Molecular Genetic Survey of Five Japanese Families With High-Molecular-Weight Kininogen Deficiency

By Hisatomo Hayashi, Fumihiko Ishimaru, Toshiaki Fujita, Naokazu Tsurumi, Takashi Tsuda, and Ikuro Kimura

Analyses of the kininogen (KGN) molecule and KGN gene status in five Japanese families with high-molecular-weight (HMW) KGN deficiency were performed by the immunoblotting method with monoclonal antibodies to HMW-KGN, and by the Southern blotting method with the cDNA for human low-molecular-weight prekallikrein. No molecular abnormality of KGN was detected in the DNA from four patients with total KGN deficiency or one patient with isolated HMW-KGN deficiency. In the former, the KGN gene appeared to be grossly normal at the level of the whole genome on Southern blotting. In isolated HMW-KGN deficiency, a partial deletion in intron 7 was found by restriction analyses of EcoRI, BamHI, HindIII, Sca I, and Bgl II fragments. This partial deletion is assumed to be related to an abnormality of the alternative RNA splicing events for HMW-prekallikrein mRNA.

© 1990 by The American Society of Hematology.

NORMAL HUMAN PLASMA contains two molecular forms of kininogens (KGNs): high-molecular-weight (HMW) KGN and low-molecular-weight (LMW) KGN. Each protein consists of three domains: an amino terminal heavy chain, a bradykinin moiety, and a carboxyl terminal light chain. The cDNA sequences for human HMW- and LMW-prekallikreins have been isolated, and the sequence determination indicated that human HMW- and LMW-prekallikrein mRNAs share an identical sequence throughout the 5'-untranslated region and the protein coding region for the signal peptide and the heavy chain up to the sequence encoding the 12 amino acids distal to the bradykinin sequence, which are common to HMW- and LMW-prekallikrein mRNAs. The two mRNAs then completely diverge from each other.1,2 The two prekallikrein mRNAs are transcribed from a single gene as a consequence of alternative RNA processing events.2

Hereditary HMW-KGN deficiency is composed of cases of isolated HMW-KGN deficiency and of both HMW-KGN and LMW-KGN deficiency (total KGN deficiency). The difference in the mechanism of appearance of these deficiencies is of great interest. We studied four Japanese families deficient in total KGN16 and one Japanese family deficient in HMW-KGN alone,1 by the Southern blotting method with the cDNA clone (phKG36) for human LMW-prekallikrein as a probe for analyzing the KGN gene status. We also analyzed the abnormalities of the KGN molecules by the immunoblotting method using monoclonal antibodies (MoAbs) to human HMW-KGN.

MATERIALS AND METHODS

Kininogen-deficient families. Patients with HMW-KGN deficiency were used in this study. Their family histories, hematologic and blood samples were kindly supplied by Drs K. Nakamura (Tottori University, School of Medicine), M. Munakata and S. Ohkubo (Kansai Medical University), K. Tsukai and H. Kohno (Tokushima Chuo Hospital). The clot-promoting activity of HMW-KGN and prekallikrein, and the content of HMW- and LMW-KGN in the plasmas of these patients, are summarized in Table 1.

Immunoblotting. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in a discontinuous buffer system was carried out by a modification of the method of Laemmli,8 using 4% to 20% gradient gel (SDS-PAG Plate 4/20, vertical type DPE120; Daiichi Chemical Corp, Tokyo, Japan). Proteins were electrophoresed at 6 V/cm for 12 hours to nitrocellulose membrane (Towbin et al,9 which immunodetection of antigens bound to the nitrocellulose membrane was achieved by a double antibody reaction: the membrane was first reacted with MoAbs directed against HMW-KGN, HKG-H12, HKG-L7, or HKG-L17 for 60 minutes. After being washed three times, the membrane was incubated with goat antiovmouse IgG (Avidin-biotin-complex; Vector, Burlingame, CA) and stained with 4-chloro-i-naphthol (Sigma Chemical Co, St Louis, MO).

Preparation of genomic DNA and Southern blotting. High molecular DNA samples were prepared by the method of Kunkel et al10 from white blood cells of 20 mL blood samples anticoagulated with heparin. DNA samples of 5 µg were digested with restriction endonucleases (Nippon Gene, Toyama, Japan; Takara Biochemicals, Kyoto, Japan; and Bethesda Research Lab, Gaithersburg, MD) at a concentration of 2 U/µL. DNA fragments of normal subjects and patients were always electrophoresed on the same gels of 0.6%, 0.8%, or 1.2% agarose and transferred onto hybridization transfer membranes (Hybond™-N, Amersham, UK) by Southern's method.11 The human LMW prekallikrein cDNA (phKG36) used as a hybridization probe for the Southern blots was labeled by Random Primed DNA Labeling Kit (Boehringer Mannheim, Penzberg, West Germany) with [32P]-d-cytidine triphosphate (dCTP). Hybridization was carried out for over 12 hours at 65°C. The filters were washed and subjected to autoradiography at −70°C for 24 hours with Fuji medical imaging RxDUP film (Fuji Photo Film Corp, Kanagawa, Japan).

