Platelets of the Wistar Furth Rat have Reduced Levels of Alpha-Granule Proteins

An Animal Model Resembling Gray Platelet Syndrome

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Abstract

Rats of the Wistar Furth (WF) strain have hereditary macrothrombocytopenia (large mean platelet volume [MPV] with increased platelet size heterogeneity and reduced platelet count). Ultrastructural studies suggest that this anomaly results from erratic subdivision of megakaryocyte cytoplasm into platelets. In this study, we have examined protein profiles of platelets of WF rats for biochemical abnormalities associated with this anomaly. Marked decreases in protein bands with an M_r of 185, 57, 53, 16, 13, and 8 kd were observed in one-dimensional reduced SDS-PAGE gels in WF platelets compared with platelets of Wistar, Long Evans, and Sprague-Dawley rats. These proteins were released into the supernatant when washed platelets were treated with thrombin suggesting that they were alpha-granule proteins. These abnormalities were not present in offspring of crosses between Wistar Furth and Wistar rats; however, they were present in platelets of offspring with large MPV derived from backcrosses of (WF × Wistar) F_1 males to WF females, but not in backcross offspring with normal platelet size. Immunoblotting confirmed decreased levels of thrombospondin, fibrinogen, and platelet factor 4 in WF platelets. Electron microscopic examination revealed that platelet alpha granules were usually smaller in Wistar Furth than in Wistar rats. In addition, immunogold electron microscopy demonstrated that the surface connected canalicular system of the large Wistar Furth platelets contained dense material composed of alpha-granule proteins, not present in Wistar platelets. From these results, we conclude that the Wistar Furth rat platelet phenotype of large mean platelet volume and decreased levels of alpha-granule proteins represents an animal model resembling gray platelet syndrome. The autosomal recessive pattern of inheritance of the large MPV phenotype and platelet alpha-granule protein deficiencies suggests that a component common to both formation of platelet alpha granules, and subdivision of megakaryocyte cytoplasm into platelets, is quantitatively or qualitatively abnormal in Wistar Furth rat megakaryocytes and platelets. (*J. Clin. Invest.* 1991. 87:1985–1991.) Key words: platelet • alpha granule • animal model • Wistar Furth rat • gray platelet syndrome

Introduction

Blood platelets are produced by subdivision of megakaryocyte cytoplasm into several thousand membrane-bounded units of relatively uniform size that are released into the circulation. The mechanisms which regulate this cytoplasmic subdivision and determine platelet size are unknown. However, we have recently discovered an animal model of hereditary macrothrombocytopenia (the Wistar Furth [WF][1] rat) (1), which has an aberrant pattern of platelet formation manifested in irregular development of the megakaryocyte-platelet demarcation membrane system, abnormal membrane complexes in both megakaryocytes and platelets, and abnormally large mean platelet volume with increased platelet size heterogeneity (1, 2).

Large mean platelet volume is a characteristic of several hereditary clinical platelet disorders and anomalies including Bernard-Soulier syndrome (3, 4), May-Hegglin anomaly (5, 6), gray platelet syndrome (7–13), Mediterranean macrothrombocytopenia (14–17), as well as other less well defined thrombocytopathies (18–25). Of these, Bernard-Soulier syndrome and gray platelet syndrome have well defined platelet biochemical abnormalities: decreased or abnormal glycoprotein Ib-IX complex in the case of Bernard-Soulier syndrome (26–32), and deficiency of alpha-granule proteins in gray platelet syndrome (9, 33, 34). The purpose of this investigation was to ascertain what biochemical abnormalities were associated with the macrothrombocytopenia of the Wistar Furth rat. In our previous report, labeling of platelet surface glycoproteins by the periodate/NaB^3H_4 procedure suggested that the major platelet surface glycoproteins including platelet glycoprotein Ib were present in normal amounts on Wistar Furth platelets (1), making it unlikely that the Wistar Furth platelet abnormality is that seen in Bernard-Soulier syndrome. Also, Dohle bodies were not detected in Wistar Furth granulocytes, suggesting that the large mean platelet volume was not akin to that seen in May-Hegglin anomaly (1). This study indicates that Wistar Furth rat platelets have decreased alpha-granule proteins, and thus resemble platelets of patients with gray platelet syndrome.

