Inhibition of Cell Adhesion by High Molecular Weight Kininogen

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Abstract. An anti-cell adhesion globulin was purified from human plasma by heparin-affinity chromatography. The purified globulin inhibited spreading of osteosarcoma and melanoma cells on vitronectin, and of endothelial cells, platelets, and mononuclear blood cells on vitronectin or fibrinogen. It did not inhibit cell spreading on fibronectin. The protein had the strongest anti-adhesive effect when preadsorbed onto the otherwise adhesive surfaces. Amino acid sequence analysis revealed that the globulin is cleaved (kinin-free) high molecular weight kininogen (HKa). Globulin fractions from normal plasma immunodepleted of high molecular weight kininogen (HK) or from an individual deficient of HK lacked adhesive activity. Uncleaved single-chain HK preadsorbed at neutral pH, HKa preadsorbed at pH > 8.0, and HKa degraded further to release its histidine-rich domain had little anti-adhesive activity. These results indicate that the cationic histidine-rich domain is critical for anti-adhesive activity and is somehow mobilized upon cleavage. Vitronectin was not displaced from the surface by HKa. Thus, cleavage of HK by kallikrein results in both release of bradykinin, a potent vasoactive and growth-promoting peptide, and formation of a potent anti-adhesive protein.

CELL attachment and spreading on extracellular matrices are central events in a variety of biological phenomena such as embryogenesis and organogenesis, tumor metastasis, wound healing, and thrombus formation. These events are recapitulated by in vitro assays in which cells attach and spread on protein-coated surfaces. Such assays have given considerable insight about the molecular basis of cell attachment and spreading and led to the identification of the integrin group of heterodimeric adhesion receptors (Hynes, 1987; Ruoslahti and Pierschbacher, 1987; Hemler, 1990). The integrins interact with specific substrate-adsorbed adhesion proteins, often by an Arg-Gly-Asp (RGD) sequence in the adhesion molecules.

Increasing attention is being paid to the molecular basis of anti-adhesion. Regulatory substances such as transforming growth factor-β and interleukin 8 influence adhesion by changing the expression of cell adhesion receptors by the adhering cell (Gimbrone et al., 1989; Heino and Massagué, 1989). Matrix-associated molecules such as tenascin (Chiquet-Ehrismann et al., 1988; Lotz et al., 1989; Spring et al., 1989), thrombospondin (Lahav, 1988; Murphy-Ullrich and Höök, 1989), and SPARC (Sage et al., 1989) inhibit adhesion when adsorbed to a potentially adhesive substrate. We were interested in whether anti-adhesive molecules other than thrombospondin are generated as a result of blood coagulation. Herein we report the purification and characterization of a potent anti-adhesive globulin from dextran sulfate–treated human plasma. This globulin inhibits adhesion of a variety of cells to vitronectin- or fibrinogen-coated substrates. Amino acid sequence analysis revealed that the globulin is the cleaved (kinin-free) form of high molecular weight kininogen (HK),[1] a well-characterized plasma protein (Takagaki et al., 1985; Kellermann et al., 1986; Müller-Esterl et al., 1986) previously implicated in the "Vroman effect" that controls adsorption to surfaces of adhesive proteins from complex mixtures (Vroman et al., 1980).

Materials and Methods

Materials and Cells

The following chemicals were purchased from Sigma Chemical Co., St. Louis, MO: dextran sulfate, benzamidine HCl, PMSF, aprotinin, barium chloride, ammonium sulfate, EDTA, BSA, leupeptin, and trypsin inhibitor type 1-S from soybean. Polyvinyldene difluoride membranes were purchased from Millipore Corp., Bedford, MA. Fresh frozen human citrated plasma was generously donated by the Badger Red Cross, Madison, WI. ^{125}I and ^{51}Cr were obtained from Du Pont Co. (Wilmington, DE). Iodogen (chloramide-1,3,4,6-tetrachloro-3α-6α-diphenylglycouril) was obtained from Pierce Chemical Co., Rockford, IL. Gelbond film was obtained from FMC Corp., Rockland, ME. CNBr-activated Sepharose CL-4B and heparin-Sepharose was obtained from Pharmacia Company, Uppsala, Sweden. Liquid chromatography columns DEAE-5PW and 300SW were from Waters/Millipore (Bedford, MA).

Human vitronectin, fibronectin, fibrinogen, and thrombospondin were purified by the methods of Dahlbäck and Podack (1985), Mosher and Johnson (1983), Mosher and Blout (1973), and Murphy-Ullrich and Mosher (1985), respectively. Antithrombin III and histidine-rich glycoprotein were obtained as byproducts of heparin-affinity chromatography described below. Platelet factor 4 was a byproduct of purification of thrombospondin

1. *Abbreviations used in this paper*: HK, high molecular weight kininogen; HKa, kinin-free HK; HKi, HKa missing its histidine-rich domain.

purification (Murphy-Ullrich and Mosher, 1985). Von Willebrand factor was purified according to the method of van Mourik and Mochtar (1970). Kinin-free high molecular weight kininogen and low molecular weight kininogen were purified by the published methods of Ohkubo et al. (1984, 1988) and Higashiyama et al. (1986). The proteins appeared >95% homogeneous by SDS-PAGE with or without reduction followed by staining with Coomassie brilliant blue.

The following cells and culture media were used: MG-63 human osteosarcoma and C-32 human melanoma cells, MEM with 10% FCS; G-361 human melanoma cells, McCoy's medium with 10% calf serum; human foreskin fibroblast, F12 with 10% FCS; and bovine aortic endothelial cells, DME with 15% FCS. Cell lines were obtained from the American Type Culture Collection (Rockville, MD). Strains of human foreskin fibroblasts were established locally. Human peripheral mononuclear cells were isolated by the method of Schwartz and Edgington (1981). Human platelets were isolated by differential centrifugation and suspended in a divalent cation-free modified Tyrode's-Hepes buffer (Schafer et al., 1986). Platelets and mononuclear cells were labeled with ^{51}Cr before use in adhesion assays (Santoro, 1986).

