Von Willebrand disease (VWD) is an inherited hemorrhagic disorder promoted by either quantitative or qualitative defects of the von Willebrand factor (VWF). The disease represents the most common human coagulopathy afflicting 1.3% of the population. Qualitative defects are subdivided into four subtypes and classified according to the molecular dysfunction of the VWF. The differential diagnosis of the VWD is a difficult task, relying on a panel of tests aimed to assess the plasma levels and function of the VWF. Here, we propose biochemical approaches for the identification of structural variants of the VWF. A bioinformatic analysis was conducted to design seven peptides among which three were representatives of specific amino acid sequences belonging to normal VWF and four encompassed sequences found in the most common VWD subtype 2B. These peptides were used to immunize mice, after which, peptide-specific immunoglobulins were purified. This resulted in four Ig preparations capable of detecting alterations in the subtype 2B VWD plus additional three antibody fractions targeting the normal VWF. The panel of antibodies could serve many applications among them (1) assessment of VWF: antigen interaction, (2) VWF multimer analysis, and (3) production of monoclonal antibodies against VWF for therapeutic purposes as in thrombotic thrombocytopenic purpura.

1. Introduction

Von Willebrand disease (VWD) is an inherited hemorrhagic disturbance related to quantitative and/or qualitative defects of the von Willebrand factor (VWF) [1, 2]. VWD prevalence varies between 0.8 and 2.0%, depending on the investigated population, being considered the most common coagulopathy afflicting humans [3].
of clinical outcomes. Through different laboratory criteria it is possible to identify three primary types of the disease [5]. Alterations on the plasma levels of VWF are associated with VWD types I and 3, whereas structural and functional defects of VWF result in VWD type 2 [3, 6–11]. VWD types 1 and 3 reflect, respectively, partial and complete deficiency of the VWF. VWD type 2 is also classified into four subtypes. In subtype 2A, there is enhanced platelet adhesion caused by the selective deficiency of high molecular mass multimers of VWF (HMW). In subtype 2B VWD, it is observed increased affinity of VWF by platelet glycoprotein Ib (GpIb) associated with loss of HMW-VWF and mild thrombocytopenia. In contrast, in subtype 2M, a pronounced reduction in platelet adhesion is described, even considering the relatively normal size of the VWF multimers. Variants of the VWF in the subtype 2N display reduced affinity by coagulation factor VIII (FVIII). Altogether these six VWD types correlate with diverse clinical outcomes each requiring adequate therapeutic interventions [4, 12]. Whilst some patients with type 2B VWD reflect, respectively, partial and complete deficiency of the VWF amino acid substitutions R1306W, R1308C, V1316M, and R1341Q are collectively found in the majority of subtype 2B VWD patients [6]. In order to design signature peptides that would represent such mutations, Homo sapiens VWF sequences were retrieved from the International Society on Thrombosis and Haemostasis database, available at http://www.vwf.group.shef.ac.uk/. Predicted peptide sequences for synthesis obeyed the following criteria: (1) peptide size should be within the 8 to 10 mer range; (2) peptides should contain one aromatic residue for estimation of peptide yield after synthesis and HPLC purification (in the absence of an aromatic residue, this was added at the peptide C-terminal); (3) the location of the peptide sequences in the crystal structure of the VWF (available at http://www.pdbj.org/), revealed by the Swiss-Pdb Viewer 3.7, should demonstrate their exposure to the solvent, meaning that highly hydrophobic peptide sequences were not considered in this study.

The selected peptide sequences were then aligned to Mus musculus VWF sequence using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/) to reveal species specific amino acid differences as predictors for successful production of anti-VWF peptide antibodies in mice.

2.3. Synthesis, Characterization, and Production of Polyclonal Antibodies against VWF Peptides. Peptides selected for synthesis were representatives of the normal and altered versions of the VWF found in the major subtypes of type 2 VWD (Table I). Peptides were synthesized using the solid-phase protocol essentially as described by Merrifield [16]. Briefly, the support matrix consisted of Rink Amide Resin HL (Merck, Germany) at 0.78 mmol/g for an expected maximum yield of 40 μM of peptides per synthesis, using Fmoc-derivatized amino acids. Synthetic peptides were then purified via reversed-phase chromatography using a C18 column (Shim-pack CLD-ODS Shimadzu, Japan) on a Shimadzu HPLC system. Selected peptide peaks were recovered for analysis through direct injection onto an electrospray-operating mass spectrometer (LCMS-IT-ToF, Shimadzu, Japan) in positive ionization mode and capillary voltage set to 4,500 V. Mass spectrometric data were acquired over 10 ms, after which m/z values obtained were compared with the expected molecular masses for the synthesized peptides.

