface of the mouse fascia graft bed. By 6 d (Fig 5B), the keratinocytes had differentiated to form a mature epidermis complete with the four layers of stratified tissue characteristic of normal skin architecture. 

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Figure 5. *LacZ*-marked keratinocytes initially form a viable epidermis that becomes encapsulated over time. (A) After 2 d, human keratinocytes (K), as identified by the occasional blue cell, were located along the surface of the mouse fascia (F). (B) High-power micrograph. (C) Low-power micrograph. By 6 d, the grafted keratinocytes had stratified and differentiated to form a characteristic epidermal architecture consisting of basal (B), suprabasal (SB), stratum granulosum (St. G), and stratum corneum (St. C) layers. The mouse mesenchyme tissue had contracted to form a thickened neodermis (ND) of fibroblasts. β-Galactosidase-positive cells were found in all layers of the epidermis (E). (D) After 2 wk, the grafted keratinocytes became engulfed by the mouse mesenchyme to form cysts of keratinocytes. Scale bars, 100 μm.

DISCUSSION

Long-term expression in the differentiated epidermis formed after grafting genetically modified keratinocytes is a prerequisite of an ex vivo approach to gene therapy. We therefore used various keratinocyte-specific enhancer elements—not previously studied as part of a gene therapy protocol—upstream of a strong CMV promoter to control expression of factor IX in retrovirally transduced keratinocytes.

Defective retroviral producer clones were isolated for each of the vector constructs. With some constructs (e.g., LNK14CIX and LNK14RCIX), the addition of the enhancer element affected the ability to isolate functional clones, whereas with others (e.g., LNPCIX, containing the HPV-16 enhancer) the viral titer of the resulting producer clones was reduced. The inclusion of nonretroviral elements, especially sequences that are not usually transcribed (e.g., upstream regulatory sequences), may affect a number of different processes in the life cycle of the virus including transcription initiation and elongation, polyadenylation, splicing, transport from the nucleus, and reverse transcription. Alternatively, heterogeneous sequence may alter the secondary structure of transcripts, which could affect mRNA stability and/or packaging. Any of these processes could reduce the levels of full-length vector mRNA available for packaging by the Psi/PA317 cell, thereby affecting the proportion of functional clones isolated and the viral titer of those clones.

After transduction by the different retroviral vectors, conditioned medium from G418-resistant keratinocytes was assayed for factor IX antigen as an approximate measure of factor IX cDNA transcriptional activity. We believe that expression from the 5’ LTR promoter is unlikely to contribute significantly to the levels of factor IX antigen, because translation of the downstream factor IX cDNA from the longer LTR-derived transcript will be at best highly inefficient (Kozak, 1991). In addition, any residual contribution to factor IX antigen would be equal for all vectors tested. Furthermore, all factor IX transcripts initiated from the internal promoter contain identical backbone sequences and should therefore be subject to the same mechanisms controlling mRNA stability, translational efficiency, and any aberrant splicing that might affect the level of factor IX antigen. Because transduction was inefficient, especially in the case of cells transduced by PA317-LNCIXc11, -LNPCIXc23, and -LNK14CIXc13, G418-resistant cells are unlikely to contain more than one proviral integration per cell. In the case of the LNPCIX vector, the levels of factor IX secreted in vitro were as high as the levels obtained using the parent LNCIX vector, indicating that the hybrid HPV-16-CMV promoter/enhancer must have been as active in keratinocytes as the CMV promoter/enhancer alone. The remaining constructs (containing the hK5 and hK14 enhancers) produced lower levels of factor IX expression in vitro, suggesting that these enhancer elements interfered with CMV promoter activity. These observations are consistent with the variation in activity with either different promoters (Cheng et al, 1993; Hafenrichter et al, 1994) or different promoter/enhancer hybrids (Dai et al, 1992; Couture et al, 1994; Okuyama et al, 1996; Wu et al, 1996).

The results with *LacZ*-marked keratinocytes suggested that transduced keratinocytes can form a normal epidermis and remain...
viable, at least initially, in vivo. The encapsulation process we observed contrasts with the results of Boukamp et al (1990) who observed encapsulation only with trunk-skin-derived and not with foreskin-derived cells, although the reasons for this discrepancy are unclear. Encapsulation may be circumvented by transplanting the factor IX-secreting keratinocytes on a collagen matrix (Boukamp et al, 1990).

When nude mice were grafted with keratinocytes transduced by the LNPCIX vector (containing the HPV-16 enhancer), recombinant factor IX was detected in plasma at levels of between 0.02 and 9 ng per ml for 4–5 wk, demonstrating that this particular combination of promoter/enhancer is capable of producing detectable levels of factor IX in vivo. This expression is longer than the 7 d of Gerrard et al (1993) and equivalent to the 23–44 d of Fenjves et al (1996). The longer duration that we observed cannot be explained simply by the increased sensitivity (from 0.1 to 0.02 ng per ml) of the ELISA over previous studies (Gerrard et al, 1993), because factor IX levels of >0.1 ng per ml were still detected beyond 30 d (Fig 3). The levels that we observed are marginally higher than the maximum levels of 2–3 ng per ml of previous studies (Gerrard et al, 1993; Fenjves et al, 1996).