Purification of Anti-Cell Adhesion Globulin (HKa)

280 ml fresh-frozen human citrated plasma was thawed at 37°C. Immediately upon thawing, dextran sulfate was added to a final concentration of 0.1% and stirred for 10 min. Then the following protease inhibitors were added to the following concentrations: benzamidine (3 mM), PMSF (2 mM), aprotinin (10 kallikrein inhibitor units/ml), and EDTA (4 mM). Subsequent steps were performed at room temperature in plastic containers. 1 M barium chloride was added to a concentration of 90 ml/liter, and adsorbed proteins were removed by centrifugation. Barium chloride-adsorbed plasma was precipitated with ammonium sulfate (30–60% saturation). The precipitate was collected by centrifugation and resuspended and dialyzed against 50 mM phosphate buffer, pH 6.3, containing 0.4 M sodium chloride, 0.1 mM PMSF, and 0.5 M EDTA (buffer A) before applying to a heparin agarose column (2.5 × 10 cm) (Fig. 1 *A*). After extensive washing with buffer A without inhibitors, bound proteins were eluted with a 0.4 liter linear gradient from 0.4 to 1.2 M sodium chloride. Fractions with peak anti-adhesive activity were pooled and extensively dialyzed with 50 mM Tris HCl, 0.02 M sodium chloride, 0.01 M citric acid, pH 8.3. The concentrated pool was applied to a DEAE-5PW column in the Waters 650 system (Waters Instruments, Inc., Rochester, MN). Proteins were eluted with a linear gradient (as described in Fig. 1 *B*). Fractions containing anti-cell spreading activity were pooled, concentrated after extensive dialysis against 20 mM Tris, 150 mM sodium chloride, pH 7.4 (TBS), and stored at −120°C until use.

Purification of HK

The protease inhibitors listed above along with soybean trypsin inhibitor (50 μg/ml) and leupeptin (0.5 μg/ml) were added immediately upon thawing. No dextran sulfate was added. Further purification steps were the same as those described above except that 0.1 mM EDTA and 0.1 mM PMSF were added to all buffers. After purification, fractions containing 140- and 120-kD proteins (as determined by SDS-PAGE) were pooled, extensively dialyzed with TBS, and stored at −120°C until use.

Purification of Degraded Anti-Cell Adhesion Globulin (HKi)

Fresh-frozen human citrated plasma was processed and ammonium sulfate precipitation, heparin-Sepharose chromatography, and DEAE chromatography were performed as described for HKa above except that protease inhibitors were omitted and the steps were done at 4°C. After DEAE chromatography, the anti-cell adhesive fraction was dialyzed against 50 mM phosphate, pH 6.4, 0.4 M sodium chloride, and then reapplied to heparin-Sepharose equilibrated in the same buffer. The heparin-Sepharose unbound fractions that contained 84-kD two-chain protein were extensively dialyzed with TBS, pH 7.4. Aliquots of degraded globulin were stored at −120°C until use.

Purification of Anti-Cell Adhesion Globulin by One-Step Chromatography on a Large Heparin-Sepharose Column

Fresh-frozen human citrated plasma was thawed and treated with dextran sulfate and protease inhibitors as described above. The dialyzed ammonium sulfate globulin fraction was applied to heparin-Sepharose (2.6 × 40 cm) and eluted with a 0.75 liter linear gradient from 0.4 to 1.0 M sodium chloride. Anti-cell adhesive globulin eluted with 0.75 M sodium chloride. Active fractions were extensively dialyzed with TBS and then aliquoted and frozen at −120°C until use.

Amino-terminal Sequence Analysis

Purified globulin was separated on SDS-PAGE without reduction and transferred to a polyvinyldene difluoride membrane. The 105-kD band was cut out and directly subjected to automated amino acid sequence analysis with a protein sequencer (model 470; Applied Biosystems, Inc., Foster City, CA).

Electroimmunoassay of Anti-Cell Adhesion Globulin

Antiserum against the purified globulin was produced in rabbits by standard techniques (Harlow and Lane, 1988). The serum was heat-treated at 56°C for 1 h and adsorbed by the globulins that did not bind to heparin-agarose. The IgG fraction from the adsorbed antiserum was purified by 33% ammonium sulfate precipitation followed by chromatography on DEAE-Sepharose. The specificities of the antibodies were analyzed by immunodiffusion, immunoelectrophoresis, and crossed immunoelectrophoresis (Laurell, 1965). The concentration of purified globulin was estimated by Laurell's "rocket" electroimmunoassay (Laurell, 1966).

HK-depleted Plasma Globulins

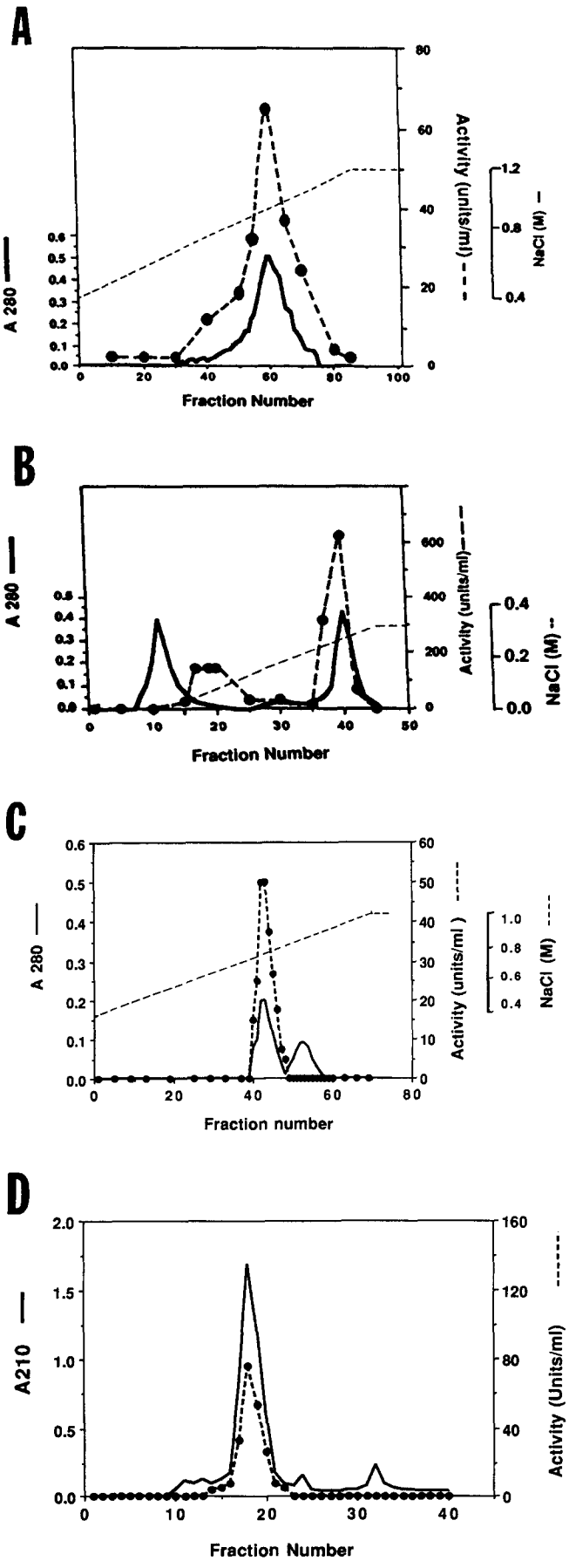
HK-depleted globulins were produced by using an affinity column containing immobilized mAb L-7 to the light chain of HK (Ishiguro et al., 1987). The affinity column was prepared by coupling L-7 to CNBr-activated Sepharose (2 mg of antibody/ml resin) as recommended by the manufacturer (Pharmacia Fine Chemicals, Piscataway, NJ). A 30–60% ammonium sulfate fraction of normal human plasma (0.5 ml) was applied to the affinity column. The unadsorbed fraction was passed over the column a second time. The unbound fraction contained no detectable antigen as assessed by immunoblotting using polyclonal antibodies described above to the anti-cell adhesive globulin. Heparin-Sepharose was used to deplete globulins of heparin-binding proteins as described above. A globulin fraction was also made from plasma from an individual lacking HK (George King Biomedical, Overland Park, KS).