RESULTS

Immunoblotting analysis of plasma in HMW-KGN deficiencies using MoAbs to HMW-KGN. MoAb HKG-H12, reacting with domains 2 and 3 of the heavy chain (a common region of HMW- and LMW-KGN), recognized two distinct protein bands of purified HMW-KGN, a major band with an apparent molecular weight (mol wt) of 120,000 and a minor one with a mol wt of 105,000, and one band of purified LMW-KGN with a mol wt of 60,000. Two bands that...
corresponded to purified HMW-KGN and one corresponding to LMW-KGN were recognized in normal plasma. No bands were detected in the plasma of total KGN deficiency patients, but a single band with a mol wt of 60,000 corresponding to LMW-KGN was identified in the plasma of isolated HMW-KGN deficiency. Two bands with a mol wt of 120,000 and 60,000 could also be seen in the plasma of a heterozygous deficiency of HMW-KGN alone (Fig 1). Other MoAbs, HKG-L7 and HKG-L17, which recognized the fragment 1-2 and the light chain of HMW-KGN, respectively, did not detect any bands in either total KGN deficiency or isolated HMW-KGN deficiency plasmas. A weak band with a mol wt of 120,000 was seen in the plasma of a heterozygous deficiency of isolated HMW-KGN (data not shown).

Southern blotting analysis of KGN gene in HMW-KGN deficiency. All restriction sites of EcoRI, HindIII, and BamHI, and relevant restriction sites of Sca I and Bgl II were elucidated in restriction mapping between the 5-terminal EcoRI and 3-terminal BamHI site of cloned human genomic DNAs containing the KGN gene.1 No abnormal fragments were seen in EcoRI, BamHI or HindIII, Sca I or Bgl II digests of DNA in four homozygous deficiencies of total KGN, as compared with normal individuals (Fig 2). The normal pattern for EcoRI consisted of five fragments with sizes of 19.0, 7.6, 6.4, 5.4, and 4.4 kb. In a patient affected with isolated HMW-KGN homozygous deficiency, the normal 7.6 kb fragment was absent, merging into the normal 6.4 kb fragment, which showed densitometrically double the intensity of fragments of the same size in normal individuals. In the heterozygous deficiency, the 7.6 kb fragment diminished at one-half of the intensity of the normal 7.6 kb fragment, and the merged 6.4 kb fragment appeared to overlap the normal fragment of the same size (Figs 2A, and 3A). The normal pattern for BamHI consisted of three fragments with sizes of 27.0, 8.8, and 3.0 kb. In the homozygous deficiency of HMW-KGN alone, the normal 8.8 kb fragment was absent, being replaced by the abnormal 8.2 kb fragment. In the heterozygous deficiency, the abnormal 8.2 kb fragment was present in addition to the normal 8.8 kb fragment at one-half of the intensity of the fragments of the same size in normal patterns (Fig 2B). However, HindIII digestion showed no abnormal fragments in either homozygous or heterozygous deficiency of HMW-KGN alone (Fig 2C).

Sca I or Bgl II digestion also showed the absence of normal fragments, being replaced by new abnormal fragments in the homozygous deficiency of HMW-KGN alone, although no abnormal fragments were seen in the homozygous deficiency of total KGN (Fig 2D and E). In the homozygous deficiency of HMW-KGN alone, the abnormal 13.0 kb fragment of Sca I digest replaced the normal 10.5 kb fragment. The normal 5.4 kb and 4.9 kb Bgl II fragments appeared to be absent, being replaced by the abnormal 4.9 kb and 4.6 kb fragments, respectively (Fig 3B). In the heterozygous deficiency, the abnormal 13.0 kb Sca I fragment was observed, in addition to the normal 10.5 kb fragment. The abnormal 4.6 kb and the