Methods

Wistar Furth, Wistar, Sprague-Dawley, and Long-Evans hooded rats were purchased from Harlan Industries, Inc. (Indianapolis, IN). Rabbit anti-rat platelet factor 4 serum was generously provided by Dr. David Kuter and Dr. Sheryl Greenberg of the Massachusetts Institute of Technology, Cambridge, MA. Rabbit antibovine platelet thrombospondin serum showing preferential binding to the heparin-binding domain was kindly provided by Dr. Daniel Walz and Dr. Rasheeda Zafar of Wayne State University, Detroit, MI. Affinity-isolated polyclonal goat anti-human fibrinogen antibody used for immunoblotting was purchased from Tago, Inc., Burlingame, CA. Rabbit anti-rat fibrinogen antiserum used for immunogold electron microscopy was obtained from Janssen, Inc., Piscataway, NJ. Alkaline phosphatase-conjugated goat anti-rabbit IgG and alkaline phosphatase substrate kits were purchased from Bio-Rad Laboratories, Richmond, CA. ^{125}I-labeled protein A and protein G were obtained from ICN Biomedicals, Inc., Costa Mesa, CA.

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1. *Abbreviations used in this paper:* MPV, mean platelet volume; WF, Wistar Furth.

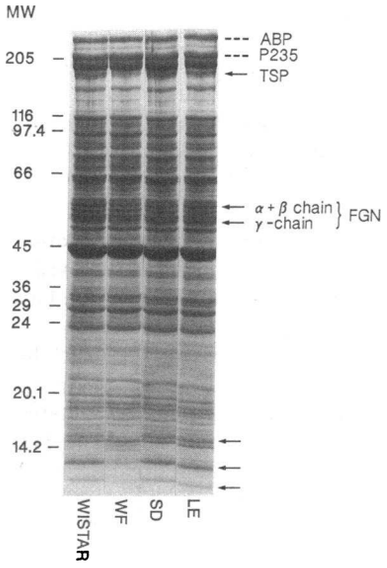


Figure 1. Comparison of protein profiles of Wistar, Wistar Furth, Sprague-Dawley (*SD*) and Long-Evans (*LE*) platelets after separation by SDS-PAGE under reduced conditions. Washed platelets were solubilized in SDS with DTT as the reducing agent and proteins separated by SDS-PAGE in 7.5–20% linear gradients of acrylamide. Gels were stained with Coomassie brilliant blue. *ABP*, actin-binding protein; *P235*, platelet P235 (talin); *TSP*, thrombospondin; *FGN*, fibrinogen. Arrows indicate protein bands deficient in WF rat platelets. *MW*, molecular weight.

Table I. Relative Deficiency of Selected Platelet Protein Bands of Wistar Furth Rats

Protein band (M_r)	Experiment number			
	1	2	3	4
kD				
185	28	26	32	28
57	35	42	55	41
53	31	34	56	53
13	23	35	55	35

Coomassie blue staining of selected protein bands in SDS-PAGE gels of Wistar and Wistar Furth rat platelets was quantified by densitometry. The values were normalized to the amount of actin staining in each case, and the quantity of the selected protein in Wistar Furth platelets was expressed as a percentage of that in Wistar platelets. The values in the table represent these percentages.