Transelution of Antibodies and Western Immunoblotting

Some purified globulin fractions contained two bands (105 and 95 kD) on nonreduced SDS-PAGE gel. Both proteins were blotted to nitrocellulose paper after SDS-PAGE. Rabbit polyclonal anti-cell adhesive globulin IgGs were incubated with nitrocellulose at 4°C overnight. After washing, antibodies against individual proteins were eluted from nitrocellulose by 0.2 M glycine HCl, pH 2.7 (Olmsted, 1987). After 1 min incubation, 1 M sodium hydroxide was added to neutralize the solution to pH 7.4 and the antibodies were extensively dialyzed with TBS. Eluted antibodies were then used to probe fresh nitrocellulose paper containing 105- and 95-kD proteins.

Cell Adhesion Assays

Cell attachment and cell spreading were measured by using the method of Grinnell (1976) with some modification. 2-cm^2 well (24-well plates; Costar Corp., Cambridge, MA) polystyrene plates were coated with 2 μg vitronectin or heat-denatured BSA (400 μl protein solution in TBS/well). Plates were coated for 3 h at 37°C and then blocked with 1 ml of 1% heat-denatured BSA in TBS for 1 h at 37°C. Plates were washed with TBS/0.2% BSA several times. MG-63 osteosarcoma or other cells were released from stock monolayers by incubation for 5 min at 37°C with trypsin (1 mg/ml) in 20 mM sodium phosphate, 135 mM sodium chloride, 0.5 mM EDTA, pH 7.4. Trypsinization was stopped with the addition of trypsin inhibitor to a final concentration of 0.5 mg/ml. The cells were pelleted twice and then resuspended in DME containing 0.2% BSA. 0.3-ml aliquots of suspended cells (3.5×10^4 cells/ml) were pipetted into coated wells, and cells were allowed to adhere to the vitronectin- or BSA-coated surface for 60–90 min at 37°C. Plates were examined by phase microscopy and sometimes photographed. Nonadherent cells were removed by washing with TBS. Adherent cells were fixed with 3% paraformaldehyde solution for 1 h and stained with 0.1% amido black solution. The number of attached cells and attached and spread cells were determined visually with a microscope equipped with



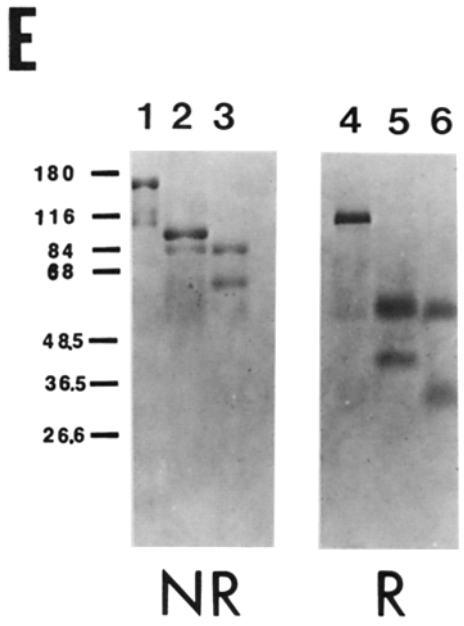


Figure 1. Chromatographic separation of the anti-cell adhesive molecule. (*A*) Heparin-Sepharose chromatography. A 30–60% saturated ammonium sulfate globulin fraction of plasma was subjected to chromatography on heparin-agarose (2.5 × 10 cm). Bound proteins were eluted with a 0.4-liter linear salt gradient from 0.4 to 1.2 M sodium chloride. The flow rate was maintained at 100 ml/h, and 3.5-ml fractions were collected. *Thick solid line*, protein concentration; *thick dotted line*, anti-cell adhesive activity; *thin dotted line*, salt gradient. The first (small) peak of activity was not seen constantly or analyzed further. (*B*) FPLC (DEAE) chromatography. Fractions with high anti-cell adhesive activity in the heparin-Sepharose chromatogram were pooled and subjected to FPLC on a DEAE column (0.8 × 7.5 cm). Proteins were eluted with a 30-ml linear salt gradient from 0.02 to 0.32 M sodium chloride. The flow rate was maintained at 1 ml/ml. *Thick solid line*, protein concentration; *thick dotted line*, anti-cell adhesive activity; *thin dotted line*, salt gradient. (*C*) Large heparin-Sepharose chromatography (one-step purification). A 30–60% ammonium sulfate globulin fraction of plasma was subjected to chromatography on large-scale heparin-Sepharose (2.6 × 40 cm). Proteins were eluted with 0.75-liter linear gradient from 0.4 to 1.0 M sodium chloride. The flow rate was maintained at 150 ml/h, and 10-ml fractions were collected. When higher salt concentrations were used to strip the column, additional protein, mostly antithrombin III, was eluted (not shown). *Thick solid line*, protein concentration; *thick dotted line*, anti-cell adhesive activity; *thin dotted line*, salt gradient. (*D*) 300SW gel filtration chromatography. The peak activity fraction in DEAE chromatography was applied to 300SW gel filtration column (0.8 × 30 cm) equilibrated with 50 mM Tris HCl, 0.15 M sodium chloride, pH 7.4. 1-ml fractions were collected. Absorbance was detected at A_{210}. *Thick solid line*, protein concentration; *thick dotted line*, anti-cell adhesive activity. (*E*) SDS-PAGE of HK (lanes *1* and *4*), the anti-cell adhesion globulin (HKa) purified by the two-step method (lanes *2* and *5*), and degraded HKa (lanes *3* and *6*) under nonreducing (*NR*) and reducing conditions (*R*) with 8% polyacrylamide.

phase contrast objectives. We counted the cells in five areas randomly chosen in the central and peripheral regions in 2-cm^2 wells. Duplicate wells were counted for each sample. Spread cells were designated as those that were polygonal and dark as determined by phase microscopy (see Fig. 3). Cell spreading was routinely determined visually. There was little ambiguity as to whether a given cell was spread or not and little variation (<10–20%) between duplicate wells or in numbers obtained by different observers.

