Prevention of spontaneous bleeding in dogs with haemophilia A and haemophilia B

T. C. NICHOLS,* R. A. RAYMER,* H. W. G. FRANCK,* E. P. MERRICKS,* D. A. BELLINGER,* N. DEFRIESS,* P. MARGARITIS,† V. R. ARRUDA,† M. A. KAY† and K. A. HIGH†,§

*Department of Pathology and Laboratory Medicine, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA; †Department of Pediatrics, University of Pennsylvania Medical Center and The Children’s Hospital of Philadelphia, Philadelphia, PA, USA; ‡Department of Pediatrics, Program in Human Gene Therapy, Stanford University, Stanford, CA, USA; and §Department of Pediatrics, Howard Hughes Medical Institute, Philadelphia, PA, USA

Summary. Dogs with haemophilia A or haemophilia B exhibit spontaneous bleeding comparable with the spontaneous bleeding phenotype that occurs in humans with severe haemophilia. The phenotypic and genotypic characteristics of haemophilic dogs have been well-described, and such dogs are suitable for testing prophylactic protein replacement therapy and gene transfer strategies. In dogs with haemophilia, long-term effects on spontaneous bleeding frequency (measured over years) can be used as an efficacy endpoint in such studies. Although complete correction of coagulopathy has not been achieved, published data show that prophylactic factor replacement therapy and gene transfer can markedly reduce the frequency of spontaneous bleeding in haemophilic dogs. Further studies are currently ongoing.

Keywords: dogs, haemophilia A, haemophilia B, spontaneous bleeding

Introduction

Dogs with haemophilia A or haemophilia B are severely deficient (<1% activity and antigen) in coagulation factor VIII (FVIII) or coagulation factor IX (FIX) respectively. These dogs exhibit a spontaneous bleeding phenotype that often occurs in joints and soft tissues in a manner that mimics humans with these disorders. When left untreated, the bleeding is severe, debilitating and can be fatal. Notably, mice with haemophilia tend not to have spontaneous bleeds, whereas haemophilia B dogs have a range of 4–6 spontaneous bleeds per year [1], and the frequency appears to be the same in the Chapel Hill strain of haemophilia A dogs (Tables 1 and 2) [2,3]. Thus, bleeding frequency can be used as an efficacy endpoint in studies designed to correct the haemophilic coagulopathy. This issue has been addressed in the haemophilic dogs both by long-term (years) prophylactic replacement with recombinant antihaemophilic factors and by long-term expression of the missing coagulation factors or canine factor VIIa (cFVIIa) mediated by successful gene transfer. This study reviews the published data on the change in bleeding frequency using these two strategies.

Molecular defects in the Chapel Hill strain of dogs with haemophilia

Haemophilia A

Haemophilia A is an inherited X-linked disorder caused by a deficiency of FVIII. The canine factor VIII (cFVIII) cDNA sequence identity is 77–92% similar to that of humans, mice, sheep and pigs, as are the homologous A1, A2, B, A3, C1 and C2 domain structures [4]. Key functional motifs are conserved between canine and human FVIII: the von Willebrand factor binding sites, three thrombin cleavage sites, the protein C cleavage site and the six tyrosines known to be sulphated on human FVIII. While it is highly likely that the cFVIII expression is
As complex as that of humans [5], very recently recombinant cFVIII was produced and shown to be safe and efficacious in haemophilia A dogs in short-term studies [6]. Using the cFVIII cDNA, researchers found that both the Chapel Hill and Queen University (Ontario, Canada) strains of haemophilia A dogs have an intron 22 inversion [3,7]. This defect faithfully replicates a causative mutation present in about 40% of humans with severe haemophilia A [8–10].

**Haemophilia B**

Haemophilia B is an inherited X-linked disorder caused by a deficiency of FIX. The canine factor IX (cFIX) cDNA is 86% conserved at the amino acid level when compared with human FIX [11]. The leader peptide, Gla domain, epidermal growth factor (EGF) domains and carboxy-terminal portion of the heavy chain all have extensive sequence conservation between dogs and humans. All glutamic acid (Glu) residues undergoing gamma-carboxylation in humans are conserved in cFIX. This 1989 description of cFIX cDNA provided the necessary tools for identification of the molecular defects in several strains of haemophilia B dogs [12–15]. Two strains have been used extensively in gene therapy studies: one with a deletion mutation in Lhasa Apso dogs that are prone to develop inhibitory antibodies to infused cFIX [14] and the other with a missense mutation that does not develop inhibitory antibodies to infused cFIX, and this latter group has been maintained in Chapel Hill since 1966 [12].