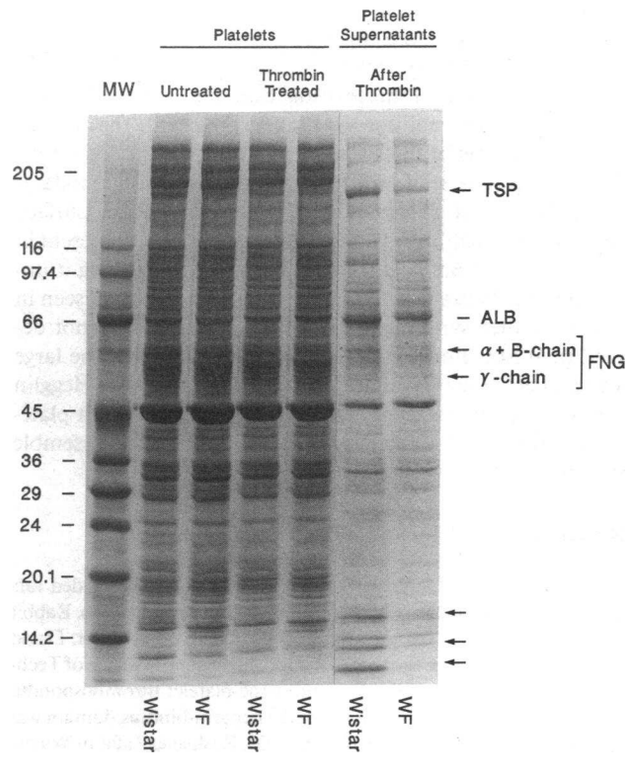


Figure 2. Analysis of proteins released from Wistar and WF rat platelets by thrombin stimulation showing that proteins deficient in WF platelets are released from Wistar platelets upon platelet activation. Washed platelets were treated with 2 U/ml thrombin for 2 min with stirring and the reaction stopped by addition of 4 U/ml of hirudin. The activated platelets were pelleted (15,850 *g*), the supernatant further centrifuged at high speed (100,000 *g*), and the 15,850 *g* platelet pellets and 100,000 *g* supernatants were solubilized in SDS for gel electrophoresis. Arrows indicate proteins noted to be deficient in untreated WF rat platelets. *TSP*, thrombospondin; *ALB*, albumin; *FGN*, fibrinogen; MW, molecular weight.

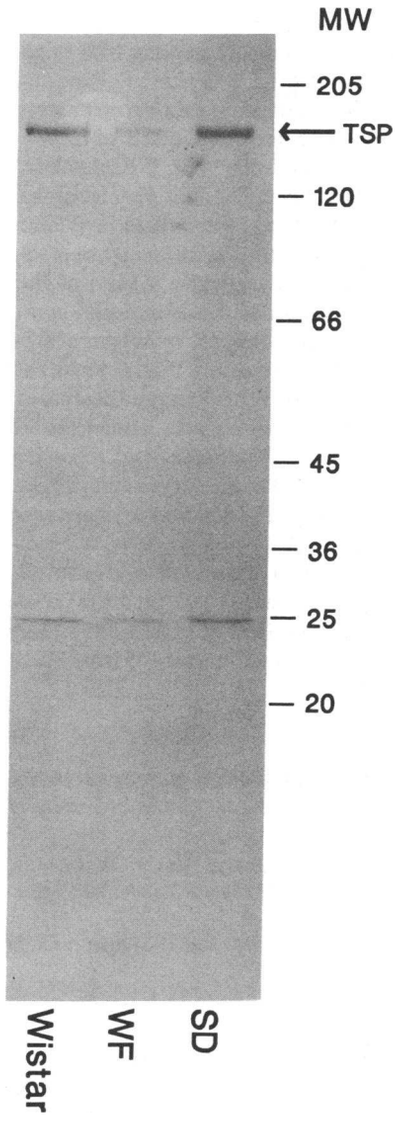


Figure 3. Immunoblot of Wistar, WF, and Sprague-Dawley (*SD*) rat platelets with antithrombospondin antiserum showing a decreased level of thrombospondin (*TSP*) in Wistar Furth rat platelets (same protein loads as in Fig. 1). Antibody binding detected with alkaline phosphatase-conjugated second antibody. MW, molecular weight.

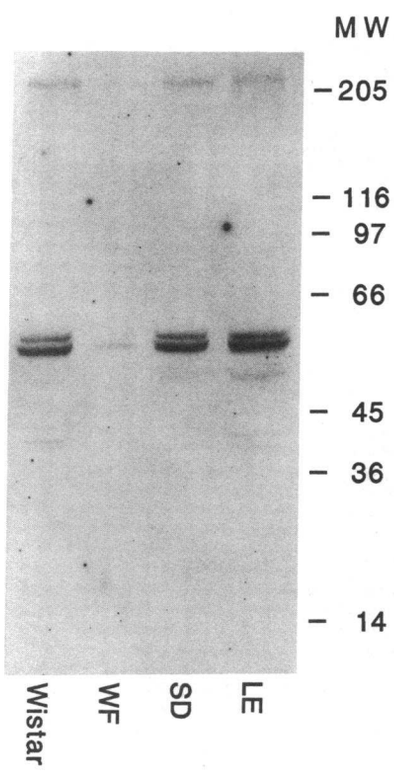


Figure 4. Immunoblot of Wistar, WF, Sprague-Dawley (SD), and Long-Evans (LE) rat platelets with antifibrinogen antibodies showing decreased fibrinogen in Wistar Furth rat platelets. Same protein loads as in Fig. 1. Antibody binding detected with ^{125}I-protein G and autoradiography. MW, molecular weight.