To validate this subjective method, in some experiments photographs were taken at 200× and analyzed by a computer image processing package (IMAGE 1®; Universal Imaging Corp., West Chester, PA) to determine cell area and diameter in collaboration with Horng-Ban Lin and Stuart Cooper of the Department of Chemical Engineering, University of Wisconsin (Madison, WI). The mean ± SD area of spread MG-63 cells on a vitronectin-coated surface was 365 ± 81 μm^2. Cells that were attached but not spread had an image area of 148 ± 28 μm^2.

1 U of anti-spreading activity in serial dilution assays was defined as the amount of factor required for 50% inhibition of spreading of MG-63 cells. The basic assay was modified to study effects of protein preadsorption, cations, etc., as explained in the text.

Adhesion of platelets and mononuclear cells was quantified using ^{51}Cr (Santoro, 1986).

Iodination of Proteins

Vitronectin was radioiodinated by the Iodogen method (Fraker and Speck, 1978). The specific radioactivity is 0.45 $\mu Ci/\mu g$. Preparations were >96% precipitable by 10% TCA, and nonreduced labeled protein migrated as a 75-kD single band under non-reducing conditions and as 75- and 65-kD single bands under reduced conditions when analyzed by SDS-PAGE and autoradiography.

Analyses of Vitronectin on Surfaces Treated with Heparin-binding Proteins

Polystyrene microtiter plates for tissue culture were coated with vitronectin at concentrations up to 2 μg/ml in TBS for 12 h at 4°C and then blocked with TBS containing 1% BSA. The wells were rinsed three times with TBS, and then various heparin-binding proteins (100 μl in TBS) were added and incubated for 1 h at room temperature. The wells were rinsed three times with TBS, and then 200 μl of rabbit antiserum (1:1,000 dilution in TBS/0.1% BSA) raised against human native vitronectin was added to the well for 2 h at 37°C. Plates were washed three times with TBS and incubated with 200 μl/well of a 1:1,000 dilution of goat anti-rabbit IgG conjugated to alkaline phosphatase in TBS. Plates were washed and incubated at 37°C with phosphatase substrate until maximum absorbance (in the wells coated with 2 μg/ml vitronectin with no additional treatment) was at least 0.7. Data are expressed as a percent of the maximum absorbance obtained in each assay and represent the mean of quadruplicate determinations. Parallel cell adhesion experiments were performed to relate retained vitronectin antigen to cell adhesive activity.

Polystyrene microtiter plates for tissue culture were coated with a mixture of nonradiolabeled vitronectin (2 μg/ml) and ^{125}I-radiolabeled vitronectin (1.0×10^5 cpm/well) for 12 h at 4°C, and blocked with TBS containing 1% BSA. After rinsing three times with TBS, 100 μl of heparin-binding proteins (10 μg/ml) were added to the wells. After 1 h at 37°C the supernatant was removed and the wells were washed. Bound protein was solubilized by incubation with 2% SDS, 1 M sodium hydroxide for 24 h at room temperature. Radioactivity was quantitated using a gamma counter. Data represent the average of six determinations.

Results

Purification and Characterization of an Anti-Cell Adhesion Molecule Globulin from Plasma

To search for anti-adhesive proteins in plasma, it is necessary to remove the adhesive proteins first. This was accomplished by preparation of globulins precipitated by 30–60% saturated ammonium sulfate: fibronectin is precipitated by <30% saturated ammonium sulfate (Mosher and Blout, 1973), whereas vitronectin requires 70–85% saturated ammonium sulfate to be precipitated (Dählback and Podack, 1985; Preissner et al., 1985). We also began with plasma that had been treated with dextran sulfate to activate the intrinsic blood coagulation system (Fujikawa et al., 1980).

An anti-cell adhesive molecule was purified from a globulin fraction of dextran sulfate-treated human plasma (Table I). When the globulin fraction was separated on heparin-Sepharose at pH 6.4, anti-adhesive activity toward human MG-63 osteosarcoma cells was eluted by 0.8 M sodium chloride in fractions that also contained histidine-rich glycoprotein and antithrombin III (Fig. 1 *A*). These fractions were separated further using a DEAE column at pH 8.3 (Fig. 1 *B*). Histidine-rich glycoprotein and antithrombin III eluted before the anti-cell adhesion molecule. The increase in specific activity from the globulin fraction was 625-fold (Table I). Although we could not measure anti-cell adhesion activity in whole plasma, no activity could be detected in globulins that did not bind to heparin-Sepharose (Table II). In subsequent purifications, it was found that anti-cell adhesive globulin could be separated cleanly from histidine-rich glycoprotein and antithrombin III in a single chromatographic step by using a larger capacity heparin-Sepharose column and a shallower salt gradient (Fig. 1 *C*). When the peak fraction from the DEAE column in the two-column purification was applied to a 300SW filtration column, protein and activity peaks coincided (Fig. 1 *D*).

Analysis by SDS-PAGE of anti-cell adhesive globulin purified by the two-step method revealed a major 95-kD band and a minor 84-kD band under nonreducing condition and 62- and 44-kD bands under reducing condition (Fig. 1 *E*). The purified globulin migrated in the α_1 zone in agarose gel electrophoresis (not shown). We purified a major 105-kD band and a minor 95-kD band under nonreducing conditions when protease inhibitors were added to the buffer of DEAE column. The 105- and 95-kD proteins could be separated on a sulphopropyl column at pH 6.5, with the 95-kD protein eluting later in a salt gradient. Peptide mapping by digestion with V8 protease and gel electrophoresis as described by Cleveland et al. (1977) revealed that the cleavage patterns of the 105- and 95-kD proteins were almost identical (not shown). Antibodies were elicited in rabbits to proteins eluted from the 105- and 95-kD positions. In immunoblots of nonreduced SDS-treated human plasma, these antibodies recognized a major band of 140 kD, a minor band of 105 kD, and a major band of 60 kD (not shown). Adsorption of the antibodies with the human globulins that did not bind to heparin-Sepharose removed the antibodies that reacted with the 60-kD band. Antibodies to the 95- and 105-kD bands reacted with one another and also with the 140-kD band when analyzed by the transelution technique (Olmsted, 1987; Thelu, 1988).

