

Hemophilia A: current treatment and future gene therapy

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Summary

The last two decades has seen significant progress in the treatment of hemophilia A. The development of highly purified and recombinant FVIII pharmaceutical products has dramatically increased the life expectancy and quality of life for many hemophiliacs. However, the high cost and short supply of these replacement products has resulted in their availability limited to less than 10% of the world's hemophiliac population. Gene therapy for hemophilia A would provide prophylactic expression of FVIII and correction of the coagulation defect. A gene therapy protocol allowing simple, infrequent vector administration may extend hemophilia treatment to remote locations worldwide that currently lack access to FVIII replacement therapy. While progress has been made with each of the gene therapy vector systems described below, each still faces obstacles to its clinical utility. However, with the efforts that are currently directed toward overcoming these limitations, gene therapy for hemophilia A will ultimately become a reality.

I. Introduction

Hemophilia A is the most common hereditary coagulation disorder and is caused by a deficiency or abnormality in blood clotting factor VIII (FVIII). This X-linked disease affects 1 in 5-10,000 males in all populations with approximately one third of the occurrences due to spontaneous genetic mutations (Sadler and Davie, 1987). Hemophiliacs suffer from uncontrolled bleeding into the joints, muscles, and internal organs and repeated joint bleeding frequently leads to a disabling arthropathy (Sadler and Davie, 1987). Hemophilia A is categorized into severe, moderate, or mild forms, with over half of the patients manifesting the severe disease (Sadler and Davie, 1987). The severity of the bleeding disorder is related to the nature of the underlying mutation of the FVIII gene (Antonarakis et al., 1995).

Current treatment involves replacing the missing clotting factor with plasma-derived or recombinant FVIII protein infusions in response to bleeding crises. While prophylactic treatment of hemophilia A has been shown to reduce the frequency and severity of bleeding, such therapy is limited by the availability and high cost of purified

difficulties associated with frequent intravenous administrations (Rosendaal et al., 1991; DiMichele, 1996). Prior to the development of recombinant FVIII and advanced viral screening and inactivation techniques, FVIII cryoprecipitates derived from pooled human plasma resulted in transmission of several human viruses, including HIV and hepatitis, to over 50% of the hemophiliac population (DiMichele, 1996).

A major complication to the treatment of hemophilia A is the development of inhibitory antibodies against the infused FVIII protein. While the majority of hemophiliac patients are immunologically unresponsive to FVIII infusions, over 20% of severe hemophiliacs develop a FVIII-specific antibody response that can become strong enough to render further FVIII administrations ineffective. Although hemophilia A therapy has progressed considerably, present treatments remain suboptimal.

Somatic cell gene therapy, which would provide constant blood levels of FVIII, would be a significant treatment improvement. Hemophilia A has been discussed widely as a candidate disease for gene therapy for several reasons (Lozier and Brinkhous, 1994; Fallaux et al., 1995; Hoehen et al., 1995; Smith, 1995; Connelly and Kaleko,

1997). The human FVIII gene has been cloned (Wood et al., 1984; Toole et al., 1984) and the protein well characterized (Kaufman, 1992; Vehar et al., 1991; Hoyer et al., 1994). Human physiological levels of FVIII are low, 100-200 ng/ml (by definition, 1U/ml), and a therapeutic benefit can be achieved with as little as 5% of normal levels (Vehar et al., 1991; Hoyer et al., 1994). As a secreted protein, FVIII expression need not be regulated or limited to a specific target organ or tissue, provided that FVIII is biologically active and absorbed into the blood. However, features of FVIII biology such as the size of the FVIII cDNA, the inhibited accumulation of the mRNA (Kaufman et al., 1989; Lynch et al., 1993; Hoeben et al., 1995; Koeberl et al., 1995) and the instability of the protein (Kaufman, 1992) have hindered efforts to develop FVIII gene transfer vectors. Despite these obstacles, significant progress has been made on the development of gene therapy for hemophilia A. Recent advances in gene transfer technology have enabled the expression of therapeutic to physiological levels of human FVIII in normal animals as well as hemophiliac mice and dogs. However, current gene transfer vectors each face limitations to their clinical utility. Hemophilia A gene therapy research is focused on improving gene transfer vehicles and delivery methods to enable sustained clotting factor expression, treatment readministration, and circumvention of the host immune response to treatment.

II. Historical perspective and current treatment of hemophilia A

Hemophilia was documented in the Talmud over 1,700 years ago, where the death of several infant boys from uncontrolled bleeding following circumcision was described (Rosner, 1969). By the early 1800s, hemophilia was characterized as a sex-linked disorder (Otto, 1803), and by 1840, whole blood transfusion was found to halt a hemophilia bleeding episode (Lane, 1840). The presence of FVIII in blood was demonstrated in 1911 by the ability of normal plasma to shorten the clotting time of hemophilic blood (Addis, 1911). In 1937, the critical role of FVIII in hemostasis was recognized, and the missing factor designated "antihemophilic globulin" (Patek and Taylor, 1937). By 1962, the blood coagulation protein was renamed factor VIII by the International Committee on Thrombosis and Haemostasis (Wright, 1962). However, a detailed biochemical and structural characterization of FVIII has been achieved only within the last 20 years. A significant advance in the understanding of FVIII biology has resulted from the isolation and expression of the FVIII gene (Gitschier et al., 1984; Toole et al., 1984; Vehar et al., 1984; Wood et al., 1984).

