RAPID COMMUNICATION

CD44 Can Mediate the Adhesion of Platelets to Hyaluronan

By Ichiro Koshiishi, Mehran Shizari, and Charles B. Underhill

CD44 represents a family of glycoproteins that are present on the surfaces of some types of lymphocytes, macrophages, and epithelial cells. In the present study, we have found that CD44 is also present on murine megakaryocytes and peripheral blood platelets as judged by immunohistochemical staining. Western blotting of platelet proteins indicated that this CD44 was predominantly of the 85-kD form. This form of CD44 also had the capacity to bind hyaluronan, because detergent extracts of platelets as well as intact platelets could bind soluble [3H]hyaluronan, and this property was blocked by antibodies directed against CD44. More importantly, isolated platelets could attach to the hyaluronan-containing extracellular matrix produced by cultured rat fibrosarcoma cells. This attachment took place in the absence of divalent cations and could be blocked by pre-treating the rat fibrosarcoma cells with hyaluronidase or by the addition of an antibody to CD44. These results suggested that CD44 was responsible for the attachment of platelets to hyaluronan. Histochemical staining also showed that hyaluronan was present immediately beneath the endothelial cells of many blood vessels of various tissues, such as the dermis, lamina propria of the intestinal tract, the lungs, and the pericardium. Thus, it is possible that CD44 plays an important role in the attachment of platelets to the surface of exposed connective tissue after injury to endothelial cells. © 1994 by The American Society of Hematology.

PLATELETS PERFORM the critical first step in hemostasis, namely the adhesion to exposed extracellular matrix underlying endothelial cells. To perform this step, platelets use different mechanisms depending on the circumstances. Under conditions of high flow rates, such as that found in arterial circulation, platelets adhere to von Willebrand factor attached to subendothelial surfaces. This interaction involves the GPIb-IX glycoprotein on the surfaces of platelets and occurs only under conditions of high shear rate. In contrast, under conditions of low flow rates associated with the venous circulation, receptors of the integrin family mediate the attachment of platelets to proteins in the extracellular matrix such as fibronectin, collagen, and laminin. In addition, nonintegrin receptors for collagen have also been identified. Some of these receptors bind ligands constitutively, whereas others require the activation of the platelets to be effective. Clearly, platelet adhesion is a complex phenomenon that involves a number of cell surface receptors.

In the present study, we present evidence that platelets from mice also express CD44, the receptor for hyaluronan. CD44 represents a family of cell surface glycoproteins ranging in size from 80 to greater than 200 kD that are present on a variety of different cell types, including both leukocytes and epithelial cells. Some, but not all, isoforms of CD44 have the capacity to bind hyaluronan; however, the structural basis for this difference is not clear. For these isoforms, the extracellular domain of CD44 specifically recognizes a six sugar sequence of hyaluronan. This property is responsible for the hyaluronan-dependent adhesion seen in several types of cells. For example, when hyaluronan is added to suspensions of pulmonary macrophages, they are induced to aggregate, and this process can be blocked by antibodies specifically directed against CD44. Similar phenomena occur in a number of other cell types as well. Thus, the fact that murine platelets express CD44 suggests that they too may be able to bind to hyaluronan. The CD44-mediated adhesion to hyaluronan may be an important aspect of platelet function, because hyaluronan is a prominent component of the subendothelial extracellular matrix in some tissues.

MATERIALS AND METHODS

Preparation of biochemicals. KM-201 is a rat monoclonal antibody (MoAb) against mouse CD44, which was originally isolated by Miyake et al. and blocks the interaction between hyaluronan and CD44. The antibody was purified from ascites fluid of nude mice by chromatography on diethyl aminomethyl (DEAE) Affi-gel blue columns as described previously. It was coupled to biotin (b-PG) with sulfo-succinimidyl 6-(biotinamido) hexanoate (Pierce, Rockford, IL) using the methods of Updyke and Nicolson. The rat IgG used as a control was purchased from Sigma (St. Louis, MO) and biotinylated in the same fashion (b-rat IgG).

The binding probe for hyaluronan (b-PG) was prepared from cartilage extracts as described previously. This probe consists of a mixture of the link protein and a fragment of the proteoglycan core protein. Previous studies have shown that the b-PG probe binds to hyaluronan with high affinity and specificity.