The well-described phenotypes and genotypes of these haemophilia A and haemophilia B dogs make them very desirable for studying the pathophysiology of haemophilia and for testing replacement therapies and gene therapy strategies.

### Prophylactic FIX replacement therapy in canine haemophilia B

To determine whether prophylactic replacement of FIX would reduce bleeding frequency in haemophilia B dogs, a group of littermates were immunologically tolerized to recombinant human FIX and then treated prophylactically to achieve trough levels of 1% and a shortened whole blood clotting time (WBCT). Compared with nontolerized haemophilia B dogs in the Chapel Hill colony monitored concurrently and treated ‘on-demand’, the tolerized dogs had a reduction in spontaneous bleeding over 3.5 years [69% during the first year of life (P = 0.0007); 49% between years 1 and 3.5 (P = 0.44); Table 2] [1]. The reduction in bleeding frequency between years 1 and 3.5 did not achieve statistical significance because of the small numbers of animals; nonetheless, 49% less bleeding events would be a considerable clinical improvement in any species. At the target level of ~1% of normal FIX, these dogs enjoyed a marked reduction in clinical bleeding; however, they still bled. Although likely, it is unknown if a higher trough level would have supported a greater reduction or ablation of bleeding in these haemophilia B dogs. Most importantly, these data establish that prophylactic administration of FIX reduces the frequency of bleeding in haemophilia B dogs.

### Gene transfer in canine haemophilia A and haemophilia B

In a series of published studies, continuous expression of cFVIII [16] and cFIX [17–21] in haemophilia A and haemophilia B dogs respectively, and cFVIIa...
PREVENTION OF SPONTANEOUS BLEEDING IN HAEMOPHILIC DOGS

[2] in both haemophilia A and haemophilia B dogs, has been achieved following successful gene transfer. The data, as reported in the original publication (vector, route of administration, vector dose, duration of follow-up, WBCT, factor levels and bleeding frequencies), are summarized in Table 3. The WBCT was shortened in the haemophilia A and B dogs expressing cFVIII and cFIX respectively, and thromboelastography parameters were corrected by cFVIIa (not shown) [2]. Also, a wide range of the respective factor levels was achieved in these studies (from ~1 to 100%). Most importantly, the bleeding frequencies showed that most, but not all, dogs had a marked reduction in bleeding frequency over several years when compared with the range of 4–6 bleeds per year reported for haemophilia A and B dogs treated ‘on-demand’. Five dogs that did not exhibit a persistent reduction in bleeding following publication of the initial gene transfer have been treated with alternative gene transfer strategies in attempts to reduce their bleeding frequencies (dogs B46, B93, B85, D31 and D32; studies in progress). In addition, dog E59 was treated by an alternative strategy, attempting to achieve a higher level of FIX produc-

Table 3. Bleeding frequencies in haemophilia A and haemophilia B dogs from published gene transfer studies.