The clinical severity of hemophilia is related to the degree of FVIII deficiency. The three degrees of hemophilia

were first noted by Legg (1872) and are designated severe, moderate, and mild. Severe hemophiliacs are defined as having <1% of normal FVIII levels and experience frequent, spontaneous bleeding into the joints and soft tissues. Patients with 2 to 5% of normal FVIII levels are considered to have moderate hemophilia. Spontaneous hemarthroses are rare, and severe bleeding into joints and tissues usually result from trauma. Mild hemophilia is defined as 6 to 50% of normal FVIII levels, and often goes undiagnosed for many years. The hemostatic defect becomes apparent only after severe trauma or surgical procedures.

Historical treatments for hemophilia included methods that are in use today, such as cautery, the application of ice, and splinting, in addition to the advice to avoid any procedure or activity that could produce trauma. However, hemophiliac mortality rates were 90% by the age of 21 years until the first half of the 20th century (DiMichele, 1996). Although the first transfusion to treat hemophilic bleeding was attributed to Lane (1840), transfusion therapy was not firmly established until 100 years later (Macfarlane, 1938). In the 1950s, fresh frozen plasma and early FVIII concentrates were developed and employed (Kekurck and Wolf, 1957). The development of a simple method for FVIII purification from human plasma by cryoprecipitation represented a milestone in hemophilia therapy (Pool and Shannon, 1965), and in the 1970s lyophilized intermediate purity FVIII concentrates were employed, each lot manufactured from more than 2000 donors. The average life expectancy and quality of life improved dramatically, for even severe hemophiliacs, from 11 years in 1921 to 60 years in 1980 in one report (Larsson, 1985). In the 1980s, the transmission of hepatitis C from these concentrates was recognized in 60 to 95% of hemophiliacs (DiMichele, 1996). Additionally, from 1979 through 1985, 55% of the hemophiliac population was infected with HIV-1 (DiMichele, 1996). Therefore, highly purified replacement products that were free from viral contamination were actively pursued in the 1980s, and the development of affinity chromatography purification, mandatory screening of all donor plasma, and improved viral inactivation methods, resulted in high purity FVIII concentrates (DiMichele, 1996). Consequently, no cases of HIV-1 transmission from FVIII products have been documented since 1986. The 1990s saw the advent of recombinant FVIII products (Lusher et al., 1993; Bray et al., 1994), and with it the hope of an end to viral transmission through replacement therapy. While the safety and purity of FVIII products has been improved dramatically, current purification techniques are not infallible as parvovirus has been reported recently in plasma-derived and recombinant, albumin-containing FVIII products (Laurian et al., 1994; Eis-Hubinger et al., 1996). In addition, the transmission of prions, the proposed

causative agents of Creutzfeldt-Jakob disease and bovine spongiform encephalopathy, through plasma-derived FVIII or bovine products employed in the production of the recombinant protein, has been debated vigorously (Arnold, 1995). Frequently, the major obstacle encountered in the late 1990s that restricts the implementation of optimal therapy for hemophilia A is the limited availability and high cost of FVIII pharmaceutical products.

The most common current treatment of hemophilia A involves infusion of plasma-derived or recombinant FVIII in response to bleeding crises. Early treatment, at the first onset of symptoms, limits both the amount of the bleeding and the extent of the ensuing tissue damage (DiMichele, 1996). However, in many cases, such therapy is not sufficient to prevent inflammation of the synovial membrane, and subsequent joint damage. Based on observations that moderate hemophiliacs rarely develop chronic arthropathy, it was theorized that adequate prevention would be accomplished by maintaining FVIII at levels of 1-5% of normal. Prophylactic treatment has been performed in Europe over the last 20 years, and, in most cases, involves protein infusion three times weekly to maintain FVIII at therapeutic levels. In a Swedish study, patients who initiated therapy at 1-3 years of age, before significant orthopedic damage occurred, had fewer than one bleeding crisis per year and normal joints over a ten year period (Nilsson et al., 1992). However, such treatment resulted in a significant increase (3-4 fold) in FVIII usage, and required the placement of a central venous catheter in young children. Prophylaxis has not been widely adopted in the United States, the major deterrent being the current lack of cost-effectiveness data regarding such therapy (DiMichele, 1996).

One of the major complications of hemophilia treatment is development of antibodies (inhibitors) against the infused FVIII (reviewed by White and Roberts, 1996). Inhibitor incidence is as great as 20% of severe hemophiliacs, and when the titer of these antibodies becomes sufficiently elevated, treatment with FVIII, even in tremendous doses, is completely ineffective. FVIII-specific antibodies may function by two general mechanisms: the inhibition of FVIII function or the clearing of FVIII from the blood. Most FVIII antibodies characterized clinically inhibit FVIII function. Several therapeutic approaches are currently available for the treatment of inhibitor patients. These include the induction of immune tolerance by infusion of large or moderate amounts of FVIII protein twice daily until inhibitor titer declines (Brackmann, 1984; Ewing et al., 1988; Mauser-Bunschoten et al., 1991). A second protocol involves the reduction of antibody titer by plasmapheresis, suppression of *de novo* antibody synthesis by the administration of cytotoxic drugs, daily infusion of FVIII, and intravenous IgG administration (Nilsson et al.,

1993). An effective treatment for patients with autoantibodies against FVIII is the infusion of intravenous gamma globulin (Sultan et al., 1994). For inhibitor patients who must be treated acutely, FVIII bypassing agents, or porcine FVIII, which may not cross react with the human inhibitor, have shown successful application (White and Roberts, 1996). For the future, it may be possible to prepare and administer synthetic peptides that mimic the FVIII epitopes recognized by the inhibitor (White and Roberts, 1996). The use of chimeric FVIII molecules, such as human/porcine hybrids, not recognized by the inhibitor, has also been investigated (Lollar, 1997).