The [3H]hyaluronan was prepared using a protocol previously described. For this, rat fibrosarcoma cells were grown in 90% Dulbecco’s modified Eagle’s medium (DMEM), 10% fetal calf serum supplemented with [3H]acetate for 1 day. The conditioned medium was collected, digested with pronase, and dialyzed extensively against distilled water. The [3H]hyaluronan was purified by precipitation with cetylpyridinium chloride and then redissolved in a saline solution. The amount of hyaluronan was determined by a uronic acid assay.

The preparation of [3H]hyaluronan used in this study had a specific activity of 8.9 x 10⁵ cpm/μg Na hyaluronan.

Isolation of platelets. Whole blood was collected from anesthetized mice, and mixed with 1/10 vol of 3.8% Na citrate. After low-speed centrifugation, the supernatant of platelet-rich plasma was carefully removed and mixed with an equal volume of ACD buffer.
(0.13 mol/L NaCl, 20 mmol/L citrate, 1.0 mg/ml glucose, pH 6.0). This was subjected to high-speed centrifugation and the precipitated platelets were resuspended in ACD buffer. The platelet preparation was washed two additional times by centrifugation followed by resuspension in ACD buffer. In some experiments, the platelets were suspended in CMF-Tyrode’s solution containing the following: 137 mmol/L NaCl, 2.7 mmol/L KCl, 11.9 mmol/L NaHCO3, 0.42 mmol/L Na2HPO4, 5.6 mmol/L glucose, 10 mmol/L HEPES, and 0.4 mg/mL bovine serum albumin, pH 7.4.

Histochemistry. The liver and other tissues of a newborn mouse were fixed overnight in a 3.7% formaldehyde solution in saline, then dehydrated in a graded series of ethanol and water solutions, and then embedded in 90% polyester wax, 10% ethanol as described by Kusakabe et al.23 Sections 7-μm thick were cut on a cryostat at 0°C, layered on a water bath, and then taken up on slides subbed with egg albumin. The slides were dried overnight at 4°C and stored at this temperature.

For staining, the sections were rehydrated in a graded series of ethanol and water solutions and then incubated for 5 to 10 minutes in 10% H2O2 to inactivate endogenous peroxidase. The slides were incubated for 1 hour with the primary reagent that was dissolved in 10% calf serum, 90% calcium- and magnesium-free phosphate-buffered saline (CMF-PBS). For the detection of CD44, the primary reagent consisted of 8 μg/mL of b-KM-201, and the background was determined by substituting an equal amount of control b-rat IgG. For the detection of hyaluronan, the primary reagent consisted of 10 μg/mL b-PG and the background was determined by preincipating the b-PG with 100 μg/mL hyaluronan. After thorough washing, the sections were incubated for 20 minutes with peroxidase-labeled streptavidin (Kirkegaard and Perry, Gaithersburg, MD) diluted 1 to 250 in 10% serum, 9% CMF-PBS. Finally, the sections were incubated for 20 to 30 minutes in a peroxidase substrate consisting of 0.03% H2O2, and 0.2 mg/ml 3-amin0-9-ethyl carbazole.22 The sections were counterstained for 1 minute in Mayer’s hematoxylin followed by 0.5 mol/L Tris, pH 8.6, for 5 minutes, and then preserved in Crystal/Mount (Biomedica, Foster City, CA). Coverslips (1.5 weight) were attached using Histomount (National Diagnostics, Highland Park, NJ).

Western blotting. The concentration of protein in the extracts was determined using the bicinecinonic acid method (Pierce). Samples containing 50 μg of protein were precipitated by the addition of 70% ethanol and then redissolved in Laemmli sample buffer in the absence of reducing agents.23 After placing in boiling water for 5 minutes, the samples, along with prestained molecular weight standards (high molecular weight; Bio-Rad, Richmond, CA), were electrophoresed on a 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel. The proteins were then electrophoretically transferred to a sheet of nitrocellulose (Immobilon; Millipore, New Bedford, MA) at 0.9 A for 30 minutes using a transblot apparatus (Idea, Corvallis, OR). The sheet of nitrocellulose was incubated in 5% nonfat milk overnight to block residual protein binding sites. The sheet was then immunostained by incubation in the following solutions: (1) 4 μg/mL of b-KM-201 in 10% calf serum, 90% CMF-PBS containing 0.05% Tween 20 (blotting buffer) for 1 hour; (2) a 1 to 500 dilution of peroxidase-labeled streptavidin in blotting buffer; and (3) a peroxidase substrate consisting of 0.03% H2O2 and 0.2 mg/ml 3-amin0-9-ethyl carbazole in 0.05 mol/L Na acetate, pH 5.0.22