Hirudin and rat fibrinogen were purchased from Sigma Chemical Co., St. Louis, MO. Bovine thrombin was obtained from Parke-Davis, Morris Plains, NJ. Platelet counts were determined by phase microscopy.

Collection and solubilization of platelets. Blood was collected from the dorsal aorta or via cardiac puncture of metofane-anesthetized rats into syringes containing Na_2EDTA or acid citrate-dextrose. Platelets were separated by differential centrifugation. Platelets were pelleted and washed three times in EDTA-Hepes-saline (EHS) buffer (0.001 M Na_2EDTA, 0.01 M Hepes buffer [Sigma Chemical Co.], and 0.15 M NaCl) (35). Less than 1 white blood cell (WBC) per 10^6 platelets was present in Wistar Furth platelet preparations. The ratio of WBC:platelets for Wistar platelet preparations was < 1:30,000. Platelets suspended in EHS buffer were boiled for 5 min in one-third volume of gel sample buffer containing 0.125 M Tris-HCl, pH 6.8, 40% glycerol, 8% SDS, 160 mM DTT, and 0.01% bromphenol blue.

Analysis of proteins released upon platelet activation. Blood was collected from the dorsal aorta of metofane-anesthetized rats into plastic syringes containing acid citrate-dextrose (28). Platelets were separated by differential centrifugation and washed three times in EHS buffer. The platelets were then suspended in EHS buffer, incubated at 37°C for 2 min before activation with thrombin (2 U/ml) for 2 min. Thrombin activation was stopped by the addition of hirudin (4 U/ml). The activated platelets were pelleted (15,850 *g* for 4 min at 4°C). The supernatant containing the released platelet proteins was removed and centrifuged a further 2 h at 100,000 *g* at 4°C (33). The 15,850 *g* platelet pellets and 100,000 *g* supernatants were solubilized in 2% SDS for analysis by SDS-PAGE.

Gel electrophoresis. One-dimensional SDS-polyacrylamide gel electrophoresis was performed in linear gradients of acrylamide using the discontinuous buffer system of Laemmli (36). Usually, ~65 μg of protein contained in 40 μl of buffer were loaded per lane. Gels were stained with Coomassie blue.

Immunoblotting of antibodies to electrophoretically separated proteins. Proteins separated in polyacrylamide gels were electrophoretically transferred to nitrocellulose sheets in a transfer buffer consisting of 25 mM Trizma base, 192 mM glycine, and 20% methanol. The nitrocellulose strips were incubated overnight with the antibody of interest, washed, and incubated with ^{125}I-labeled protein A or G as appropriate, or with alkaline phosphatase-conjugated goat anti-rabbit IgG. 2% casein was included in all incubations and washes to reduce nonspecific binding (37). The strips were dried and autoradiograms prepared using Kodak XAR-5 film when ^{125}I-protein A or protein G was used as the antibody label. Alkaline phosphatase substrate kits were used to detect binding of alkaline phosphatase-conjugated antibodies. The antithrombospondin antibody was affinity purified from nitrocellulose transfers of Wistar rat platelets reacted with antiserum to thrombospondin, which was detected with alkaline phosphatase conjugated second antibody, the thrombospondin (185 kD) band excised, and the antibody eluted with glycine-HCl.

Immunogold electron microscopy. Blood was collected into acid citrate with prostaglandin E_1 (8 μM) and platelets were separated by differential centrifugation. The platelets were fixed in 1% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 1 h at 22°C, then washed three times in 0.1 M phosphate buffer (pH 7.4) and embedded in glycol-methacrylate as previously described (13). Thin sections were floated for 2 h on diluted (1:10) primary antisera (antiplatelet Factor 4 and antifibrinogen), extensively washed in Tris-buffered saline, incubated with goat anti-rabbit immunoglobulin fractions coupled to 5-nm colloidal gold particles (GAR G5) (Janssen Pharmaceutica, Beerse, Belgium) for 1 h, washed, and counterstained with uranyl acetate and lead citrate. The preparations were observed with a Philips CM 10 electron microscope (Philips Electronic Instruments Co., Eindhoven, The Netherlands).