<table>
<thead>
<tr>
<th>Dog</th>
<th>A/B</th>
<th>M/F</th>
<th>Vector</th>
<th>Route</th>
<th>Vector dose</th>
<th>Months follow-up</th>
<th>WBCT (min)</th>
<th>cFIX, cFVIII, or cFVIIa</th>
<th>Bleeds per year post gene transfer</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Herroz et al. 1999 [17]</td>
<td>B45</td>
<td>B</td>
<td>M</td>
<td>AAV-2-CMV-cFIX</td>
<td>i.m.</td>
<td>$1.3 \times 10^{11}$ vp kg$^{-1}$</td>
<td>16</td>
<td>20 ± 5</td>
<td>2.6 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>B46</td>
<td>B</td>
<td>M</td>
<td>AAV-2-CMV-cFIX</td>
<td>i.m.</td>
<td>$1.1 \times 10^{12}$ vp kg$^{-1}$</td>
<td>14</td>
<td>20 ± 2.5</td>
<td>12 ± 2</td>
</tr>
<tr>
<td></td>
<td>B93</td>
<td>B</td>
<td>M</td>
<td>AAV-2-CMV-cFIX</td>
<td>i.m.</td>
<td>$3 \times 10^{10}$ vp kg$^{-1}$</td>
<td>12</td>
<td>15 ± 1.5</td>
<td>21 ± 2</td>
</tr>
<tr>
<td></td>
<td>B48</td>
<td>B</td>
<td>F</td>
<td>AAV-2-CMV-cFIX</td>
<td>i.m.</td>
<td>$3.4 \times 10^{12}$ vp kg$^{-1}$</td>
<td>12.5</td>
<td>16 ± 1.5</td>
<td>17 ± 2</td>
</tr>
<tr>
<td></td>
<td>B83</td>
<td>B</td>
<td>F</td>
<td>AAV-2-CMV-cFIX</td>
<td>i.m.</td>
<td>$8.5 \times 10^{12}$ vp kg$^{-1}$</td>
<td>11</td>
<td>17 ± 2</td>
<td>69 ± 6</td>
</tr>
<tr>
<td>2. Snyder et al. 1999 [18]</td>
<td>B84</td>
<td>B</td>
<td>F</td>
<td>AAV2-MFG-cFIX</td>
<td>PV</td>
<td>$2 \times 10^{12}$ vp</td>
<td>4</td>
<td>12–20</td>
<td>30–95</td>
</tr>
<tr>
<td></td>
<td>B89</td>
<td>B</td>
<td>M</td>
<td>AAV-2-MFG-cFIX</td>
<td>PV</td>
<td>$2 \times 10^{12}$ vp</td>
<td>4</td>
<td>10–25</td>
<td>10–45</td>
</tr>
<tr>
<td>3. Mount et al. 2002 [19]</td>
<td>E34</td>
<td>B</td>
<td>F</td>
<td>AAV2-hAAT-cFIX</td>
<td>PV</td>
<td>$8 \times 10^{11}$ vg kg$^{-1}$</td>
<td>12</td>
<td>11 ± 2.5</td>
<td>262 ± 92</td>
</tr>
<tr>
<td>4. Arruda et al. 2004 [20]</td>
<td>E57</td>
<td>B</td>
<td>M</td>
<td>AAV-1 CMV PK9</td>
<td>i.m.</td>
<td>$2.4 \times 10^{11}$ vg kg$^{-1}$</td>
<td>33</td>
<td>18.1*</td>
<td>104</td>
</tr>
<tr>
<td></td>
<td>E35</td>
<td>B</td>
<td>F</td>
<td>AAV-1-CMV-PK9</td>
<td>i.m.</td>
<td>$1 \times 10^{12}$ vg kg$^{-1}$</td>
<td>9.5</td>
<td>19.1*</td>
<td>87</td>
</tr>
<tr>
<td></td>
<td>D31</td>
<td>B</td>
<td>F</td>
<td>AAV-2-CMV-cFIX</td>
<td>i.m.</td>
<td>$8.5 \times 10^{12}$ vg kg$^{-1}$</td>
<td>27</td>
<td>18.4</td>
<td>39</td>
</tr>
<tr>
<td></td>
<td>D32</td>
<td>B</td>
<td>M</td>
