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Genetic Polymorphism of the A Subunit of Human Coagulation Factor XIII

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SUMMARY

Utilizing a fluorescent technique for the localization of transglutaminase activity after electrophoresis on thin layer agarose gels, we observed a new polymorphism of coagulation factor XIII in both platelets and plasma. The electrophoretic pattern was that of a dimeric protein. Homozygotes gave a single band, while heterozygotes presented a three banded pattern. The polymorphism was found to be due to variation of the A subunit. Data from Australian blood donors indicate that the A subunit of factor XIII has an autosomal locus.

INTRODUCTION

Factor XIII (fibrin stabilizing factor) is the precursor of the enzyme fibrinoligase. This enzyme has transglutaminase activity which forms intramolecular γ-glutamyl-ϵ-lysine cross links between fibrin molecules [1]. Cross-linking of fibrin stabilizes clot structure and plays a significant role in hemostasis. Factor XIII is found in plasma, platelets, prostate gland, placenta, uterus, and liver [2]. It is thought that factor XIII from plasma is comprised of two A subunits joined as a dimer and two B subunits, (A_2B_2) [3]. The B subunits do not have transglutaminase activity and may serve as a carrier molecule in plasma, since factor XIII from platelets does not have B subunits and is comprised simply of A_2 dimers [3].

Factor XIII is activated to the transglutaminase form when a small peptide (mol. wt. 4,000) is cleaved from the A subunits by thrombin [4]. The transglutaminase activity of activated factor XIII is also dependent on the presence of Ca^{++}, thus the complete activation of factor XIII to a functional form requires two steps, as follows [5]:

$$\text{zymogen} \xrightarrow{\text{thrombin}} \text{zymogen}' \xrightarrow{Ca^{++}} \text{enzyme}.$$

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Numerous cases of factor XIII deficiency have been reported since the original description of this syndrome in 1960 by Duckert et al. [6]. Although some have suggested that factor XIII deficiency may be sex-linked [7, 8], more recent evidence suggests that it is an autosomal recessive disorder [1, 9, 10].

Using thin layer agarose electrophoresis and a specific transglutaminase staining technique, we have discovered a genetic polymorphism of the A subunit of factor XIII in the Australian population.

MATERIALS AND METHODS

Sample Collection and Preparation

Blood samples were drawn onto heparin or ethylenediaminetetra acetic acid (EDTA) as anticoagulants. Plasma was obtained in 3 hr by centrifugation and used immediately or stored frozen until use. Erythrocytes obtained at this time were washed three times in ice cold 150 mM NaCl and stored frozen as packed cells until use.

Platelets were obtained from expired packs of concentrated platelets prepared by the Canberra Red Cross blood transfusion service, or by differential centrifugation of 20 ml blood samples collected onto EDTA [11]. Platelets were washed three times in ice cold 150 mM NaCl and stored as packed platelets at 4°C until use within 72 hr. Platelet extracts were prepared by combining 1 vol of 1% (w/v) sodium dodecylsulfate (SDS) with 3 vol of packed platelets. The platelet/SDS mixture stood at room temperature for 30 min before use. Ultrasonicated platelets could also be used, but the SDS extract is easier to prepare and gives better electrophoretic resolution.

Electrophoresis

Electrophoresis was carried out on thin layer agarose gels prepared on 15 × 17 cm glass plates. The plates were rinsed in ethanol, warmed to 60°, and precoated with a thin film of 1% (w/v) agarose (Sigma type II, St. Louis, Mo.) in the gel buffer. After drying at 60°C, 35 ml of 1% (w/v) agarose in the gel buffer was spread over each plate and allowed to solidify at room temperature for 20 min in a humid environment. Sample slots (14/gel) were cut by pressing a plastic slot former into the gel 3 cm from the cathodal end of the plate. Samples of up to 6 μl of thawed packed erythrocytes, platelet extract, or plasma were then applied to the gel. Electrophoresis was carried out horizontally with the gel placed on a cooling block between two electrode tanks. After electrophoresis of the samples for 5 min, the sample slots were overlaid with 1% agarose (w/v) dissolved in the gel buffer, and electrophoresis continued for the appropriate time period. As described subsequently, electrophoresis was carried out using two different buffer systems.