Results

Protein profiles of Wistar Furth rat platelets obtained by one-dimensional SDS-polyacrylamide electrophoresis performed

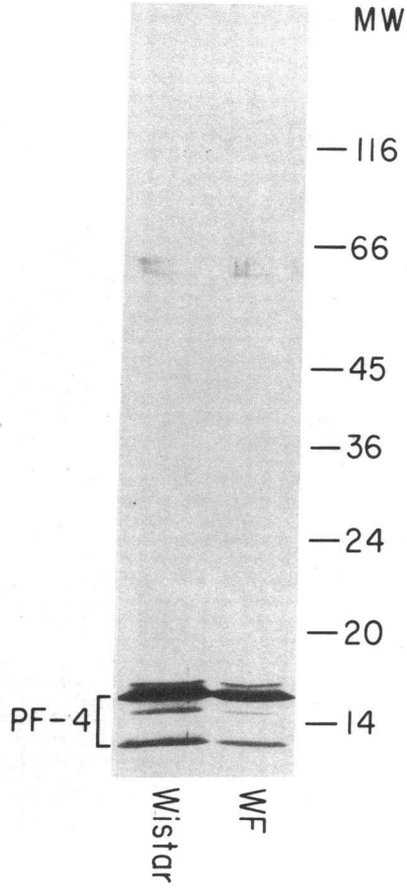
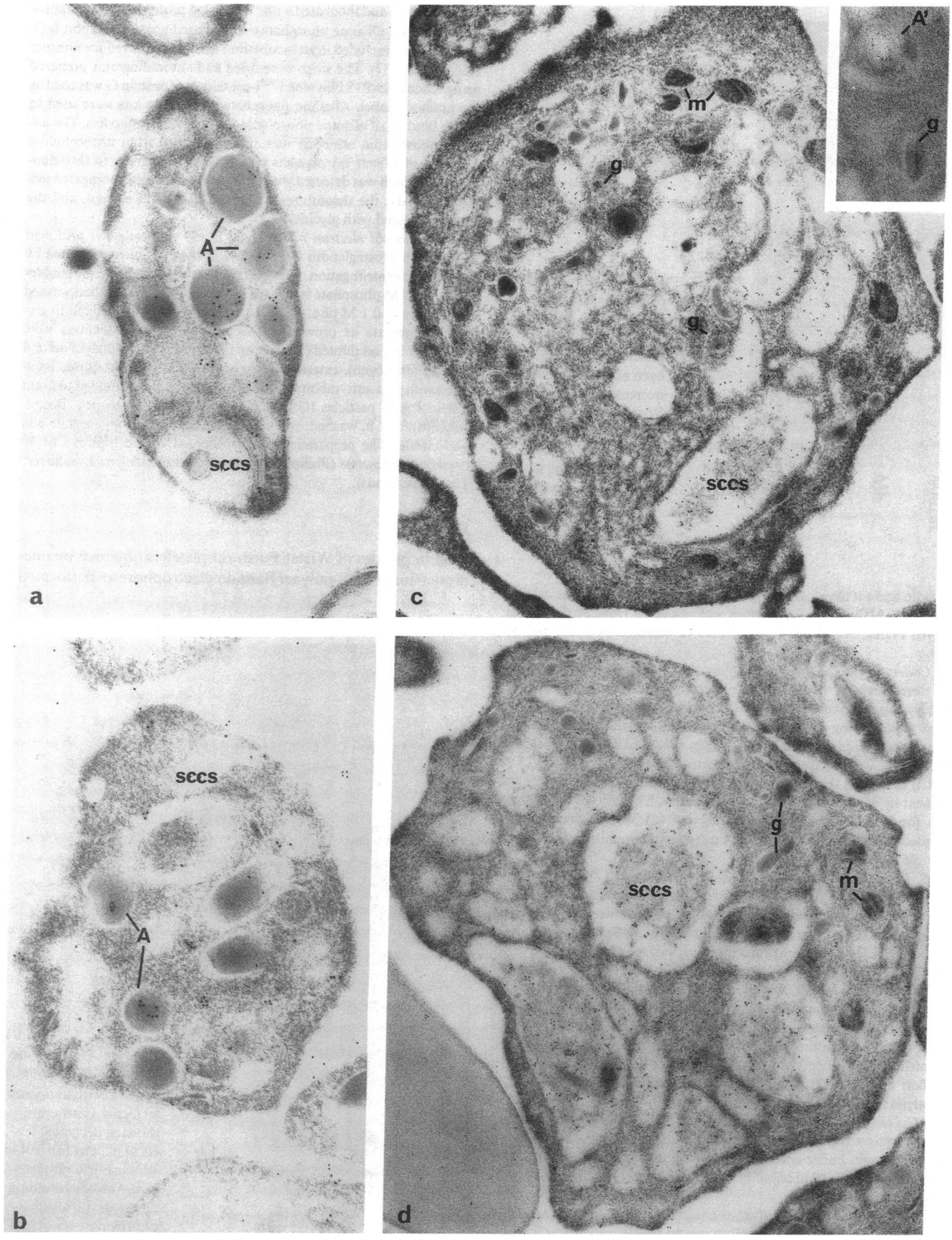