III. The role of factor VIII in blood coagulation

Normal blood coagulation requires the rapid activation of a series of sequential enzymatic reactions in which plasma proteins and proteins released from damaged cells have essential roles (reviewed by Davie, 1995). The lack or deficiency of any of the proteins involved in this cascade blocks the propagation of the initial stimulus. Blood clotting begins with injury to a blood vessel. The damaged vessel wall causes adherence and accumulation of platelets, which, in turn, activate the plasma proteases in the intrinsic pathway of coagulation leading to the localized generation of thrombin and the conversion of fibrinogen to fibrin. The deposit of insoluble fibrin stabilizes the platelet plug and impedes blood flow through the damaged vessel. Thrombin generation requires the interaction of proteases, cofactors, and substrate zymogens, which assemble on a phospholipid surface (reviewed by Kaufman, 1992). FVIII functions in the blood coagulation cascade as a cofactor accelerating the activation of factor X by activated factor IX (factor IXa). FVIII, in turn, is activated by factor Xa and thrombin cleavage. The initial activation of FVIII may be caused by a trace amount of factor Xa generated by the tissue factor-factor VIIa complex (Hoyer, 1994). The formation of factor Xa by this mechanism is rapidly restrained by tissue factor pathway inhibitor, however. Therefore, to sustain hemostasis, the activation of factor X by factor IXa, accelerated through thrombin activation of FVIII, is required (Hoyer, 1994). Subsequently, factor Xa acts in the presence of activated factor V, negatively charged phospholipids, and calcium to convert prothrombin to thrombin. The mechanism by which FVIII functions in the factor Xa-generating complex remains poorly understood.

FVIII circulates in the plasma in a noncovalent complex with von Willebrand factor (vWF) and has binding sites for factor IXa, factor X, calcium, phospholipid, and vWF (Kaufman, 1992). vWF is an adhesive glycoprotein that is essential for platelet aggregation and adhesion to the vessel wall in response to

vascular injury (Kaufman, 1992). Major functions of vWF are to protect FVIII from proteolysis and to concentrate FVIII at the sites of active hemostasis (Kaufman, 1992). The association of FVIII with vWF was misunderstood for many years, and the distinction between the two coagulation factors was not realized until the mid-1970s (Hoyer, 1994).

IV. Structure and function of factor VIII

The isolation of the FVIII gene in 1984 (Gitschier et al., 1984; Toole et al., 1984; Vehar et al., 1984; Wood et al., 1984) represented a significant advance in the understanding of FVIII biology and structure (**Figure 1**). The human FVIII gene maps to the most distal band of the long arm of the X chromosome, Xq28. The genomic DNA is 186 kb and contains 26 exons, making it one of the largest human genes identified to date. The exon lengths vary considerably, from 69 to 262 base pairs (bps) with the exception of the 3106 bp exon 14 (encoding the B-domain, see below), and the 1958 bp exon 26 (reviewed by Antonarakis et al., 1995). The FVIII mRNA is approximately 9 kb, 7053 nts of which is the coding region (**Figure 1**). Interestingly, two additional RNA transcripts, initiated in intron 22, have been identified (Levinson et al., 1990; 1992). One transcript, the 1.8 kb F8A, is transcribed in the opposite orientation from that of FVIII (Levinson et al., 1990). The F8B transcript is 2.5 kb and is transcribed in the same direction as FVIII (Levinson et al., 1992). The function of F8A and F8B mRNAs and their potential protein products are unknown. Notably, the F8A gene and several kb of surrounding sequence are duplicated elsewhere on the X chromosome (Levinson et al., 1990; Freije and Schlessinger, 1992).

FVIII mRNA is expressed in the liver, spleen, kidney, and lymph nodes, but not in peripheral blood lymphocytes or endothelial cells (Wion et al., 1985). Within the liver, the hepatocyte is the cell type that synthesizes FVIII (Wion et al., 1985; Zelechowska et al., 1985).

The FVIII protein is synthesized as a 2351 amino acid (aa), single-chain precursor having the domain structure A1-A2-B-A3-C1-C2 (**Figure 1**; Toole et al., 1984; Vehar et al., 1984). The 19 aa signal peptide is removed upon translocation to the endoplasmic reticulum. Upon transit to the Golgi, FVIII is cleaved specifically within the B-domain to generate the heavy chain, composed of domains A1-A2-B, and the light chain, composed of domains A3-C1-C2 (Kaufman, 1992). The large B domain has no detectable homology to any known genes. The A domains share homology with ceruloplasmin and factor V, while the C domains are homologous to factor V, and discoidin I, a phospholipid-binding protein (Antonarakis et al., 1995).