The Anti-Cell Adhesion Globulin Is Kinin-free HK

Estimates of the plasma concentration of the globulin as determined by Laurell's electroimmunoassay (Laurell, 1966) were of the order of 50–70 μg/ml, making it likely that this globulin was an already described plasma protein. We therefore carried out microsequencing of ~100 pmol of the 105-kD band. A single sequence, Ser-Ser-Arg-Ile-Gly-Glu-Ile-Lys, was obtained. This corresponds to the amino terminus of the light chain of kinin-free HK; i.e., HKa or HK that has been cleaved twice by kallikrein to release bradykinin and

Table I. Purification of an Anti-Cell Adhesion Globulin from Human Plasma

Fraction	Total protein	Total units*	Specific activity	% of recovery	Fold purification
	mg		*U/mg*		
Plasma	15,176	<280‡	<0.02‡	–‡	–‡
30–60% saturated $(NH_4)_2SO_4$ fraction	1,587	3,228	2.0	[100]‡	[1.0]‡
Heparin-Sepharose bound	8.75	3,059	350	94	175
FPLC (DEAE) peak	1.80	2,248	1,249	70	625

* A unit (U) was defined as the amount in 1 ml required for 50% inhibition of cell spreading.
‡ We could not measure inhibition of cell adhesion in whole plasma and therefore have related the purification to the globulin fraction.

create a two-chain protein held together by a disulfide bond (Takagaki et al., 1985; Kellermann et al., 1986; in the numbering system for the mature protein of Kellermann et al. [1986] the observed sequence begins at Ser 372). The amino terminus of single-chain HK and of the heavy chain of HKa is blocked by pyroglutamic acid (Kellermann et al., 1986) and would not give rise to a detectable sequence. All of the original data on the purified globulin are compatible with its identity to kinin-free HK (HKa) (Fig. 2). Treatment of plasma with dextran sulfate is known to cause activation of kallikrein and release of bradykinin from HK (Fujikawa et al., 1980). The 140-kD band recognized by our antibodies in blots of untreated plasma corresponds to single-chain HK. The major 60-kD band in untreated plasma recognized by our unabsorbed antibodies corresponds to low molecular weight kininogen (LK), a second product generated by differential splicing of the kininogen gene (Takagaki et al., 1985). The 95-kD band probably corresponds to an additional cleavage between Arg 418 and Lys 419 (Tait and Fujikawa, 1986).

To corroborate further the identity between our purified globulin and HKa, we found that human HKa purified by a published method (Ohkubo et al., 1984, 1988) had anti-cell adhesion activity and that anti-HK antibodies kindly given to us by John Griffin of the Research Institute of Scripps Clinic (La Jolla, CA) recognized our purified globulin in immunoblots (not shown). Also, we found that globulins produced from plasma from an individual deficient in HK or immunodepleted of HK by a mAb (Ishiguro et al., 1987) lacked anti-cell adhesive activity (Table II).

Table II. Anti-Cell Adhesive Activity of 30–60% Saturated Ammonium Sulfate Globulin Fraction

Proteins	Concentration	Cell spreading
	μg/ml	*cells/15 mm²*
Globulin from normal plasma	370	<1
Globulin depleted of heparin-binding protein	370	375 ± 5
Globulin immunodepleted of HK	370	390 ± 5
Globulin from HK-deficient plasma	370	357 ± 7
Bovine albumin	370	384 ± 6
Low molecular weight kininogen	10	382 ± 2
HKa	10	<1

The proteins were tested in a 1-h adhesion assay to vitronectin-coated wells. Values represent mean ± range of duplicated wells. LK, low molecular weight kininogen.

Further Characterization of the Anti-adhesive Activity of HKa

Our principal model system was the MG-63 cell line from which integrin adhesion receptors for fibronectin (Pytela et al., 1985*a*) and vitronectin (Pytela et al., 1985*b*) were characterized. Spreading of these cells on vitronectin-coated tissue culture plastic was prompt and robust (Fig. 3 *A*). In the presence of HKa, 1–10 μg/ml, cells were rounded (Fig. 3 *B*). After washing, few cells remained attached to the substratum when HKa had been present.

To elucidate the mechanism(s) of these effects, HKa was introduced into the cell adhesion assay in three different ways (Fig. 4). First, vitronectin-coated wells, after blocking with

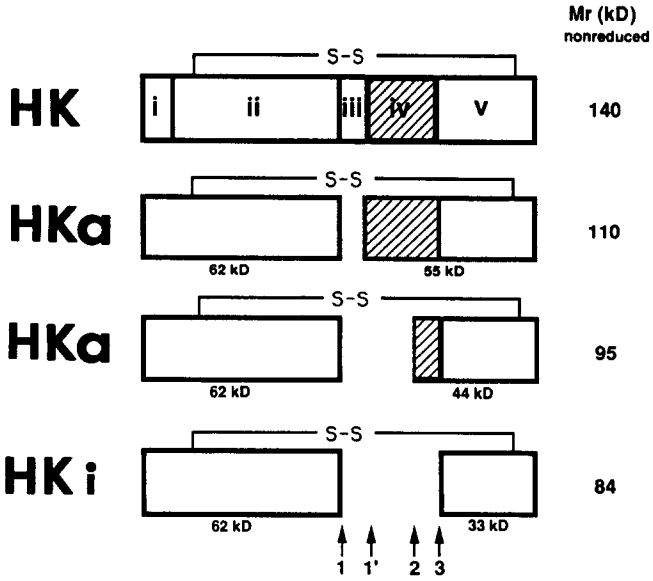


Figure 2. Schematic of the various forms of HK. The domains, *i–v*, of intact HK are described in the Discussion. Cleavages 1 and 1' by kallikrein release bradykinin and produce the two-chain active HKa molecule. Kallikrein also cleaves at 2 without loss of anti-cell adhesive activity. Cleavage at 3, presumably by Factor XIa, releases the histidine-rich domain and results in loss of anti-cell adhesion activity. This model has been adapted from Scott et al. (1985).