<td>AAV-2-CMV-cFIX</td>
<td>i.m.</td>
<td>$5.6 \times 10^{12}$ vg kg$^{-1}$</td>
<td>30.5</td>
<td>17</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>B14</td>
<td>B</td>
<td>M</td>
<td>AAV-2-CMV-cFIX</td>
<td>i.m.</td>
<td>$1.1 \times 10^{13}$ vg kg$^{-1}$</td>
<td>39.5</td>
<td>21.2*</td>
<td>30</td>
</tr>
<tr>
<td>5. Arruda et al. 2005 [21]</td>
<td>F37</td>
<td>B</td>
<td>M</td>
<td>AAV2-CMV-cFIX</td>
<td>ILP</td>
<td>$1.7 \times 10^{12}$ vg kg$^{-1}$</td>
<td>27</td>
<td>18.2</td>
<td>260 ± 52</td>
</tr>
<tr>
<td></td>
<td>D99</td>
<td>B</td>
<td>F</td>
<td>AAV2-CMV-cFIX</td>
<td>ILP</td>
<td>$3.7 \times 10^{12}$ vg kg$^{-1}$</td>
<td>39</td>
<td>13.9</td>
<td>730 ± 60</td>
</tr>
<tr>
<td></td>
<td>H08</td>
<td>B</td>
<td>M</td>
<td>AAV2-CMV-cFIX</td>
<td>ILP</td>
<td>$3.0 \times 10^{12}$ vg kg$^{-1}$</td>
<td>8</td>
<td>19.9</td>
<td>210 ± 16</td>
</tr>
<tr>
<td></td>
<td>E60</td>
<td>B</td>
<td>F</td>
<td>AAV2-CMV-cFIX</td>
<td>ILP</td>
<td>$3.9 \times 10^{12}$ vg kg$^{-1}$</td>
<td>10</td>
<td>16.3</td>
<td>&lt;1–100</td>
</tr>
<tr>
<td></td>
<td>E59</td>
<td>B</td>
<td>F</td>
<td>AAV2-CMV-cFIX</td>
<td>ILP</td>
<td>$2.9 \times 10^{12}$ vg kg$^{-1}$</td>
<td>37</td>
<td>16.8</td>
<td>31–78</td>
</tr>
<tr>
<td>6. Xu et al. 2005 [16]</td>
<td>H22</td>
<td>A</td>
<td>F</td>
<td>RVhAAT-cFVIII-WPRE</td>
<td>i.v.</td>
<td>~0.8 × 10^{10} TU kg$^{-1}$</td>
<td>16</td>
<td>9.7</td>
<td>101 ± 4%</td>
</tr>
<tr>
<td></td>
<td>H18</td>
<td>A</td>
<td>M</td>
<td>RVhAAT-cFVIII-WPRE</td>
<td>i.v.</td>
<td>~0.8 × 10^{10} TU kg$^{-1}$</td>
<td>16</td>
<td>9.8</td>
<td>129 ± 7%</td>
</tr>
<tr>
<td>7. Margaritis et al. 2009 [2]</td>
<td>J10</td>
<td>B</td>
<td>M</td>
<td>AAV8-hAAT-cFVIIia</td>
<td>PV</td>
<td>$2.06 \times 10^{13}$ vg kg$^{-1}$</td>
<td>34</td>
<td>37.8</td>
<td>≤0.5</td>
</tr>
<tr>
<td></td>
<td>J55</td>
<td>A</td>
<td>M</td>
<td>AAV8-hAAT-cFVIIia</td>
<td>PV</td>
<td>$6.25 \times 10^{13}$ vg kg$^{-1}$</td>
<td>18</td>
<td>26.2</td>
<td>1.3–2.6</td>
</tr>
<tr>
<td></td>
<td>J57</td>
<td>A</td>
<td>F</td>
<td>AAV8-hAAT-cFVIIia</td>
<td>PV</td>
<td>$1.25 \times 10^{14}$ vg kg$^{-1}$</td>
<td>15</td>
<td>23.9</td>
<td>1.3–2.6</td>
</tr>
<tr>
<td></td>
<td>E66</td>
<td>A</td>
<td>M</td>
<td>AAV8-hAAT-cFVIIia</td>
<td>PV</td>
<td>$1.25 \times 10^{14}$ vg kg$^{-1}$</td>
<td>12</td>
<td>29.8</td>
<td>1.3–2.6</td>
</tr>
</tbody>
</table>