In plasma, FVIII consists of a heterodimer composed of a heterogeneously sized heavy chain polypeptide extending up to 200 kDa in a metal ion complex with the 80 kDa light chain (**Figure 1**). FVIII circulates, in a complex with vWF, as an inactive cofactor. On exposure to thrombin or factor Xa, the heterogenous heavy chain is first cleaved into a 92 kDa fragment, followed by further cleavage into 54 and 44 kDa fragments, both of which are required for procoagulant activity (Hoyer, 1994). Concurrently, a small fragment is cleaved from the light chain to disassociate vWF. These processing steps generate the activated FVIII heterotrimer, FVIIIa. FVIIIa is an unstable molecule that rapidly loses cofactor function, due to subunit dissociation (Hoyer, 1994).

V. Molecular etiology of hemophilia A

Since the identification of the FVIII gene, the DNA of hemophilia A patients has been examined extensively for molecular defects (reviewed by Antonarakis et al., 1995), and a data base of FVIII mutations has been established (Tuddenham et al., 1991). As expected, a variety of FVIII mutations have been identified, although their characterization has been impeded by the large size of the FVIII gene (see above). Initial studies were performed by restriction analysis of patient DNA, and revealed that most families carried distinctive mutations, and that approximately one third of hemophilia A cases are the result of new mutations (Antonarakis et al., 1995). Approximately 5% of severe hemophiliacs have large deletions of the FVIII gene, and 24% have point mutations resulting in missense or nonsense mutations. Many of these base changes were identified by the alteration of *TaqI* restriction sites (TCGA) within the FVIII gene. *TaqI* sites are established hot spots for the occurrence of point mutations because they contain CpG dinucleotides in which cytosine can be methylated and subsequently deaminated to thymine (Yousoufian et al., 1986). More than 80 different missense mutations have been identified. These base changes usually involve single aa substitutions at sites critical for FVIII function and are associated with normal or reduced levels of FVIII antigen and the production of a dysfunctional FVIII molecule. However, until 1993, the mutation causing severe hemophilia A in approximately 50% of patients remained elusive. Lakich et al. (1993) and Naylor et al. (1993) discovered that these patients have a partial inversion of the FVIII gene caused by homologous recombination between the region within intron 22 encoding the F8A gene, and one of the two other homologous regions located elsewhere on the X chromosome. These inversions originate almost exclusively in male meiosis (Rossiter et al., 1994), suggesting that nearly all mothers of hemophiliacs with inversions are carriers.