Binding of [3H]hyaluronan. Hyaluronan binding activity was determined on both detergent-extracted and intact platelets. In the case of the detergent-extracted platelets, the binding assay was performed as previously described.14,19 Purified platelets were dissolved in DOC buffer (0.1% Na deoxycholate, 0.5 mol/L NaCl, and 0.02 mol/L Tris, pH 8.0), and 460-μL aliquots of this extract were mixed with 2 μg of [3H]hyaluronan and the volumes were adjusted to 500 μL. After shaking for 20 minutes, 500 μL of saturated (NH4)2SO4 was added to each tube followed by 50 μL of nonfat milk, and the samples were centrifuged at 9,000g for 5 minutes. The tubes were rinsed twice with 50% saturated (NH4)2SO4, and the pellets were dissolved in water and processed for scintillation counting. The background level of binding was determined by including an excess of nonlabeled hyaluronan (200 μg) in the assay mixture. In some experiments, antibodies (KM-201 and nonspecific rat IgG) were included in the assay mixture.

In the case of intact platelets, the binding assay was performed by a modification of a previously described technique.23 The purified platelets were suspended in CMF-Tyrode’s solution and aliquots were distributed into microfuge tubes (1 to 2 × 109 platelets in 500 μL), to which was added 2 μg of [3H]hyaluronan. After 30 minutes of incubation, the samples were centrifuged (9,000g for 2 minutes) and washed twice with the appropriate buffer. The cell pellets were finally solubilized in a detergent solution and processed for scintillation counting. The background level of binding was determined by including a large excess of nonlabeled hyaluronan in the assay mixture. The protein content was determined from representative samples extracted in detergent.

Attachment of platelets to rat fibrosarcoma cells. Rat fibrosarcoma cells that produce a large extracellular coat of hyaluronan were cultured in 90% DMEM, 10% fetal calf serum in an atmosphere of 5% CO2, 95% air.24 The cells were transferred to chambered slides (3 to 5 × 103 cells/cm2, 4 cm2/chamber; Nunc, Naperville, IL) and grown for 2 days before fixation with 3.7% formaldehyde in PBS for 10 minutes. In some cases, the cell surface hyaluronan was digested by incubating the cells with Streptomyces hyaluronidase (117 U/200 μL/chamber; Sigma) at 37°C for 1 hour. Mouse platelets were fluorescently tagged by 5 minutes of incubation in CMF-Tyrode’s solution containing 10 μmol/L 5-(and-6)-carboxy-2′,7′-dichlorofluorescein diacetate succinimidyl ester (Molecular Probes, Eugene, OR).25 The platelets were then centrifuged, washed with ACD solution, and resuspended in CMF-Tyrode’s solution. The platelet suspension was transferred to the chambers (500 μL/chamber) containing the fixed rat fibrosarcoma cells and incubated for 1 hour. In some cases, 100 μg of the KM-201 MoAb was included in the assay. The chambers were gently washed and then fixed with 3.7% formaldehyde in PBS for 10 minutes. The attachment of platelets to the hyaluronan-containing matrix surrounding the rat fibrosarcoma cells was evaluated by fluorescent microscopy.

RESULTS

Histochemical localization of CD44. In the course of an immunohistochemical survey for CD44, we observed that this protein was associated with a group of large cells with lobulated nuclei present in the livers of new-born mice (Fig 1a and b show specific and control staining, respectively). Similar cells were also found in bone marrow smears of adult mice (data not shown). Based on their location and morphology, these cells were identified as megakaryocytes. It was interesting to note that, in these megakaryocytes, the CD44 was not restricted to the cell surface, but was present throughout the cytoplasm. This finding suggests that CD44 may be associated with the demarcation system, an internal network of membranes continuous with the plasma membrane.