Synthetic peptides representatives of both normal and altered VWF were individually coupled to the highly immunogen carrier protein keyhole limpet hemocyanin (KLH, Sigma) at 1:1 ratio (1 mg peptide: 1 mg KLH) using glutaraldehyde as the crosslinking agent [17]. Polyclonal anti-KLH peptide antibodies were raised in male Swiss mice aged ten weeks. Immunization regimen consisted of three intraperitoneal administrations of 50 μg of the KLH–peptide conjugate prepared in 10% aluminium hydroxide as adjuvant, each at a 15 days interval. Blood was withdrawn after 45 days post-immunization.

2.4. Immobilization of Synthetic Peptides on Sepharose 4B and Purification of Anti-VWF Specific Immunoglobulins. Affinity columns (0.2 mL) containing immobilized synthetic peptides, at approximately 10 mg/mL, were produced as
Table 1: Proposed synthetic peptides for detection of the major qualitative alterations found in VWD type 2B.

<table>
<thead>
<tr>
<th>Major qualitative alterations found in VWD type 2B</th>
<th>Designed 8 mer peptides for synthesis</th>
<th>Theoretical [M + H]*</th>
<th>Observed [M + H]*</th>
<th>Respective peptide from M. musculus VWF</th>
</tr>
</thead>
<tbody>
<tr>
<td>R1306W</td>
<td>MEWLRISY</td>
<td>1097.54</td>
<td>1113.56*</td>
<td>MERLHIS</td>
</tr>
<tr>
<td>R1308C</td>
<td>MERLCSISY</td>
<td>1014.47</td>
<td>1014.50</td>
<td></td>
</tr>
<tr>
<td>Respective peptide found in H. sapiens VWF: MERLRSY</td>
<td>1067.56</td>
<td>1082.57*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>V1316M</td>
<td>SQKWVRMA</td>
<td>1005.52</td>
<td>1020.54*</td>
<td>SQKRIRVA</td>
</tr>
<tr>
<td>Respective peptide found in H. sapiens VWF: SQKWVRMA</td>
<td>973.55</td>
<td>972.57</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R1341Q</td>
<td>RPSELQRY</td>
<td>1048.55</td>
<td>1048.57</td>
<td>RPSELRR</td>
</tr>
<tr>
<td>Respective peptide found in H. sapiens VWF: RPSELRR</td>
<td>1076.59</td>
<td>1076.62</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Peptide mass containing an oxidized methionine.

described previously [18]. The antisera from mice immunized with KLH coupled to altered VWF peptides were loaded individually in affinity columns bearing the corresponding normal versions of the VWF peptide. This procedure aimed the subtraction of antibodies targeting the normal VWF. Approximately 100 μL of the unbound fraction was collected for analysis during the first chromatographic step, whilst the remaining were immediately submitted to further six rounds of chromatography to guarantee complete removal of antinormal VWF antibodies. For each chromatographic step bound fractions were eluted in 5 mL 0.1M glycine pH 2.6 and collected in tubes containing 5 mL of 0.4M Tris-HCl pH 8.0 for pH neutralization. Alkaline phosphatase labeled anti-mouse IgG, at 1:2000 dilution, was used to detect the presence of IgGs found in both nonretained and retained fractions from each chromatographic step, using the western blotting technique [19].

The next approach involved confirmation that antibodies targeting the normal VWF have been thoroughly subtracted after the six passages in affinity columns containing immobilized altered VWF peptides. For this purpose, 1 mL of pooled plasma from human healthy donors (n = 5) was first precipitated in 20% ethanol to enrich for normal VWF. Approximately 10 μg aliquots of the precipitated plasma were resuspended in protein loading buffer and loaded in three different lanes for separation on a 7% SDS-PAGE. The gel was transferred to a PVDF (polyvinylidene fluoride) membrane and each lane western blotted with either a 100 μL fraction obtained from the crude antisera or the two 100 μL non-retained fractions recovered from the first and sixth chromatographic steps.

The combined eluates from each chromatographic step containing immunoglobulins targeting a given altered VWF peptide were lyophilized, resuspended in 1 mL of phosphate buffer pH 7.4, and individually loaded onto the Sepharose-4B affinity column bearing the respective altered VWF peptide. Bound IgGs targeting the altered VWF peptide were eluted exactly as described above.