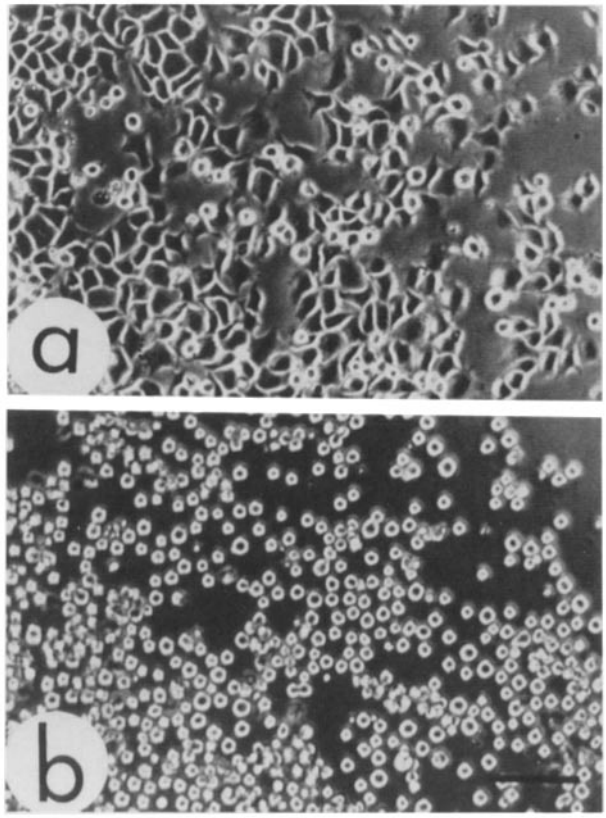


Figure 3. Spreading of Mg-63 cells. Wells were coated with vitronectin as described in Materials and Methods. MG-63 cells in medium containing 0.2% BSA were incubated with vitronectin-coated wells in the absence (*a*) or presence (*b*) of 10 μg/ml HKa. After 60 min incubation at 37°C, cells were photographed. Few of the round cells in *b* remained when the plate was washed, whereas those in *a* were not washed away. Bar in lower right corner of *b*, 40 μm.

0.2% albumin, were incubated for 1 h with various concentrations of HKa, after which the HKa-containing solutions were removed and cells were added. Second, cells and various concentrations of HKa were added simultaneously to the vitronectin-coated wells. Third, cells and various concentrations of HKa were incubated together for 1 h and added to vitronectin-coated wells. HKa was most effective when preincubated with the vitronectin-coated wells (Fig. 4). Preincubation of HKa with cells also resulted in greater anti-adhesive activity than if HKa and cells were added together to the vitronectin substratum.

A study was performed to learn the minimal time of conditioning surfaces with HKa. Cell spreading was inhibited in vitronectin-coated wells exposed to HKa, 10 μg/ml, for as little as 5 s (Fig. 5). Exposure of the wells to HKa before coating with vitronectin also resulted in a surface that did not support cell adhesion (Fig. 6). Histidine-rich glycoprotein and antithrombin III, two other heparin-binding proteins, did not inhibit cell adhesion to vitronectin, either when added in soluble form to adhesion assays (Fig. 1 *B* and additional experiments, not shown, with purified proteins) or when used to coat wells (Fig. 6). As a further test of specificity, it was found that incubation of HKa with polyclonal immune IgGs before addition to adhesion assays neutralized the anti-adhesive activity of HKa (Fig. 7).

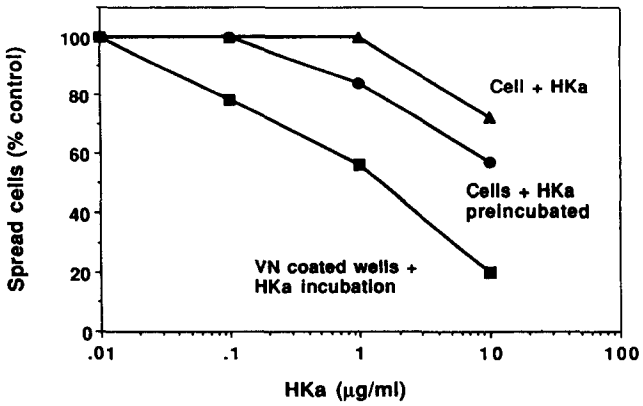


Figure 4. Effect of assay conditions on HKa-dependent anti-cell adhesion. Anti-cell adhesion activity was analyzed in assays in which varying concentrations of HKa were introduced by the following protocols: MG-63 cells and HKa were added simultaneously to vitronectin-coated wells (▲); cells were preincubated for 60 min with HKa and added to vitronectin-coated wells (●); and HKa was preincubated with vitronectin-coated wells and, after washing the wells with TBS, cells were added to the wells (■).

Comparison of the Anti-adhesive Activities of HKa, Uncleaved HK, and HKi

The anti-adhesive activity of HKa was compared with the activities of HK, the single chain precursor of HKa (140- and 120-kD bands under nonreducing conditions and 120-kD band under reducing conditions), and an 84-kD two-chain degraded form of HKa (HKi). SDS-PAGE analyses of these proteins are shown in Fig. 1 *E*. HK was purified in the presence of protease inhibitors. Even so, ~10% of purified HK was degraded to the two-chain form (Fig. 1 *E*) as previously described (Ohkubo et al., 1988). The 84-kD HKi protein arose spontaneously if protease inhibitors were not included. It did not bind to heparin-Sepharose and therefore could be separated easily from HKa by heparin-affinity chromatography. We could not obtain reliable sequence data on the degradation product but we think that the 84-kD protein probably arose from cleavage of HKa between Lys 523 and Thr 524, a hypothesis compatible with the observed reduction in the size of the light chain (Fig. 1 *E*), known glycosyla-

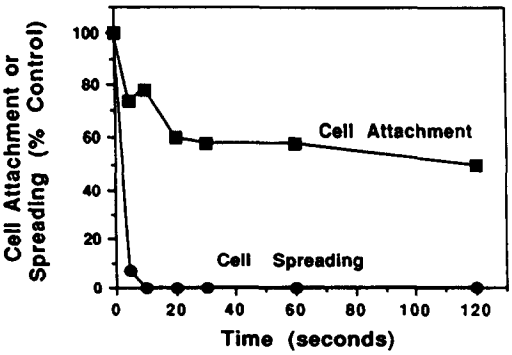


Figure 5. Effect of precoating time on HKa activity. Vitronectin-coated wells were preincubated with HKa (10 μg/ml) for various lengths of time. Plates were washed with TBS, then blocked with TBS containing 2% BSA. MG-63 cells were added in medium containing 0.2% BSA. Cell attachment (■) and cell spreading (●) were ascertained after 60 min incubation at 37°C.