The fact that megakaryocytes expressed large amounts of CD44 suggested that platelets derived from these cells may also possess large amounts of CD44. To test this possibility, platelets were isolated from the blood of adult mice and...
histochemically stained for CD44. As shown in Fig 1c, the isolated platelets were uniformly positive for CD44 (Fig 1c and d show both specific and control staining of platelets, respectively).

Physical characteristics of platelet CD44. CD44 comes in a wide variety of isoforms that differ in their molecular weight and other physical characteristics. The isoform of CD44 expressed by platelets was identified by Western blotting. As shown in Fig 2, the majority of CD44 immunoreactivity of platelets was associated with a discrete band of 85 kD. Higher molecular weight isoforms were not observed.

To determine whether this isoform of CD44 could bind hyaluronan, binding studies were conducted on detergent extracts of platelets. Platelets isolated from mouse blood were solubilized in a detergent solution of high ionic strength and then examined for [3H]hyaluronan binding by (NH4)2SO4 precipitation as described in the Materials and Methods. As shown in Fig 3, the extract of platelets did bind the isotopically labeled hyaluronan and this binding was blocked by the addition of either an excess of nonlabeled hyaluronan or by the KM-201 MoAb directed against CD44. However, nonspecific rat IgG had no effect on the binding. Thus, the CD44 present in the platelets was capable of binding hyaluronan.

Binding of platelets to hyaluronan. To determine whether platelets could bind hyaluronan under physiologic conditions, intact platelets were suspended in CMF-Tyrode’s solution and mixed with [3H]hyaluronan. After centrifugation, the amount of label present in the pellet was determined. Figure 4 shows that the intact platelets could bind the [3H]hyaluronan under physiologic conditions, and this binding was again blocked by both nonlabeled hyaluronan and the KM-201 MoAb. It should be noted that the amount of binding was significantly lower than that observed after detergent
solubilization because the assay was performed under conditions of low ionic strength. Previous studies have indicated that binding of hyaluronan is a direct function of the ionic strength of the medium.

We then examined the binding of the platelets to insoluble hyaluronan. For this examination, we used the rat fibrosarcoma cell line that produces a large pericellular coat of hyaluronan. Previous studies have indicated that hyaluronan is covalently linked to the surface of these cells and is retained after fixation with formaldehyde. Figure 5a shows hyaluronan on the surfaces of fixed rat fibrosarcoma cells after staining with the b-PG probe derived from cartilage. Most of this staining is lost after treatment with Streptomyces hyaluronidase (Fig 5b). Interestingly, much of the hyaluronan on the surface of the rat fibrosarcoma cells was associated with microvilli-like structures that projected from the surface of the cells (see arrows in Fig 5a and c). These structures may account for the large extent of pericellular coat associated with these cells.

To measure the binding of platelets, the rat fibrosarcoma cells were grown on slides and then fixed with formaldehyde. Next, platelets that had been labeled with fluorescent dye were added to the fixed rat fibrosarcoma cells and allowed to settle. After 1 hour, the cultures were gently washed and the attached platelets were examined by fluorescent microscopy. Figure 6a shows that a large number of platelets attached to the fixed cultures of rat fibrosarcoma cells, and most of this binding occurred on the microvilli that projected from the surfaces of these cells. However, if the rat fibrosarcoma cells were pretreated with Streptomyces hyaluronidase, then the attachment of platelets was diminished (Fig 6b), suggesting that hyaluronan was required for the attachment. Furthermore, the addition of KM-201 greatly diminished the extent of binding (Fig 6c), whereas the addition of control rat IgG had no apparent effect on this binding (Fig 6d). These results indicate that CD44 mediates the attachment of platelets to hyaluronan on the rat fibrosarcoma cells.