The question whether the presence of the chimeric HPV-16 enhancer contributed to the higher levels and longer duration of factor IX expression in vivo remains open. This was not systematically investigated, although in the single control animal (mouse 11, see Results) grafted with cells transduced by the parent (CMV promoter/enhancer alone) vector, factor IX expression was of short duration (7 d). This may not be a representative experiment, however, because of the wide variation in the in vivo results observed for mice 1–10 (see Results and Fig 3).

Consistent with earlier evidence (Gerrard et al, 1993) showing a correlation between the presence of a stratified human epidermal graft and human factor IX in plasma, we found that grafts retrieved after factor IX levels had fallen to baseline showed no evidence of a stratified epithelium (Fig 4B), although we believe that it is certain that an epithelium did form initially. Because the declining levels of recombinant factor IX correlated with the timing of the encapsulation process observed for lacZ-marked keratinocytes, it is likely that the factor IX-secreting keratinocytes suffered a similar fate, and this is probably the major factor contributing to the loss of factor IX expression. Because mouse 11, sacrificed after 7 d, showed only the remnants of a stratum corneum along the surface of the graft (Fig 4A), however, it is possible that terminal differentiation of the transduced keratinocytes was an additional factor leading to the loss of expression. Because of a poor transduction efficiency with PA317-LNCIXc11 and -LNCIXc23, G418 selection may have reduced the number of clonogenic stem cells below the threshold required for maintaining a grafted epithelium. Furthermore, G418 is known to be cytotoxic to keratinocytes (Stockschläder et al, 1991), thus, inducing or accelerating differentiation. Transduced keratinocytes were therefore grafted after the minimum time in culture to maximize the opportunity of transplanting a stem cell population and to obtain at least short-term in vivo data.

To ensure the long-term maintenance of genetically modified keratinocytes in the grafted epithelium, the efficiency of stem cell transduction must be improved, possibly by increasing the viral titer of defective vectors, for instance by pseudotyping the vectors with the envelope G-glycoprotein of vesicular stomatitis virus (Chen et al, 1996). By replacing the neomycin phosphotransferase selective marker gene with that of histidinol dehydrogenase, the problems associated with G418-induced terminal differentiation may be overcome (Stockschläder et al, 1991). Alternatively, enriching the population of keratinocyte stem cells in culture prior to transduction, for instance by selecting for high levels of B integrins (Jones and Watt, 1993), may improve targeting of stem cells. Targeting the skin by in vivo gene transfer procedures is an attractive alternative to the ex vivo methods explored herein. Initial reports, however, using liposomes and sensitive reporter assays (Alexander and Akhurst, 1995; Li and Hoffman, 1995), although demonstrating feasibility, give little indication of efficiency. Further studies using a therapeutic cDNA of interest are required if such methods are to become a practical reality.

Although the decrease in transgene expression after keratinocyte transplantation (Flowers et al, 1990; Gerrard et al, 1993; Jensen et al, 1994; Stockschläder et al, 1994; Chaoate et al, 1996; Fenjves et al, 1996) has commonly been attributed to promoter down-regulation, part of this decrease may have been caused by a declining survival of genetically modified cells over time. In superficial grafting studies (Fenjves et al, 1996), this may be a result of graft shrinkage caused by competing host epidermis. Alternatively, when keratinocytes are transplanted without selecting for transduced cells (Chaoate et al, 1996), a survival advantage of rare nontransduced cells may lead to a decreased proportion of transduced cells within the graft. In our study, transplanted cells were protected from competing host epidermis and transduced cells were selected in G418. By using the transplantation chamber, we observed higher levels of factor IX and were able to show that the subsequent decline in expression was due to encapsulation and possibly terminal differentiation of the grafted keratinocytes. The fact that the HPV-16 enhancer vector produced levels of factor IX in vitro comparable to the parent vector and that these levels were sufficiently high to enable detection of recombinant factor IX in plasma indicates that this enhancer may be a useful addition to expression vectors for keratinocyte gene therapy. It must be emphasized that although the formation of a stable epithelium after transplantation of keratinocytes to a xenogenic (e.g., human to mouse) host has been demonstrated before (Boyce et al, 1996), long-term survival (>8 wk) using transduced keratinocytes has still not been demonstrated.

Even if the maximum levels of 9 ng of human factor IX per ml detected in plasma in the mouse model herein could be maintained, this is only equivalent to ~0.2% of factor IX in normal human plasma, which is inadequate as a therapeutic dose for hemophiliacs (Hedner and Davie, 1989). The graft area of ~100 mm² available beneath the transplantation chamber, however, is only ~1% of the total surface area of the mouse (Alexander et al, 1995). Therefore, increasing the graft to 10% of the total surface area would theoretically raise the clotting activity to ~2%, which approaches the ≥4% factor IX that physicians presently aim for with conventional factor IX replacement therapy regimens (Hedner and Davie, 1989). This, together with the recent evidence that factor IX expressed by retrovirally transduced keratinocytes is essentially completely biologically active (Gerrard et al, 1996), suggests that the epidermis remains an attractive target tissue for the gene therapy of hemophilia B.

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REFERENCES


Miller AD, Bortinme C: Rescued of a retrovirus packaging cell lines to cell recombination leading to helper virus production. Mol Cell Biol 6:2895–2902, 1986