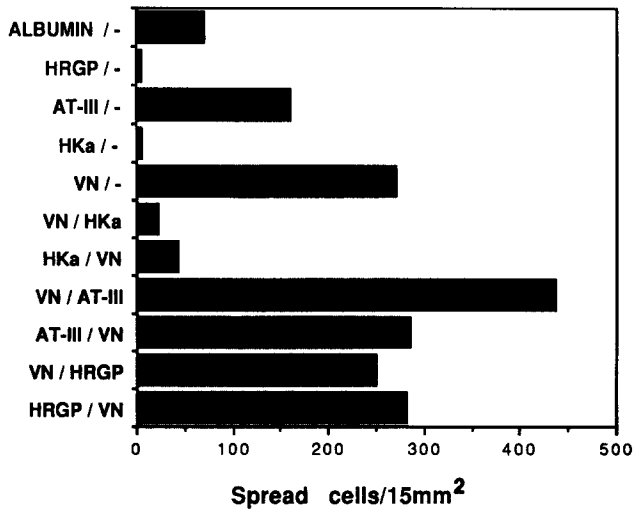


Figure 6. Comparison of the effects of HKa and other heparin-binding globulins on cell adhesion. Vitronectin and/or other proteins were adsorbed to wells. VN/HKa indicates that vitronectin (2 μg/ml) was incubated in the well, and the well was then blocked with 2% BSA, washed with TBS, and incubated with HKa (10 μg/ml). After a 1-h incubation, wells were washed with TBS, MG-63 cells (8 × 10^3 cells) were added, and spread cells were counted. HKa/VN indicates that HKa and vitronectin were adsorbed in the reverse order. Control heparin binding proteins, antithrombin III (AT III, 10 μg/ml) and HRGP (10 μg/ml) were also tested, either alone or in sequential adsorptions. (−) indicates that no second protein was adsorbed. Bars represent means of duplicate wells; all duplicates agreed to within 15% of values shown.

tion of Thr 524 (Kellerman et al., 1986) that would make sequencing difficult, and the previous characterization of a similar degradation product (Kato et al., 1981; Ohkubo et al., 1988). The missing sequence, Ser 372-Lys 523, is extremely rich in histidine and also contains short stretches with multiple arginines and lysines, explaining the loss of affinity for heparin (Takagaki et al., 1985; Kellermann et al., 1986). LK, which lacks the light chain completely, had no anti-adhesive activity (Table II).

MG-63 cells spread vigorously on vitronectin-coated wells preincubated at pH 7.4 with HKi using conditions in which cell spreading was inhibited by HKa (Fig. 8). Intact HK inhibited cell adhesion to vitronectin but was not as active as HKa (Fig. 8). Further investigation revealed that the pH at which HKa and HK were preincubated with the wells had a profound effect on subsequent anti-adhesive activity (Fig. 9). Vitronectin-coated wells conditioned with HK or

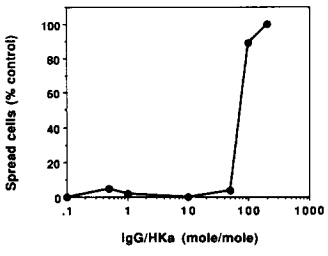


Figure 7. Neutralization of anti-cell adhesive activity with polyclonal antibody against HKa. HKa (10 μg/ml) was incubated at 22°C for 30 min with the IgG fraction of rabbit anti-HKa at a molar ratio of 0.5:1 to 200:1. The mixtures were incubated with the vitronectin substratum. MG-63 cells were added and spread cells were quantified. Note loss of HKa activity (return of cell spreading to control values) when IgGs were present at 100–200 molar excess.

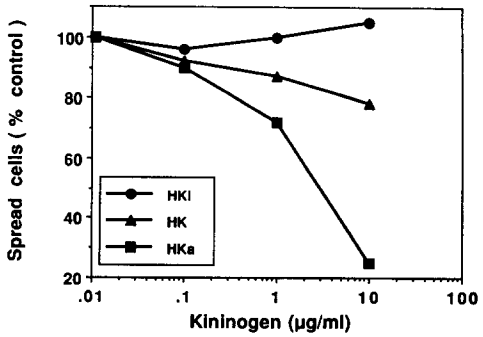


Figure 8. Effect of HK, HKa, and HKi on cell adhesion of MG-63 cells to vitronectin. Vitronectin (2 μg/ml) was coated onto wells, and, after blocking with 1% BSA in TBS, various concentrations of HK (▲), HKa (■), or HKi (●)were added followed by MG-63 cells in DME/0.2% BSA. Cell spreading was quantified at 1 h.

HKa at pH <7.0 did not support cell spreading; i.e., HK or HKa were active inhibitors of adhesion. Coating with HK at pH >7.6 and with HKa at pH >8.0 resulted in wells with full adhesive activity. The pH dependency suggests that histidines are important for expression of anti-adhesive activity and is consistent with the critical role of the histidine-rich region suggested by the experiments described above.

Effects of HKa on the Attachment and Spreading of Various Cells on Various Substrates

Cell spreading of bovine aortic endothelial cells on fibrinogen-coated wells was inhibited by HKa in a concentration-dependent manner (Fig. 10). The concentration of HKa that caused half-maximal inhibition was ~1 μg/ml and was independent of the concentration of fibrinogen used to coat the wells and the number of cells spreading on fibrinogen in the absence of HKa. HKa also inhibited spreading of endothelial cells on von Willebrand factor (Table III).

HKa inhibited attachment of platelets and peripheral mononuclear cells to fibrinogen and vitronectin, and spreading of human melanoma cells to vitronectin (Table III). HKa did not inhibit spreading of MG-63 cells on fibronectin (Ta-

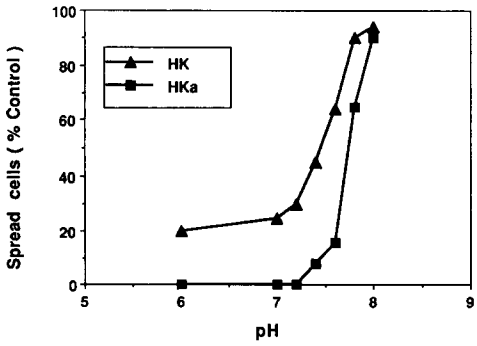


Figure 9. Effect of pH during precoating of HK or HKa. Vitronectin-coated wells were incubated with 10 μg/ml of HK (▲) or HKa (■) at different pH in 20 mM phosphate buffer containing 0.15 M sodium chloride for 1 h. After washing with TBS, pH 7.4, MG-63 cells were added and cell spreading was quantified after 1 h. There was little variation (<10%) between duplicate wells. This experiment is representative of the four times that the experiment was done.