Figure 5. Immunoblot of Wistar and WF rat platelets with antiplatelet factor 4 antiserum showing decreased levels of platelet factor 4 in Wistar Furth rat platelets. Antibody binding detected with alkaline phosphatase-conjugated second antibody. MW, molecular weight.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under reducing conditions showed decreases in five protein bands with an M_r of 185,000, 57,000, 53,000, 16,000, 13,000 and 8,000, respectively, compared with those of Wistar, Sprague-Dawley, and Long Evans rats (Fig. 1). During the course of these studies, one-dimensional analysis of 26 different preparations of WF platelets have been performed: all showed the same protein deficiencies. Assessment of the relative deficiencies of four of these proteins by densitometry revealed individual levels in Wistar Furth platelets of one-fourth to one-half those of Wistar platelets (Table I). The 16,000-, 13,000-, and 8,000-M_r bands also were deficient when WF platelets were electrophoresed under nonreduced conditions (data not shown).

Offspring of matings between Wistar and WF rats had normal platelet size and had normal levels of these platelet proteins. Backcrosses of WF × Wistar hybrid males with WF females yielded offspring with a 1:1 ratio of large mean platelet volume (MPV) phenotype to normal MPV phenotype (1), suggesting that a single allele was responsible for the large platelet phenotype in WF rats. Platelet protein profiles of backcross offspring were therefore examined to determine whether the decreases in platelet proteins detected in WF platelets would be associated with the large MPV phenotype in offspring of these backcrosses. Samples from 41 backcross offspring (17 with large platelet size phenotype and 24 with normal MPV) were examined by one-dimensional SDS-PAGE. All of the protein reductions present in WF platelets were present in platelets of the backcross offspring with large platelet phenotype, but not in backcross offspring with normal platelet size (data not shown).

Proteins with the same electrophoretic mobilities as the proteins deficient in WF platelets (Fig. 1) were released into the supernatant after treatment of washed Wistar and Wistar Furth rat platelets with thrombin, suggesting that they were alpha-granule proteins (Fig. 2). Smaller amounts of these proteins were released from Wistar Furth platelets than from Wistar rat platelets.

Immunoblotting of reduced SDS-PAGE-separated rat platelet proteins with antithrombospondin antibody revealed a deficiency of thrombospondin in Wistar Furth rat platelets, suggesting that the 185-kD protein deficient in Coomassie blue-stained gels of Wistar Furth rat platelets was thrombospondin (Fig. 3). The faint reactivity with a band of an M_r of 25 kD is apparently the heparin binding domain of degraded thrombospondin. Immunoblotting of reduced SDS-PAGE-separated rat platelet proteins with antifibrinogen antibody revealed deficiencies of two bands (M_r of 57 and 53 kD, respectively [Fig. 4]); rat Aα- and Bβ-fibrinogen chains had been reported to migrate with the same M_r in SDS-PAGE gels using the Weber and Osborn buffer system so that the three rat fibrinogen chains were resolved into only two bands (38). We confirmed this finding with purified rat fibrinogen electrophoresed in the Laemmli buffer system. Immunoblotting of Wistar and Wistar Furth platelets with platelet Factor 4 antiserum demonstrated a deficiency of the two molecular weight species of rat platelet factor 4 (39) in Wistar Furth compared with Wistar rat platelets (Fig. 5). This platelet Factor 4 antiserum also detected another minor band intermediate in M_r between the two rat platelet Factor 4 species reported by Greenberg et al. (39), which also was decreased in WF platelets.