Buffer I. The gel buffer was comprised of 7.5 mM potassium phosphate (pH 6.7) containing 20 μl/35 ml mercaptoethanol. The electrode buffer contained 0.2 M potassium phosphate (pH 6.7) and was connected to the gel via double layers of Whatman 3 MM chromotography paper. Electrophoresis was carried out at 13 V/cm for either 30 min or 3.5 hr.

Buffer II. The second buffer system was essentially the discontinuous system described by Ashton and Braden [12]. This buffer was made each day immediately before use. Electrophoresis was carried out at 18 V/cm until a hemoglobin marker migrated at least 10 cm down the gel.

Localization of Transglutaminase Activity

Monodansyl cadaverine has previously been shown to be an excellent substrate for transglutaminase in the presence of casein [13]. In the procedure described here, monodansyl cadaverine is covalently linked to casein in areas of transglutaminase activity. The subsequent treatment with trichloroacetic acid precipitates the casein in the gel, thus fixing the fluorescent monodansyl cadaverine/casein complex at the site of transglutaminase activity. Elution of

unreacted monodansyl cadaverine under running water removes the background fluorescence and leaves clearly defined fluorescent zones.

To identify the zones of transglutaminase activity, the gels were overlaid with a filter paper strip (Whatman No. 1) soaked in a reaction mixture containing, 1 M Tris/HCl (pH 8.0) 1 ml; 0.05 M $CaCl_2$, 1 ml; casein (Sigma purified powder, St. Louis, Mo.) 10 mg/ml dissolved in 0.2 M Tris/HCl (pH 8.0) 3 ml; monodansyl cadaverine 0.025 M, 1 ml; and β mercaptoethanol 20 μl. It was found necessary to initially dissolve the monodansyl cadaverine in a small amount of 0.1 M HCl and then make up the volume with 0.1 M Tris/HCl (pH 8.0). To differentiate between transglutaminase activity and activated factor XIII transglutaminase activity, 2 U of human thrombin (Ortho Diagnostics, Raritan, N.J.) were either deleted or added to the reagent mixture. The gel was then incubated at 37°C for 3.5 hr for erythrocyte and platelet extracts, and 12–16 hr for plasma samples. To prevent dessication during incubation, the plates were kept in covered dishes containing enough water-soaked absorbent paper to maintain a humid environment.

After incubation, the filter paper containing the staining reagents was removed and the gel fixed by application of a filter paper overlay containing 7.5% (w/v) trichlorocetic acid for 5 min. After fixing, the gel is again overlaid for a further 5 min with another filter paper strip soaked in 0.3 M Na_2HPO_4. The gel is subsequently washed under running tap water for 1–2 hr to elute the unincorporated monodansyl cadaverine. During this elution process, the appearance of areas of transglutaminase, or activated factor XIII activity, can be followed if the gel is viewed under short wave (254 mM) UV light.

Immunofixation

After electrophoresis using buffer II, factor XIII was specifically localized by overlaying the gel with a 30% solution (v/v) of rabbit anti-human factor XIII subunit A anti-serum (Behringwerke Marburg, W. Germany). The diluted anti-serum was spread over the gel with a glass rod, taking care not to touch the gel surface, and the gel was incubated at room temperature in a humid environment for at least 1 hr. After immunofixation, the gel was rinsed briefly under running tap water, covered with a wet filter paper, and compressed for 5 min under approximately 1 cm of absorbent paper covered by a perspex plate and a 2 kg weight. After compression, the gel was soaked overnight at 4°C in at least 1 L of 150 mM NaCl to remove non-immunoprecipitated protein. To facilitate staining, the gel was again compressed and air dried. The dried plates were stained for 5 min with 0.2% coomassie brilliant blue R, dissolved in a 9:2:9 mixture of methanol, acetic acid, and water. The background was destained by rinsing the gel plate in a solution of 2.5% acetic acid and 2.5% Teepol.