### Table 1. HMW-KGN Deficiency in Japan

<table>
<thead>
<tr>
<th>Cases</th>
<th>Clot-Promoting Activity</th>
<th>HMW-KGN (%)</th>
<th>PKK (%)</th>
<th>HMW-KGN (µg/ml)</th>
<th>LMW-KGN (µg/ml)</th>
<th>Total KGN (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total KGN deficiency</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B1. Hayashi's first case</td>
<td>&lt;1</td>
<td>26</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
<td></td>
</tr>
<tr>
<td>(Fujishara trait)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B2. Nakamura's case</td>
<td>&lt;1</td>
<td>25</td>
<td>&lt;0.01</td>
<td>NR</td>
<td>0.24</td>
<td></td>
</tr>
<tr>
<td>(Tachibana trait)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B3. Munakata's case</td>
<td>&lt;1</td>
<td>17</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>B4. Hayashi's second case</td>
<td>&lt;1</td>
<td>34</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
<td></td>
</tr>
<tr>
<td>Isolated HMW-KGN deficiency</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H1. Tsukai's case</td>
<td>&lt;1</td>
<td>39</td>
<td>0</td>
<td>2.82</td>
<td>NR</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: PKK, prekallikrein; BK, bradykinin; NR, not reported.
Fig. 2. (A, B, and C)
Fig 2. Southern blotting analysis of KGN gene in HMW-KGN deficiency after digestion of restriction endonuclease. (A) Eco RI; (B) BamHI; (C) HindIII; (D) Sca I; (E) Bgl II. All restriction sites of Eco RI, BamHI, and HindIII were elucidated in restriction mapping between the 5-terminal EcoRI and 3-terminal BamHI site of cloned human genomic DNAs fragment containing the kininogen gene. Lanes defined as in Fig 1. The sizes (kb) of the normal bands (○, △) and the abnormal bands (←, --) are indicated on both sides.
Fig 3. Densitometrical analysis of Southern blots of KGN gene in HMW-KGN deficiency after digestion of EcoRI or Bgl II. (A) EcoRI: peak a, 19 kb band; b, 7.6 kb band; c, 6.4 kb band; d, 5.4 kb band; e, 4.4 kb band. (B) Bgl II: peak a, 13.0 kb band; b, 5.4 kb band; c, 4.9 kb band; d, 4.6 kb band; e, 3.7 kb band. NR, normal plasma; H1, isolated HMW-KGN homozygous deficiency of Tsukai's case; H10, heterozygous deficiency of Tsukai's case.

4.9 kb Bgl II fragment with double the intensity due to duplication by the normal 4.9 kb and the abnormal 4.9 kb fragments were also shown, in addition to the normal 5.4 kb fragment.

Restriction analysis using a number of other enzymes containing Taq I, Pst I, Pvu II, Hae III, Alu I, Rsa I, and Xba I did not show any other abnormal fragments in total KGN deficiencies. From the results described above, it was considered that no large deletions or insertions of the KGN DNA were recognized in patients affected with total KGN deficiency, suggesting that the KGN gene status of these patients was grossly normal. Thus, analysis of DNA digestion in this deficiency by Alu I, Hpa I, or Sca I, which possesses the restriction site around exon 1 with the 5'-
untranslated region and the protein-coding region for the signal peptide and the N-terminal 65 amino acid of the heavy chain, was also performed. No abnormal fragments were seen in these enzyme digests in these deficiencies as compared with normal individuals (data not shown).

Msp I polymorphism of HMW-KGN gene. Genomic DNA samples from 15 unrelated healthy Japanese were screened to be digested with the above restriction enzymes for their ability to produce restriction fragment length polymorphism (RFLP) (Fig 4A). Except Msp I, no other enzymes used in this study revealed RFLP. An Msp I digest of individuals showed three constant bands and two variable bands of 3.2 kb and 2.5 kb. We estimated from analysis of a total of 48 DNA samples (23 males and 25 females) that the 3.2 kb and 2.5 kb bands detected occurred at frequencies of 0.70 and 0.30 in the normal population, respectively. However, we have envisaged that Msp I polymorphism would not be useful clinically in the diagnosis of the homozygous and heterozygous deficiency of HMW-KGN in the families of Hayashi’s first case (Fujinara trait) and second case, Nakamura’s case (Tachibana trait), further examinations of other family members will be needed, because a variable band of 3.2 kb showed in a normal subject was not detected in homozygous and heterozygous deficiencies (Fig 4B).
DISCUSSION

The presence of an abnormal KGN molecule in the plasma of patients with total KGN deficiency or isolated HMW-KGN deficiency was examined using MoAbs for the domains constituting HMW-KGN. Although no analysis using the MoAb directed against the light chain of LMW-KGN was performed, no bands in plasma samples from patients with total KGN deficiency or isolated HMW-KGN deficiency were detected with other MoAbs, including HKG-H12 directed against domains 2 and 3 of the heavy chain, a common region of HMW- and LMW-KGN; HKG-L7 directed against the fragment 1,2 of HMW-KGN, a unique histidine-rich region; and HKG-L17 directed against the light chain of HMW-KGN. A single band with a mol wt of 60,000 corresponding to that of normal LMW-KGN in isolated HMW-KGN deficiency, was detected with HKG-H12. Thus, together with the data shown in Table 1, the lack of any molecular abnormalities of KGNs in either deficiency state was confirmed. Our results regarding the KGN gene status in the total KGN deficiency state indicated that no large deletions or insertions of the KGN gene were present, in spite of extensive restriction analysis using EcoRI, HindIII, and BamHI, for which the restriction map of the KGN gene has been clarified.