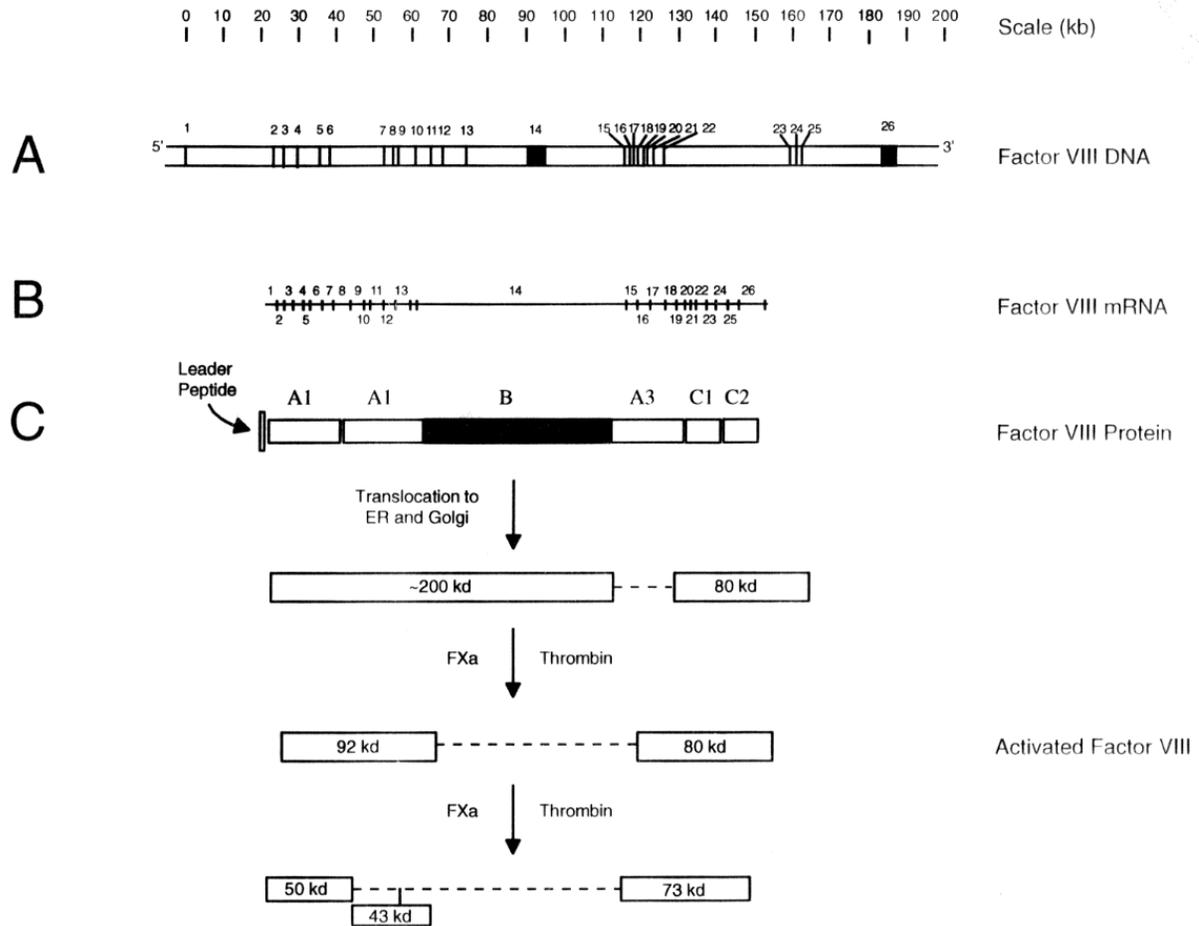


Figure 1. Schematic representation of the structure of the factor VIII gene, mRNA and protein. A) Factor VIII (FVIII) gene structure. The double horizontal line depicts the human FVIII gene, with exons represented by vertical lines or solid boxes. The scale is drawn in kilobases (kb). The human FVIII gene is 186 kb and contains 26 exons (Gitschier et al., 1984; Toole et al., 1984; Vehar et al., 1984; Wood et al., 1984). B) FVIII mRNA structure. The single horizontal line represents the FVIII mRNA coding region. The exon boundaries are depicted as vertical lines. The human FVIII mRNA is approximately 9 kb, 7053 nts of which is the coding region. C) FVIII protein structure. The 19 amino acid secretory leader peptide, the three A domains, A1, A2, and A3, the B domain, and the two C domains, C1 and C2 are represented by open boxes. The 2351 amino acid single-chain precursor is displayed. The leader peptide is removed upon translocation to the endoplasmic reticulum (ER). In the Golgi, FVIII is cleaved specifically within the B-domain to generate the heavy chain, A1-A2-B, and the light chain A3-C1-C2. In plasma, FVIII circulates as a heavy chain and light chain heterodimer in a complex with von Willebrands factor (vWF). Activation of FVIII upon exposure to thrombin or activated factor X (FXa), results in cleavage of the heavy chain into a 92 kd fragment, followed by further cleavage into 50 and 43 kd fragments. The light chain is concurrently cleaved into a 73 kd fragment, resulting in the release of vWF. Activated FVIII (FVIIIa) functions as a cofactor in the intrinsic blood coagulation cascade. FVIIIa is rapidly inactivated by subunit dissociation (Hoyer, 1994).

VI. Gene therapy for hemophilia A

Gene therapy for hemophilia A, the transfer and expression of a functional FVIII cDNA or gene to hemophiliac patients, remains a viable treatment option for the future. Gene therapy would provide a significant treatment benefit by providing constant, prophylactic blood levels of FVIII and correction of the coagulation

defect. Two basic gene therapy strategies, *ex vivo* and *in vivo*, have been employed to date. *Ex vivo* gene transfer involves the isolation of host cells, expansion and genetic modification of the cells in culture, and reimplantation of the transduced cells into the host. Alternatively, the *in vivo* approach involves the direct delivery of the gene transfer vehicle, in most cases, a viral vector, to the patient.

The success of an *ex vivo* gene therapy strategy would require that FVIII-transduced cells exhibit prolonged survival, sustained FVIII expression, and allow efficient entry of FVIII into the blood. To date, most *ex vivo* gene transfer strategies have employed retroviral vectors derived from murine retroviruses. Retroviral vectors can infect a broad spectrum of cell types and stably integrate into the genome allowing long-term persistence of the transgene and transfer to all progeny cells. A disadvantage of retroviral vectors is that host cell division is necessary for vector transduction and integration (Miller et al., 1990), thus limiting retroviral-mediated gene therapy to actively dividing host cells.

Until recently, the development of hemophilia A gene therapy was focused almost exclusively on *ex vivo* strategies utilizing retroviral vectors for FVIII gene transfer and expression (Dwarki et al., 1995; Lynch et al., 1993; Chuah et al., 1995; Hoeben et al., 1990; 1992; 1993; Israel and Kaufman, 1990). For use in the development of FVIII-encoding retroviral vectors, the FVIII cDNA was modified by deletion of the B-domain. Removal of the B-domain reduces the FVIII cDNA from >7 kb, too large to be effectively packaged into most viral vectors for gene transfer, to 4.5 kb (Eaton et al., 1986; Toole et al., 1986). Removal of the B-domain coding region from the FVIII cDNA has no effect on FVIII function, activity, or immunogenicity (Eaton et al., 1986; Toole et al., 1986; Pittman et al., 1993). However, the inclusion of the FVIII cDNA into retroviral vectors was demonstrated to dramatically decrease vector titer (Lynch et al., 1993; Chuah et al., 1995; Israel and Kaufman, 1990). The identification of RNA accumulation inhibitory sequences within the FVIII cDNA (Lynch et al., 1993; Koeberl et al., 1995; Chuah et al., 1995), reported to function as a transcriptional silencer (Hoeben et al., 1995) or as a block to transcriptional elongation (Koeberl et al., 1995), were cited as the cause of the decreased vector titer. Furthermore, conservative mutagenesis of the entire 1.2 kb inhibitory region described by Lynch et al. (1993) failed to increase FVIII expression or retroviral vector titer (Chuah et al., 1995).