Immunogold electron microscopy demonstrated that in Wistar rat platelets, platelet Factor 4 (Fig. 6*a*), and fibrinogen (Fig. 6 *b*) were localized in the matrix of alpha granules. Almost no labeling was found in other platelet structures, i.e., plasma membrane, cytoplasm, surface connected canalicular system, or mitochondria. In Wistar Furth rat platelets, which tended to be much larger, alpha granules, identified by specific immunogold labeling for platelet Factor 4 (Fig. 6*c*) and fibrinogen (Fig. 6*d*), were less numerous and usually smaller than in Wistar rat platelets. Dense material was present in the dilated channels of the surface connected canalicular system of occasional unstimulated Wistar Furth rat platelets (Fig. 6, *c* and *d*), but not in Wistar rat platelets. Immunogold labeling demonstrated that this dense material contained platelet Factor 4 (Fig. 6 *c*) and fibrinogen (Fig. 6 *d*). The small alpha granules of Wistar Furth rat platelets also were positive for these two alpha-granule proteins.

Discussion

Platelets of Wistar Furth rats have abnormally large mean platelet volume with increased platelet size heterogeneity inherited as an autosomal recessive trait (1). Both megakaryocytes and platelets of this rat strain contain ultrastructural abnormalities including large membrane complexes (1, 2) and tubular structures (2). Megakaryocytes show haphazard arrangement of the demarcation membrane system (1, 2) and an uneven distribution of granules (2). In this study, decreases in platelet proteins with an M_r of 185,000, 57,000, 53,000, 16,000, 13,000, and 8,000 were detected by reduced one-dimensional gel electrophoresis. The proteins which were decreased in WF rats appear to be alpha-granule proteins since they were released after thrombin stimulation of Wistar and WF platelets, and the relative amounts of three alpha-granule proteins, thrombospondin, fibrinogen, and platelet Factor 4, were found to be decreased in WF platelets by immunoblotting. Immunoelectron microscopy revealed that alpha granules of Wistar Furth rat platelets tended to be smaller than those of Wistar platelets. In addition, channels of the surface connected canalicular system of unstimulated Wistar Furth rat platelets contained fibrinogen and platelet Factor 4, which were not present in surface connected canalicular system channels of Wistar rat platelets.

Figure 6. Immunogold labeled electron micrographs of Wistar (*a* and *b*) and Wistar Furth (*c* and *d*) rat platelets. (*a*) Wistar rat platelet immunogold labeled for platelet Factor 4; gold particles are present in the alpha granules (*A*) and absent from the channels of the surface connected canalicular system (*SCCS*). Magnification, 25,500. (*b*) Wistar rat platelet immunogold labeled for fibrinogen; gold particles are mainly localized in the alpha granules. SCCS and mitochondria are devoid of labeling. Magnification, 25,500. (*c*) Wistar Furth rat platelet immunogold labeled for platelet Factor 4 with gold particles present within the SCCS and vacuoles. A few small granules (*g*) as well as some partially empty granules of normal size (*A'*) can be identified as abnormal alpha granules by their specific labeling for platelet Factor 4. Mitochondria (*m*) are negative. Magnification, 11,500 (*inset*, 25,500). (*d*) Wistar Furth rat platelet immunogold labeled for fibrinogen shows large fibrinogen deposits in the SCCS, and rare small labeled granules (*g*). Magnification, 11,500.

The platelet characteristics of increased mean platelet volume, reduced platelet number, decreased content of platelet alpha-granule proteins, decreased alpha granule size, and presence of alpha-granule proteins in channels of the platelet surface connected canalicular system of WF rats resemble those of patients with gray platelet syndrome (7–13, 33, 34, 40). In addition, platelets and megakaryocytes of the Wistar Furth rat contain large membrane complexes like those seen in platelets (8, 10, 12, 13) and megakaryocytes (41) of patients with gray platelet syndrome, and described by Breton-Gorius in a number of macrothrombocytopenias (42). Wistar Furth rat platelets also contain large membranous inclusions (2) that have been observed in platelets of patients with gray platelet syndrome (8, 10). The primary difference between platelets of the WF rat and those of patients with gray platelet syndrome resides in the degree of alpha-granule protein deficiency, with the reduction less severe in the WF rat, which probably explains why platelet aggregation to collagen and ADP was not defective (1) in this animal model. Raccuglia (7) and others (8, 10–13) have also reported marked vacuolization of platelets from patients with gray platelet syndrome. Some of these "vacuoles" are alpha granules largely devoid of alpha-granule proteins (13, 40, 43).