There is strong circumstantial evidence that CpG dinucleotides are hot spots for mutation in man. The proportion of CpG in sequenced genes is 31% of that expected. A usually high frequency of polymorphism has been found using restriction enzymes whose recognition sequences contain a CpG dinucleotide. In fact, most cases of inherited disorders of blood coagulation, such as hemophilia A (reported so far as nonsense mutations to a stop codon), have been detected with Taq I, which has the dinucleotide CpG in its recognition sequence. However, no abnormal bands could be detected upon Taq I digestion in either deficiency state.

Abnormal bands noted with these enzymes were also detected in the heterozygous state of this deficiency. The fact that abnormal bands were noted with so many restriction enzymes suggested a partial deletion in intron 7 from restriction mapping with EcoRI and BamHI for the human KGN gene, rather than abnormalities in the formation of each cleavage site for each enzyme (Fig 5). The fact that no abnormal band was detected with HindIII and no deletion was confirmed may be due to only a small part of exon 7 being present at the cleavage site of HindIII including intron 7, and the lack of hybridization of the cDNA used as a probe. It is known that the human KGN gene is composed of 11....
exons separated by 10 introns. The nine 5'-terminal exons encode the 5'-untranslated region and the protein-coding region for the signal peptide and the heavy chain, which are common to the HMW- and LMW-prekininogen mRNAs. Exon 10 consists of the common sequence for bradykinin and the immediately adjacent unique sequence for HMW-prekininogen mRNA. Exon 11 is located adjacent to a 90-nucleotide sequence downstream from exon 10 and precisely specifies the sequence unique to LMW-prekininogen mRNA. HMW- and LMW-prekininogen mRNAs are produced from a single gene as a consequence of alternative RNA-processing events.1

There are still many unknown aspects of this alternative splicing mechanism and the transacting factors involved. It has been reported that the switch from membrane-bound to secreted IgM with the differentiation of B cells is accomplished by producing alternative forms of mRNA from a single μ-heavy-chain gene.22 Although this process is assumed to be controlled at any of three steps; ie, transcription termination, RNA splicing, or RNA cleavage/poly(A) addition, it has been proved that the recognition sequence for the cleavage and polyadenylation of secreted mRNA plays an essential role in regulating the balance between secreted and membrane-bound mRNA.22

We suggest that the deletion site of intron 7 detected in isolated HMW-KGN deficiency may have some influence on the mechanism of alternative splicing. Although very little is known about the function of introns, a preceding intron probably influences the next splicing, as is known to occur in the regulation of the yeast mitochondrial gene specifying cytochrome b.23 Here, the second intron encodes a trans-acting protein “mRNA maturase” responsible for the splicing and maturation of cytochrome b mRNA. This protein is interlaced with the cytochrome b exon sequence for exact splicing of the remaining intron, thus controlling the splicing by negative feedback, which may constitute a regulatory mechanism for the expression of split genes. It is unknown how universal this mechanism is. As to the mechanism of alternative splicing, it has been pointed out that the secondary structure (hairpin loop) of the mRNA precursor is important in the alternative splicing pattern.24 Thus, the partial deletion in intron 7 detected in isolated HMW-KGN deficiency may also influence the secondary structure, so insufficient formation of HMW-prekininogen may occur.

ACKNOWLEDGMENT

We thank the members of the HMW-KGN-deficient family and their physicians for participating in this study. We also thank Dr Naomi Kitamura, Kansai Medical University, Osaka, Japan, for his gift of human LMW-prekininogen cDNA, and Dr Iwao Ohkubo, Department of Biochemistry, Nagoya City University Medical School, Nagoya, Japan, for his gift of MoAbs against HMW-kininogen and purified HMW- and LMW-kininogens.

REFERENCES

19. Orkin SH, Sexon JP, Cheng TC, Goff SC, Giardina PJV, Lee...


22. Danner D, Leder P: Role of an RNA cleavage/poly(A) addition site in the production of membrane-bound secreted IgM mRNA. Proc Natl Acad Sci USA 82:8658, 1985


Molecular genetic survey of five Japanese families with high-molecular-weight kininogen deficiency

H Hayashi, F Ishimaru, T Fujita, N Tsurumi, T Tsuda and I Kimura