RESULTS

Preliminary Observations

Short term electrophoresis (30 min) of platelet extracts in the phosphate buffer system (buffer I) revealed a very fast region of transglutaminase activity. This electrophoretically fast zone migrated as a single anodal band and required the presence of Ca^{++} (but not thrombin) in the staining reagent mixture. A similar zone of activity with identical electrophoretic mobility and staining requirements was seen in hemolysates (fig. 1). Prolonged electrophoresis (3.5 hr) of platelet extracts in buffer I revealed additional anodal migrating components which were dependent on the presence of both Ca^{++} and thrombin for the development of transglutaminase activity (not shown). These components were not seen in similarly treated erythrocyte samples and were therefore considered to indicate the activity of activated factor XIII.

Since the thrombin-activated zone of activity from platelets showed evidence of variation in electrophoretic mobility between different individuals, these components were investigated further using an alternate buffer system (buffer II). Under improved

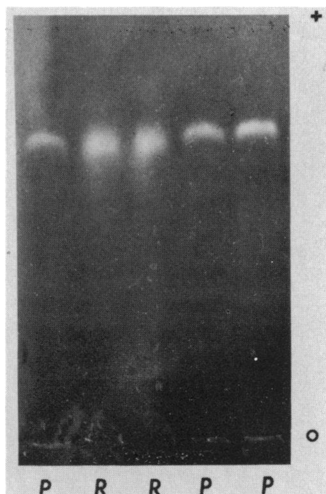

FIG. 1.—Electrophoresis of platelet (*P*) and red cell (*R*) calcium-dependent transglutaminase (buffer I, 30 min at 13 v/cm).

conditions, the electrophoresis of platelet extracts from normal Australian blood donors revealed three different electrophoretic patterns of activated factor XIII activity (fig. 2). These were either (1) a single anodal fast band, (2) a single anodal slow band or (3) a three-banded anodal pattern composed of both fast and slow bands and an additional intermediate band with increased staining intensity. The most anodal factor XIII activity zone migrated approximately 2 cm behind hemoglobin which was normally run at the side of the gel as a standard migration marker. Samples taken repeatedly over 6 months consistently produced patterns identical to the originals.

FIG. 2.—Electrophoresis of platelet thrombin-activated, calcium-dependent transglutaminase (factor XIII) (buffer II, 5 hr at 18 v/cm).

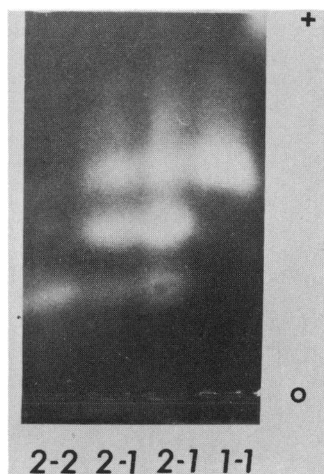

FIG. 3.—Electrophoresis of plasma thrombin-activated, calcium-dependent transglutaminase (factor XIII) (buffer II, 5 hr at 18 v/cm).

Electrophoresis of platelet-free plasma samples gave identical results (fig. 3). Individuals showing a fast or slow platelet factor XIII component gave an identical pattern when their plasma was used. However, the factor XIII activity is much lower in plasma than in platelet extracts, and it was necessary to incubate the electrophoresis plates with the stain overlay for up to 12 hr before the zones of plasma factor XIII could be easily observed. Plasma collected with either heparin or EDTA as an anticoagulant is suitable, but serum fails to develop any factor XIII activity.