A/B, haemophilia A or haemophilia B genotype; M/F, male/female; Vector: AAV-n, adeno-associated virus (and n refers to serotype number); cFVIIa, canine factor VIIa; cFVIII, canine factor VIII; cFIX, canine factor IX; CMV, cytomegalovirus promoter; hAAT, human alpha-1-antitrypsin promoter; MFG, viral MFG promoter; RV, retrovirus; WPRE, woodchuck post-transcriptional regulatory element; Route: ILP, isolated limb perfusion; i.m., intramuscular; i.v., intravenous; PV, portal vein; Vector dose: TU, transducing units; vg, vector genomes; vp, vector particles; WBCT, whole blood clotting time.

cFIX in ng mL$^{-1}$ (in 1, 2, 3, 4 and 5), cFVIIa as percentage of normal (in 6), or cFVIIa μg mL$^{-1}$ (in 7).

*Values before these dogs developed inhibitory antibodies to canine FIX at which time their WBCT was >60.

© 2010 The Authors
Journal Compilation © 2010 Blackwell Publishing Ltd Haemophilia (2010), 16 (Suppl. 3), 19–23
tion (study in progress). Thus, the severe spontaneous bleeder phenotype coupled with a significantly longer lifespan of dogs when compared with mice (>10 years vs. ~2 years; Table 1) allows for assessment of the degree of phenotypic correction, including monitoring for the clinically relevant endpoint of reduction in spontaneous bleeds.

Discussion

Antihaemophilic replacement products are often tested in haemophilic dogs during short-term (1–2 weeks) infusion studies to document pharmacokinetic parameters and the degree of correction of the haemophilic coagulopathy that can be achieved. This short-term study design models ‘on-demand’ therapy and does not address the clinically relevant endpoint of reduction in spontaneous bleeding frequency. In contrast, prophylactic administration of the missing coagulation protein or continuous expression following gene transfer allows for determining the effect on bleeding frequencies over years. When trough FIX levels were maintained at ~1% with prophylactic FIX administration, a significant but not complete reduction in bleeding frequency was achieved. A higher trough level may provide greater protection from spontaneous bleeding. Likewise, continuous expression of antihaemophilic factors following gene transfer was accompanied by a marked reduction in bleeding frequency, but did not provide complete correction. The advantage of gene therapy is that many different vectors and routes of administration are being developed. These are now being exploited to determine whether additional treatments with alternative gene therapy strategies will ameliorate spontaneous bleeding if the first gene transfer approach is unsuccessful or has limited success.

Although short-term (2-week) pharmacokinetic and pharmacodynamic infusion studies with antihaemophilic factors are not designed to address reduction in bleeding frequency, the results have nonetheless been of considerable interest and importance. Indeed, early studies with plasma products established the basis for such replacement therapy in dogs and humans with haemophilia [22–24]. When human plasma-derived and recombinant FVIIa [25], FVIII [26–28] and FIX [29] replacement products are tested in the haemophilia A and B dogs and shown to be safe and to correct the haemophilic coagulopathy, these products have consistently proven to be safe and efficacious in humans, with comparable pharmacokinetics. Likewise, the recently produced recombinant cFVIII [6] has provided outstanding haemostasis in the Chapel Hill haemophilia A dogs in spontaneous, traumatic and surgical bleeding without inducing immune responses (Valder R. Arruda, Katherine A. High, Timothy C. Nichols personal communication). The positive predictive accuracy of these studies is a major strength of performing such studies in dogs in comparison with other species (Table 1). Consequently, many advisory boards strongly encourage investigators contemplating new therapies for haemophilia A and haemophilia B to demonstrate safety and efficacy in bleeder dogs before initiating studies in humans [30] [Recommendations #137 and #160 of the Medical and Scientific Advisory Council (MASAC) of the National Hemophilia Foundation of the United States [31]].

Spontaneous bleeding has been a recognized phenotype in humans with severe haemophilia from its earliest descriptions [32]. A comparable severe phenotype with spontaneous bleeding is present in the Chapel Hill strain of haemophilia A and haemophilia B dogs. Careful monitoring of these dogs for spontaneous bleeding is essential for their survival and has provided a valuable endpoint for assessing the success and limitations of prophylactic protein replacement therapy and gene transfer studies.

Acknowledgements

This work was supported by NIH grants HL063098 to Timothy C. Nichols, HL64274 to Mark A. Kay and P01 HL074124 to Katherine A. High.

Disclosures

Dr Nichols has acted as an unrestricted speaker and received honoraria from Novo Nordisk. Dr High has acted as a consultant for Novo Nordisk.

References

PREVENTION OF SPONTANEOUS BLEEDING IN HAEMOPHILIC DOGS

23