Despite these difficulties, the development of retroviral vectors encoding the human B-domain deleted FVIII cDNA demonstrated the feasibility of retrovirus-mediated transfer and expression of human FVIII (Hoeben et al., 1990; Israel and Kaufman, 1990). Transduction of mouse 3T3 fibroblasts resulted in secretion of biologically active FVIII at peak levels of 56 mU/ml (Israel and Kaufman, 1990). Similarly, transduction of murine fibroblasts and primary human skin fibroblasts resulted in expression of FVIII at levels of 120 mU/ml/10⁶ cells/day and 25 mU/ml/10⁶ cells/day, respectively (Hoeben et al., 1990). However, subcutaneous implantation of collagen matrices containing transduced rodent fibroblasts or primary human

fibroblasts into immune-deficient nude mice did not result in expression of detectable levels of human FVIII (Hoeben et al., 1993). The genetically modified cells persisted *in vivo*, and cells capable of secreting FVIII could be rescued from the implants for up to two months (Hoeben et al., 1993). Furthermore, the transplantation of transduced murine bone marrow into lethally irradiated mice also did not result in FVIII expression in the plasma although the vector was detected in individual hemopoietic progenitor cell-derived spleen colonies (Hoeben et al., 1992).

A significant advance in retroviral titer and FVIII expression was achieved by the addition of an intron into vectors encoding the B-domain deleted cDNA (Chuah et al., 1995; Dwarki et al., 1995). These vectors were based on the MFG vector system comprised of the Moloney murine leukemia virus (MMLV) splice donor and acceptor sites incorporated upstream of the transgene cDNA (Krall et al., 1996). Inclusion of the intron was demonstrated to significantly increase vector titer and FVIII expression up to 40-fold (Chuah et al., 1995). Dwarki et al. (1995) constructed a similar retroviral vector which mediated expression of high levels of FVIII (peak of 2000 ng/ml/10⁶ cells/24 hrs) in transduced primary human fibroblasts. Intraperitoneal implantation of the vector-transduced cells on neo-organs consisting of polytetrafluoroethylene coated with collagen into SCID (severely combined immunodeficiency) mice resulted in high level expression of FVIII (100 ng/ml) in the mouse plasma at two days (Dwarki et al., 1995). However, by day 13, FVIII expression levels had declined to background. Limited survival of the transduced cells within the neo-organ implants and transcriptional inactivation of the FVIII expression cassette may have contributed to the cessation of FVIII expression. Notably, a similar strategy using a transduced myoblast cell line and muscle implantation was not successful suggesting that the secreted FVIII was poorly absorbed into the circulation (Dwarki et al., 1995).

As an alternative approach for hemophilia A gene therapy, a non-viral transfection strategy for delivery and expression of human FVIII has been described (Zatloukal et al., 1994). Using receptor-mediated, adenovirus-augmented gene delivery, primary mouse fibroblasts were transfected with a B-domain deleted FVIII expression plasmid and then surgically implanted into mouse spleens. Low-level FVIII expression (peak of 17 ng/ml) was detected one day after intrasplenic administration, but expression persisted less than 48 hrs.

Recently, *in vivo* gene therapy approaches to hemophilia A treatment have been described. A facile, intravenous administration of a FVIII-encoding vector would provide a more benign and cost-effective treatment than *ex vivo* protocols involving surgical procedures.

means to transfer an exogenous gene to target cells *in vivo*. Most adenoviral vectors are derived from human adenovirus serotype 5 and rendered replication-deficient by removal of critical viral regulatory elements (Berkner, 1988; Trapnell and Gorziglia, 1994). Adenoviral vectors can transduce a broad spectrum of cell types and, unlike retroviral vectors, do not require target cell proliferation for gene transfer and expression. In addition, the adenovirus chromosome remains episomal in the transduced cell, thus avoiding the possibility of insertional mutagenesis (reviewed by Gingsberg, 1984; Horwitz, 1990). The main disadvantage of adenoviral vectors is that the host immune response, in general, appears to limit the duration of transgene expression and the ability to readminister the vector.