2.5. Production of Carrier Albumins Bearing Altered VWF Synthetic Peptides and Their Recognition by Anti-VWF Specific Antibodies. Aiming to produce a model sample for detection of altered VWF through the western blotting technique, purified albumin was conjugated to two representative synthetic peptides (SQKWVRMA and RPSELQRY, Table 1) [20] at a 1:1 ratio, respectively. Briefly, to each 1 mg of the respective peptides it was added 17 μL of N'-diisopropylcarbodiimide, diluted 1:25 in dimethylformamide (DMF), and 20 μL of 1M N-hydroxysuccinimide (also diluted in DMF). Reaction proceeded during 5 min at room temperature, after which 50 μL of 0.4 M sodium acetate (in DMF) was added. After further 2 min incubation, at room temperature, 1 mL of bovine serum albumin (BSA, Sigma), at 1 mg/mL, prepared in 0.05 M ammonium bicarbonate pH 7.5, was combined. Coupling reactions occurred during 1h followed by dialysis of each preparation in 0.1 M ammonium acetate over 24 h. Peptide-derivatized albumins were lyophilized and resuspended in 100 μL of saline.

Five μg aliquots of peptide-conjugated albumin were loaded into three lanes and separated through 12% SDS-PAGE, followed by Coomassie staining. A replica gel was produced and transferred to a PVDF membrane. Antipeptide-specific IgG purified through affinity chromatography, at approximately 0.15 μg/μL, was used as the primary antibody, at a final dilution of 1:500. The reaction was allowed to proceed for 3 h at room temperature. Development of the reactive band was achieved using alkaline phosphatase labeled anti-mouse IgG (Sigma), at 1:2000 dilution for 2h, followed by addition of the alkaline phosphatase substrates nitroblue tetrazolium/5-bromo-4-chloro-3-indolyolphosphate (NBT/BCIP, Sigma) as per the manufacturer’s instructions.

3. Results and Discussion

In this study four amino acid sequences, here termed altered VWF peptides (MEWLRISY, MERLCSY, SQKWVRMA, and RPSELQRY), were synthesized for generation of antibodies in mice. These were intended for recognition of the most common qualitative defects of VWF found in subtype 2B VWD. Three additional peptide sequences MERRISY, SQKWVRMA, and RPSELRR, representatives of the respective normal versions of the VWF peptides, were also produced and immobilized onto Sepharose 4B, for depleting
Figure 1: SDS-PAGE and western blotting approaches featuring the major steps involved in the production of a panel of anti-(KLH-peptide) antibodies for detection of VWD subtype 2B. (a) Lane 1, 10% SDS-PAGE profile of pooled human plasma after enrichment for normal VWF using ethanol precipitation; Lane 2, western blotting for detection of normal VWF at approximately 250 kDa, using the commercially available antibody against VWF/FVIII—note the presence of additional protein bands being recognized, particularly at lower mass; Lane 3, a representative Western blotting reaction obtained with the generated panel of anti-(KLH-peptide) antibodies, targeting both normal and altered VWF. (b) Detection of normal VWF by western blotting following the subtractive affinity chromatography; Lane 4, detection of VWF prior to depletion of antibodies against the normal factor; Lanes 5 and 6, detection of VWF using the nonretained fractions from the first and sixth chromatographic steps, respectively—not that 6 column passages proved sufficient for complete removal of anti-(KLH-peptide) antibodies. (c) Lane 7, 10% SDS-PAGE profile representative of the eluates obtained after affinity purification of antibodies targeting specifically altered VWF peptides—note the presence of IgG heavy and light chains at approximately 50 and 25 kDa, respectively; Lanes 8, 9, and 10, 10% SDS-PAGE profile of nonderivatized BSA, BSA-(RPSELRR) and BSA-(SQKRIRVA), respectively; Lanes 8', 9', and 10', corresponding western blotting reactions obtained using control sera, anti-(KLH-RPSELRR) and anti-(KLH-SQKRIRVA), respectively.

Out immunoglobulins targeting the normal VWF from the antisera raised with the altered VWF peptides. Given that the corresponding Mus musculus VWF peptides are MERLHIS, SQKRIRVA, and RPSELRR, we anticipated that the observed amino acid differences between human and mice VWF would justify the peptides capabilities for generating specific antibodies in mice (Table 1).

After immunization, the obtained antisera were tested for their ability to specifically recognize the VWF enriched from plasma samples of human healthy donors. Figure 1(a), lane 3 is a representative reactivity obtained for all antisera raised with either the altered or normal versions of VWF peptides. A unique band at approximately 250 kDa was observed coinciding exactly with a major band, at the same mass, observed in Figure 1(a), lane 2. This was probed with a commercially available antiserum against VWF-FVIII. In contrast, the latter antibody preparation do recognize other protein bands (particularly of lower masses) in the sample.
4. Conclusions

In this study, a combination of bioinformatic analysis, peptide synthesis, and affinity chromatography allowed the generation of antipeptide specific antibodies capable of recognizing the most common qualitative defects associated with type 2B VWD. These should provide speed and innovation when potentially applied to the diagnosis of human VWD.

**Abbreviations**

VWF: Von Willebrand factor  
VWD: Von Willebrand disease.

**Conflict of Interests**

Authors do not declare conflict of interests.

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