Albumin, another alpha-granule protein (40, 44), is only moderately decreased in platelets of patients with gray platelet syndrome as compared with other alpha-granule proteins (33, 40). Albumin was not noticeably decreased in Wistar Furth rat platelets. Why albumin is not decreased to the same extent as other alpha-granule proteins in platelets of WF rats and patients with gray platelet syndrome remains unknown. Recent studies have shown that megakaryocytes, and to a lesser extent platelets, can endocytose plasma proteins such as albumin (45), immunoglobulin G (45), and fibrinogen (45, 46), suggesting that uptake from the plasma by megakaryocytes may be the primary source of these proteins in platelet alpha granules. This endocytosis of plasma proteins shows specificity in that lactoferrin was not incorporated into megakaryocytes, suggesting that this process may be receptor mediated (45). Platelet glycoprotein IIb/IIIa complex, a fibrinogen receptor (47), is found on the internal face of alpha-granule membranes (43) as well as on the plasma membrane and on the surface of the open canalicular system (43), raising the possibility that fibrinogen uptake and delivery to alpha granules may be facilitated by the glycoprotein IIb/IIIa complex. Consistent with this idea, platelet fibrinogen is severely deficient in patients with Type I Glanzmann's thrombasthenia (48, 49), which is characterized by a deficiency of platelet glycoprotein IIb/IIIa complex (50). Belloc et al. have reported that endogenously synthesized platelet fibrinogen is rapidly degraded in patients with Type I Glanzmann's thrombasthenia (51), again suggesting that glycoprotein IIb/IIIa complex plays a role in the transport and/or storage of platelet fibrinogen in alpha granules. However, the presence of these plasma proteins in alpha granules must represent more than just uptake and transport by specific receptors, because the level of fibrinogen in the platelets of the WF rat reported here and of patients with gray platelet syndrome (33) is much lower than that of albumin, in spite of the observation that the level of surface glycoprotein IIb/IIIa complex appears normal both in the WF rat (1) and in patients with gray platelet syndrome (33). Furthermore, glycoprotein IIb/IIIa complex is present on the internal face of the large vacuoles (43), thought to be defective alpha granules (13, 40, 43), in platelets of patients with gray platelet syndrome. The Wistar Furth rat provides an animal model with which to examine why some alpha granule proteins are substantially decreased in gray platelet syndrome, while others are not.

The presence of alpha-granule proteins in channels of the surface connected canalicular system of platelets of patients with gray platelet syndrome has been attributed to leakage or release when these proteins are not properly assembled into alpha granules (13). The presence of alpha-granule proteins in surface connected canalicular system channels of Wistar Furth rat platelets presents an opportunity to analyze this phenomenon in more detail.

Myelofibrosis also has been associated with gray platelet syndrome (10, 41), and is postulated to result from leakage of platelet-derived growth factor and/or platelet Factor 4 from megakaryocytes because of their inability to assemble or retain these proteins in alpha granules (10, 41). Myelofibrosis was not detected in femoral marrow sections of Wistar Furth rats stained and examined for reticulum. Marrows from six one-year-old Wistar Furth rats were evaluated along with marrows from the other three rat strains. This lack of myelofibrosis in Wistar Furth rats may be due to the less severe reduction of platelet alpha-granule proteins in this animal model as compared with that in patients with gray platelet syndrome.

Animal models with deficiencies of platelet dense granules have been reported (52–59); however, to our knowledge, the WF rat represents the first animal model with decreased platelet alpha-granule proteins. The combination of large MPV with reduced platelet number and decreased alpha-granule proteins suggests that formation of alpha-granules, and subdivision of megakaryocyte cytoplasm into platelets employ a common biochemical pathway that is defective in WF rats and patients with gray platelet syndrome. This animal model should be useful in studies aimed at defining this biochemical pathway.

Acknowledgments

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