Considerable progress has been made recently in the development of adenoviral-mediated gene therapy of hemophilia A (Connelly et al., 1995, 1996a, 1996b, 1996c, 1998). Adenoviral vectors are an efficient system for *in vivo* FVIII gene delivery since a peripheral vein injection in mice (Smith et al., 1993; Kozarsky and Wilson, 1993; Connelly et al., 1995) and dogs (Connelly et al., 1996c) results in efficient transduction of hepatocytes, cells capable of secreting FVIII directly into the blood (Kaufman, 1992). The transduction of human hepatoma cells with an adenoviral vector in which a liver-specific, albumin promoter directed expression of a human B-domain deleted FVIII cDNA resulted in secretion of high levels of biologically active human FVIII, >2,400 mU/106 cells/24 hrs (Connelly et al., 1995). Intravenous administration of the vector to normal C57BL/6 mice, via the tail vein, resulted in expression of human FVIII in the mouse plasma at levels averaging 300 ng/ml one week postinjection. Therapeutic plasma levels of FVIII were sustained for several weeks and the human FVIII expressed in the mice was biologically active (Connelly et al., 1995). The inclusion of an untranslated exon and intron from the human apolipoprotein 1 gene (Swanson et al., 1992) upstream of the FVIII cDNA in a second, more potent FVIII vector, boosted *in vivo* FVIII expression approximately 10-fold (Connelly et al., 1996b). Administration of low, non-toxic doses of this vector to normal, adult mice resulted in expression of FVIII at levels 4-fold above the human therapeutic range sustained for at least five months (Connelly et al., 1996a). In contrast, when high, hepatotoxic doses of the vector were administered, FVIII expression declined rapidly to background levels suggesting that dose-dependent vector toxicity limited vector persistence (Connelly et al., 1996a). Similarly, using a human α_1 -antitrypsin-encoding adenoviral vector, it was observed that high viral doses limit the duration of transgene expression (Morral et al., 1997). Furthermore, FVIII expression, directed by the albumin promoter, was demonstrated to be liver-specific

(Connelly et al., 1996a and 1996c) thus providing a potential margin of safety for the use of adenoviral vectors to treat hemophilia. Although no problems are anticipated from ectopic expression of FVIII, the consequences of expression in organs other than the liver are presently unknown.

The achievement of phenotypic correction in FVIII-deficient dogs, a large, clinically relevant animal model of hemophilia A demonstrated the potential utility of adenoviral vectors for the treatment of hemophilia A (Connelly et al., 1996c). Peripheral vein administration of a FVIII adenoviral vector resulted in normalization of the clinical clotting parameters and expression of human FVIII in the canine plasma at levels well above therapeutic (peak levels of 8000 mU/ml). However, phenotypic correction in the treated dogs was transient, as the animals developed a strong antibody response directed to the human protein (Connelly et al., 1996c). In contrast to human FVIII, the canine FVIII protein is less immunogenic in hemophiliac dogs (Tinlin et al., 1993). Therefore, the establishment of sustained phenotypic correction in hemophiliac dogs may require the development of vectors that encode the canine cDNA.

The recent generation of FVIII-deficient mice, by gene disruption techniques, provides the first small animal model of hemophilia A (**Figure 2**; Bi et al., 1995). Affected mice have FVIII activity levels that are <1% of normal and display lethal bleeding after the trauma of tail biopsy (Bi et al., 1995). The mice frequently are anemic, exhibit prolonged bleeding after routine procedures such as ear tagging, and occasionally develop joint bleeds (S.C. unpublished data). Therefore, the murine phenotype is similar to that of human hemophiliacs (Sadler and Davie, 1987). Treatment of the hemophiliac mice with a FVIII adenoviral vector resulted in expression of biologically active human FVIII sustained at levels well above the human therapeutic range for over nine months (Connelly et al., 1998). Furthermore, a tail-clip survival study demonstrated that FVIII vector-treated mice readily survived tail clipping with minimal blood loss, while mice that received a similar dose of a β -galactosidase-encoding vector and untreated hemophiliac mice suffered 70-95% mortality. These data directly demonstrate sustained phenotypic correction of murine hemophilia A by *in vivo* gene therapy (Connelly et al., 1998). Notably, human B-domain deleted FVIII expressed endogenously in the vector-treated mice was not immunogenic, while hemophiliac mice injected intravenously with human full-length FVIII protein rapidly develop a potent anti-FVIII antibody response (Qian et al., 1996). These observations represent preliminary evidence to suggest that constant level, endogenous expression of human FVIII may be less immunogenic than intermittent, intravenous protein administration.



Figure 2. Hemophiliac mice. The factor VIII (FVIII)-deficient hemophiliac mice bleed severely from scratches and routine procedures such as ear tagging. Application of topical thrombin controls the bleeding. Two genotypes of factor VIII (FVIII)-deficient hemophiliac mice were generated by disruption of exon 16 or exon 17 of the murine FVIII gene (Bi et al., 1995). Affected mice of both genotypes have FVIII levels <1% of normal and display lethal bleeding after trauma. The hemophiliac mice suffer from joint bleeds, subcutaneous bleeding, and spontaneous death indicating a similarity to the pathophysiology of human hemophilia A. Notably, hemophiliac females of both genotypes survive pregnancy and birth (Bi et al., 1996; Connelly et al., 1998).

The treatment of human patients with an adenoviral vector will require that the vector efficiently transduce and express in human hepatocytes. Cultured primary human hepatocytes were exposed to low doses (10, 100 and 1000 particles/cell) of vectors encoding β -galactosidase or a B-domain deleted human FVIII. Hepatocyte transduction efficiency was high, 50%, 90% and 100%, respectively, and FVIII was secreted into the tissue culture media at levels of 300, 2500, and 3000 mU/ml per 10^6 cells per 60 hrs, respectively (S.C., manuscript in preparation). Additionally, the cultured primary human hepatocytes were used to test the potency of a recently generated adenoviral vector that encodes the full-length FVIII cDNA. Transduction with this vector yielded biologically active FVIII at levels 10-fold lower than those obtained with the B-domain deleted FVIII vector (S.C., manuscript in preparation). These data are consistent with previous studies with transfected COS cells in which the B-domain deleted FVIII was expressed at a higher level than the full-length protein (Toole et al., 1985). Processing and secretion of the B-domain deleted FVIII protein may be more efficient than that of the full-length protein as they follow different secretory pathways (Dorner et al., 1987).