**Distribution of hyaluronan in subendothelial connective tissue.** One important consideration is whether platelets have the opportunity to come in contact with hyaluronan after injury to endothelial cells. To address this issue, we examined the distribution of hyaluronan in a variety of vas-
Fig 5. Hyaluronan containing extracellular matrix surrounding cultured rat fibrosarcoma cells. In each case, the cultured cells were stained with the b-PG probe followed by counterstaining with hematoxylin. (a) Cultured rat fibrosarcoma cells were stained for hyaluronan with the b-PG probe. The arrows point to microvilli-like structures that project from the cell surface. (b) Rat fibrosarcoma cells treated with Streptomyces hyaluronidase show a decreased staining for hyaluronan. (c) A higher magnification view of a cell in (a) showing the microvilli-like structures projecting from the surface of the rat fibrosarcoma cells, which stain positively for hyaluronan (indicated by arrows). Bar = 25 μm.

Fig 7. Distribution of hyaluronan in various tissues. (A) Liver. The arrows point to microvilli-like structures that project from the cell surface. (B) Spleen. The arrow points to a microvilli-like structure. (C) Heart. The arrow points to a microvilli-like structure.
PLATELET ADHESION TO HYALURONAN

Fig 6. The binding of platelets to hyaluronan. Fixed rat fibrosarcoma cells were incubated for 1 hour with fluorescently labeled mouse platelets, rinsed, and then examined under a fluorescent microscope. (a) In the absence of additional treatments, the fluorescently tagged platelets attached to hyaluronan containing matrix surrounding the fixed rat fibrosarcoma cells. (b) Pretreating the rat fibrosarcoma cells with Streptomyces hyaluronidase (117 U/chamber) reduced the attachment of platelets. (c) The addition of the KM-201 MoAb (100 μg/chamber) also reduced the number of attached platelets. (d) The addition of control rat IgG (100 μg/chamber) did not prevent the attachment of platelets to rat fibrosarcoma cells. Bar = 50 μm.

The level of hyaluronan circulating in the blood is maintained at low levels.20-34 Although hyaluronan in the body is turned over at a rapid rate, most of it is removed from the lymph by the lymph nodes before reaching the blood, and that which does enter the blood is rapidly removed by endothelial cells lining the sinusoids of the liver.34 It is possible that this system has evolved to remove hyaluronan, so that it does not interfere with the interaction of platelets with hyaluronan in the subendothelial connective tissue.

Along these lines, Bracey et al35 have described a patient with Wilms' tumor who had abnormally high levels of hyaluronan circulating in the blood (8.5 mg/mL). The platelets of this patient did not function normally, resulting in a severe clotting disorder. This disorder was resolved when the tumor was removed and the level of circulating hyaluronan returned to normal. A similar type of disorder was obtained when rabbits were injected with hyaluronan.35 Although part of the platelet dysfunction was attributed to the viscosity of the hyaluronan, it is possible that an inhibition of the platelet adhesion system could also have been involved.

Fig 7. The distribution of hyaluronan in subendothelial connective tissue. Sections of the (a) skin, (b) intestine, (c) lung, and (d) heart were stained for hyaluronan with the b-PG probe. The arrows in each panel point to the hyaluronan found immediately beneath endothelial cells of veins and venules. In (b), the arteriole of the right has a layer of smooth muscle that is deficient in hyaluronan. Please note that the open spaces surrounding the blood vessels in (a) and (b) are artifacts caused by tissue shrinkage during processing. Bar = 25 μm.
In conclusion, the CD44-mediated adhesion mechanism should be considered as part of a multifunctional system that platelets use to initiate hemostasis. Platelets use different mechanisms of adhesion depending on the flow conditions and the structure of the blood vessel. Given the distribution of hyaluronan, the CD44-mediated adhesion of platelets is most likely to occur in the venous circulation of the skin, intestines, heart, and other organs containing a hyaluronan-rich extracellular matrix.

ACKNOWLEDGMENT

The authors thank Dr Martine Culty and Prasit Pavasant for their help in performing this study.

REFERENCES

33. Amannaka K, Mitsui Y: Hyaluronic acid synthesis is absent in normal human endothelial cells irrespective of hyaluronic acid synthetase inhibitor activity, but is significantly high in transformed cells. Biochim Biophys Acta 1092:336, 1991
CD44 can mediate the adhesion of platelets to hyaluronan

I Koshiishi, M Shizari and CB Underhill