Genetic Studies

The study of the electrophoretically very fast zone of Ca^{++} dependent transglutaminase activity (buffer I) in hemolysates and platelets from 200 Australian blood donors, revealed no evidence for genetic variation at the locus controlling that enzyme. In comparison, the patterns of Ca^{++} and thrombin-dependent factor XIII activity, observed in both platelets and plasma (buffer II) (figs. 2 and 3) are characteristic of the electrophoretic variation of a dimeric protein with two alleles. We therefore propose to refer to this system as the *F-XIII* locus with the two alleles termed $F\text{-}XIII^1$ for the fast anodal component, and $F\text{-}XIII^2$ for the slow anodal component.

Based on this genetic model and the nomenclature noted above, observed and expected phenotyped numbers in a population of normal unrelated Australian blood donors are given in table 1. Observed phenotype numbers do not differ significantly from those expected under an assumption of a Hardy-Weinberg equilibrium. The close agreement between the observed and expected distributions of the three phenotypes and the identification of 37 heterozygote (F-XIII 2–1) males, clearly indicates that the F-XIII locus is autosomal. Family data (fig. 4) also indicate that the observed variation of factor XIII is genetically transmitted.

TABLE 1

F-XIII PHENOTYPES FOR UNRELATED AUSTRALIAN BLOOD DONORS

	F-XIII 1	F-XIII 2-1	F-XIII 2	
Determined from plasma:*				
Male				
Observed	73	37	5	
Expected	72.7	37.8	4.7	χ^2 = .026
Female				
Observed	39	21	4	
Expected	38.2	22.6	3.2	χ^2 = .333
Pooled				
Observed	112	58	9	
Expected	111	60	8	χ^2 = .208
Determined from expired platelets:†				
Observed	128	70	6	
Expected	130.2	65.6	8.2	χ^2 = .892

* Gene frequency $F\text{-}XIII^1$ = .79; $F\text{-}XIII^2$ = .21. No. = 179.
† Gene frequency $F\text{-}XIII^1$ = .80; $F\text{-}XIII^2$ = .20. No. = 204.

Molecular aspects

Because this polymorphism is observed in platelets, it is evident that the structural variation giving rise to the differences in electrophoretic mobility of the two allelic products must be located on the A subunit of factor XIII, since the B subunits are not found in the platelet factor [3]. Activation of factor XIII to the transglutaminase form results from the cleaving of a small (mol. wt. 4,000) fragment from the A subunit [4]. We normally included thrombin in the staining reagent mixture. However, to determine if the observed variation in the A subunit of factor XIII was located on the activation peptide, we pretreated platelet extracts with thrombin prior to electrophoresis. The thrombin-activated extracts resulted in the same patterns of factor XIII activity as untreated extracts (not shown), indicating that the variation giving rise to the electrophoretic polymorphism lies on the activated A subunit and not on the activation peptide fragment.

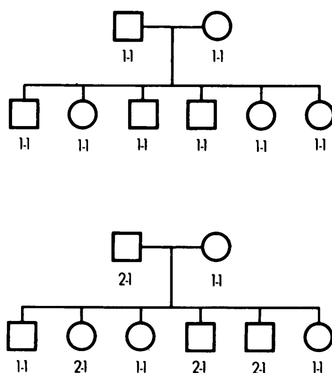

FIG. 4.—Family data showing genetic transmission of the $F\text{-}XIII^1$ and $F\text{-}XIII^2$ alleles.

Although the transglutaminase activity we observed on the gels was dependent on the incorporation of Ca^{++} and thrombin in the stain reagent mixture (both essential for factor XIII activation), we also utilized specific rabbit anti-human factor XIII A subunit anti-serum to confirm the identification of the observed polymorphism. Figure 5 shows the immunofixation products of platelet extracts. There was complete agreement between the phenotypes identified by the histochemical and immunological techniques, and similar results were obtained using plasma.