Although sustained expression of FVIII has been

may be limited as expression is not likely to be life-long and an antibody response directed to the viral capsid prevents repeated administration. The duration of expression is limited by at least two aspects of vector biology. The vector remains episomal and may be lost as the hepatocytes slowly proliferate. In addition, a cytotoxic T lymphocyte (CTL) response, directed against vector backbone gene products, may result in elimination of the transduced hepatocytes (Yang et al., 1994a, 1994b). Current efforts are directed towards removing viral backbone genes to diminish the host immune response and to increase the duration of gene expression (Armentano et al., 1995; Wang et al., 1995; Yeh et al., 1996; Gao et al., 1996; Gorziglia et al., 1996; Kochanek et al., 1996; Clemens et al., 1996; Lieber et al., 1996; Haecker et al., 1996; Kumar-Singh and Chamberlain, 1996; Fisher et al., 1996; Zhou et al., 1996; Hardy et al., 1997; Morral et al., 1997).

A major limitation in the application of adenoviral vectors to the treatment of hemophilia is the block to repeated administration. Immunosuppressive strategies designed to prevent the formation of antibodies to the viral capsid have been successful in mice (Smith et al., 1996; Yang et al., 1995; 1996). Smith et al. (1996) have demonstrated that the immune response to a systemically administered adenoviral vector is dose-dependent and can be modulated by transient immunosuppression with cyclophosphamide or deoxyspergualin (DSG) at the time of initial vector injection to allow effective repeated treatment. More recently, using low dose combination immunotherapy, at least three efficacious adenoviral vector treatments were achieved (TAG Smith, personal communication). However, an immunosuppressive protocol that is clinically relevant to the treatment of human disease will require a means of further diminishing vector immunogenicity either through tolerization or by capsid modification.

Several other viral vector systems are currently under development which may be applicable to the treatment of hemophilia A. Among the most promising are recombinant adeno-associated viral vectors (AAV). AAV is a nonpathogenic, defective parvovirus that establishes a latent infection by integrating into the host genome (Kotin, 1994). Vectors derived from AAV have been shown to transduce several tissues *in vivo* including muscle (Xiao et al., 1996), brain (McCown et al., 1996), lung (Halbert et al., 1997), and liver (Snyder et al., 1997). Following portal vein infusion of a purified human factor IX (FIX)-encoding AAV vector into normal mice, human FIX was detected in mouse plasma for at least 36 weeks in one animal (Snyder et al., 1997). An AAV vector encoding the B-domain deleted human FVIII cDNA has been described (Gnatenki et al., 1996), although *in vivo* expression data has not yet been reported.

The use of MMLV retroviral vectors for *in vivo* gene delivery has been described recently. The development of complement resistant vectors, in addition to improved methods of vector concentration and purification have allowed *in vivo* hepatocyte delivery (Bosch et al., 1996). High dose intravenous infusion of a purified retroviral vector to juvenile animals resulted in transduction of 1% of hepatocytes (Greengard et al., 1997). Peripheral vein administration of a high dose of a vector encoding a human B-domain deleted FVIII cDNA to rabbits resulted in expression of therapeutic levels of FVIII in 50% of the animals, sustained for at least one year (Greengard et al., 1997). Similar treatment of two normal dogs resulted in FVIII expression in one animal, sustained for at least two months (Greengard et al., 1997).

A novel retroviral vector system derived from lentiviruses has emerged recently (Naldini et al., 1996a;

1996b) and may be well suited for the treatment of hemophilia A. The lentivirus life cycle, the prototype for which is HIV, is distinguished from that of murine retroviruses in that the capsid is readily transported into the nucleus thus enabling the efficient transduction of nondividing cells (Naldini et al., 1996a). An HIV-derived vector pseudotyped with VSV G protein demonstrated localized, efficacious transduction of rat neurons *in vivo* and transgene expression sustained for at least three months (Naldini et al., 1996a, 1996b). Present efforts are aimed at the generation of stable vector packaging cell lines (Corbeau et al., 1996) and safe, clinically acceptable vectors.

Finally, nonviral or synthetic vectors are receiving increasing attention as gene transfer vehicles. Synthetic vectors have the potential to be less immunogenic than viral vectors, and can be assembled in cell-free systems from well defined components. The elimination of viral components from the vector system may diminish patient anxiety in a population ravaged by viral illnesses. Currently, the most efficient synthetic system for gene transfer to hepatocytes following intravenous injection is composed of DNA/polylysine/asialoglycoprotein conjugates which utilize hepatocyte receptors for targeting and gene delivery (Wu and Wu, 1988; Perales et al., 1994). Using an optimized human FVIII expression cassette, this gene transfer strategy yielded therapeutic plasma levels of FVIII in mice sustained for at least 30 days (Ill et al., 1997). Current challenges faced with this approach include achieving a consistent formulation, demonstrating reproducibility of transgene expression and delivery, and developing nonimmunogenic conjugates suitable for repeated treatments.

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