DISCUSSION

A method utilizing the same staining principle and polyacrylamide disc electrophoresis has been previously described [5]. The previous report was only concerned with purified factor XIII activity, so the authors were unaware of the variation to be found between individuals.

Previous reports [14, 15] have suggested that platelets may contain a native transglutaminase in addition to the transglutaminase activity of activated factor XIII. We have certainly confirmed that suggestion. Our findings clearly indicate that platelets contain a low molecular weight transglutaminase which does not require activation by thrombin and has an electrophoretic mobility similar to the transglutaminase found in erythrocytes (fig. 1). Since the electrophoretic mobility of the platelet and erythrocyte transglutaminase was invariant in over 200 normal blood donors, in which factor XIII showed significant variation, it is evident that transglutaminase and factor XIII are the product of separate loci.

That this observed polymorphism is of activated factor XIII is clearly indicated by the dependence of the reaction on the presence of both Ca^{++} and thrombin. The presence of the polymorphism in platelets, as well as plasma, indicates that the polymorphism is associated with the A subunit, since platelets do not contain the B subunits found in plasma factor XIII [3]. Both these findings are supported by the

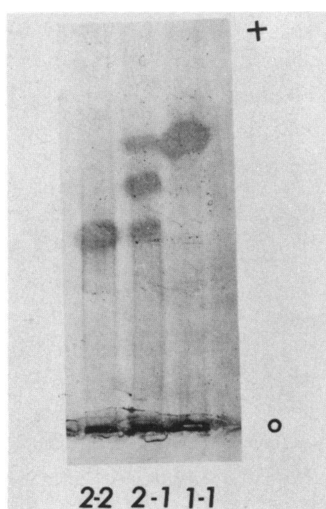

FIG. 5.—Immunofixation of platelet factor XIII A subunits (buffer II, 5 hr at 18 v/cm).

identification of the polymorphism by specific immunofixation of A subunits from platelets and plasma. Activation of platelet factor XIII by thrombin before electrophoresis did not alter the relative difference in mobility of the different phenotypes. This suggests that the polymorphism is the result of a difference in the major component of the A subunit, and not the small activation fragment.

The mode of inheritance of factor XIII deficiency has been the subject of some discussion. Some reports have suggested the possibility of a sex-linked mode of inheritance [7, 8] but recent reports have all indicated that deficiency of the A subunit of factor XIII is inherited as an autosomal recessive disorder [1, 9, 10]. Our data clearly show that the A subunit locus is on an autosomal chromosome.

The frequency of $F\text{-}XIII^2$ in the Australian population is clearly high enough for the *F-XIII* locus to be described as being polymorphic. Since individual phenotypes can be readily determined from plasma samples by an inexpensive technique, the *F-XIII* locus should be of considerable value as a genetic marker for population genetic studies. Preliminary investigations have already indicated that this polymorphism is found in New Guinean, Samoan, Chinese, and Australian Aboriginal populations.

Judging by the gel fluorescence, there is probably little difference between the activity of factor XIII from homozygotes for either of the two alleles. Since only 1%–5% of normal activity is required for clot stabilization [1], there is probably little direct physiological advantage or disadvantage gained by either allele.

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Workshop on Bioethics and Public Policy

The Institute of Society, Ethics, and the Life Sciences will sponsor a workshop this summer on "Bioethics and Public Policy," at the University of Colorado in Boulder, July 15–22, 1979. The workshop will examine health policy alternatives: national health insurance, the current cost containment effort, and health planning techniques. Participants in the national policy making effort will interact with those focusing on the ethical analysis of the health policy alternatives. Other topics will include public policy issues related to abortion, death and dying, involuntary commitment, policies on homosexuality, the ethics of behavior control, the policy dimensions of human genetics, as well as the ethical and legal positions on informed consent. A brochure describing detailed workshop agenda, registration, and costs is available from the Hastings Center, 360 Broadway, Hastings-on-Hudson, NY 10706, or call (914) 478-0500.