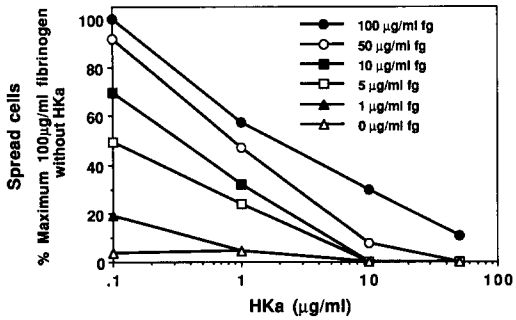


Figure 10. Effect of HKa on spreading of BAE to fibrinogen substratum. Various concentrations of fibrinogen (*fg*) were coated on the polystyrene dishes. After blocking with 1% BSA in TBS, various concentrations of HKa were added followed by endothelial cells in serum-free DME, and cell spreading was quantified. Results are expressed as maximum spreading seen in wells receiving 100 µg/ml fibrinogen; i.e., 210 spread cells per 7.5 mm². The same results were found in a second complete experiment and in two experiments in which only selected concentrations were tested.

ble III), even when used at a concentration of 20 µg/ml or when precoated at pH 6.5 (data not shown).

Effects of Divalent Cations on HKa Activity

Zn^{2+} enhances the binding of HK to platelets (Greengard and Griffin, 1984). Cellular adhesion is also dependent on divalent cations and is especially enhanced by Mn^{2+} (Evans and Jones, 1982). The effects of divalent cations on the spreading of cells on vitronectin in the presence of HKa were therefore investigated. MG-63 cell spreading supported by Ca^{2+} was inhibited by HKa (Fig. 11 *A*). In contrast, cell spreading supported by >0.3 mM Mn^{2+} was not overcome by HKa. To learn the mechanism of its contrary action to HKa, Mn^{2+} was incubated with vitronectin or HKa during coating of the substrate or with cells during the adhesion assay (Fig. 11 *B*). Only when Mn^{2+} was present during the adhesion assay was it able to counteract the anti-adhesive effects of HKa. In studies not shown, Zn^{2+} did not support cell adhesion and had no effect on the anti-adhesive effects of HKa.

Effects of HKa on Substrate-adsorbed Vitronectin

Vroman et al. (1980) and subsequent investigators (Brash et al., 1988) noted that HK can displace fibrinogen from artificial hydrophilic surfaces. HKa did not, however, displace ^{125}I-labeled vitronectin from tissue culture plastic (Fig. 12 *A*). This is compatible with the known resistance of vitronectin to the "Vroman effect" (Bale et al., 1989). The antigenicity of adsorbed vitronectin, as assessed by a high titer polyclonal antiserum, was decreased in a dose-dependent manner by HKa when measured by ELISA using polyclonal antibodies (Fig. 12 *B*). When a surface coated with 2 µg/ml vitronectin was exposed to 10 µg/ml of HKa, the antigenicity fell to the level corresponding to a surface coated with 0.25 µg/ml vitronectin and not treated with HKa (Fig.

Table III. Inhibition by HKa of Adhesion of Various Cells to Substratum Coated with Vitronectin (VN), Fibronectin (FN), Fibrinogen (Fg), or Von Willebrand Factor (vWF)

	Concentration of HKa causing 50% inhibition*			
Cells	Substratum: VN	Fg	FN	vWF
		µg/ml		
Bovine aortic endothelial cell line (BAE)	7.5	1.5	ND	3.0
(MG-63) human fibrosarcoma cell line	1.4	ND	>20	ND
(C-32) human melanoma cell line	0.9	ND	ND	ND
(G-361) human melanoma cell line	6.0	ND	ND	ND
Human peripheral mononuclear cell	0.7	1.5	ND	ND
Human platelet	0.7	0.2	ND	ND

* HKa was assayed as in Figs. 8 and 10.

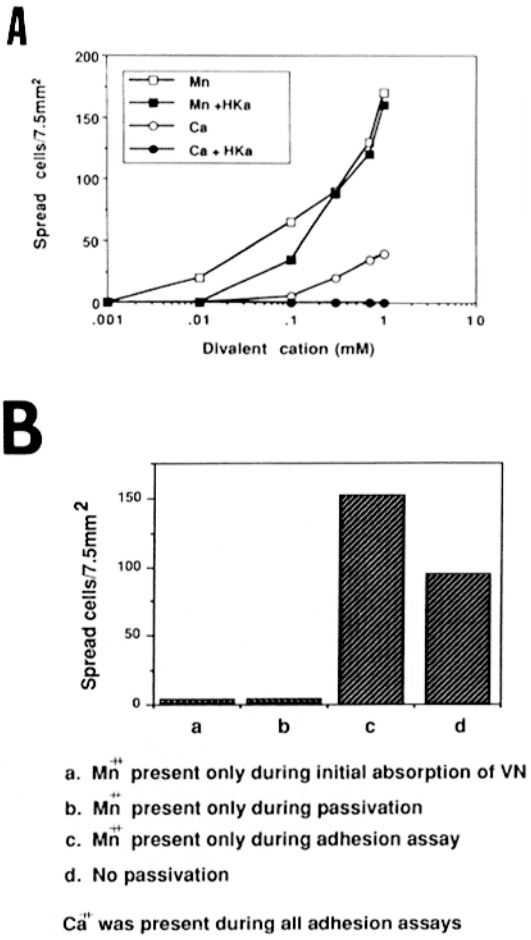


Figure 11. Effect of divalent cations on cell adhesion to the vitronectin substratum with or without HKa. (*A*) Various concentrations of calcium or manganese ions dissolved in TBS with or without HKa (final concentration 10 µg/ml) and MG-63 cells (8×10^3 cells) in HBSS were added together on the vitronectin-coated wells, and cell spreading was quantified. (*B*) Four different protocols (*a–d*) were tested: (*a*) 1 mM Mn^{2+} dissolved in TBS was incubated in the wells with vitronectin for 1 h, the wells were washed with TBS, HKa (final concentration of 10 µg/ml) was incubated with the wells, the wells were washed, and then MG-63 cells (8×10^3) were added to the wells. (*b*) Mn^{2+} was incubated with HKa during the passivation of the vitronectin-coated wells, and wells were washed before the spreading assay. (*c*) Mn^{2+} was incubated with the cells during the spreading assay. (*d*) Cells were incubated with vitronectin-coated wells in the absence of HKa as a control. 1 mM Ca^{2+} was present during spreading assay in all experimental condition (*a–d*). Thus, the comparison of *c* to *d* is similar to the comparison of Mn^{2+} alone to Ca^{2+} alone in Fig. *A*.

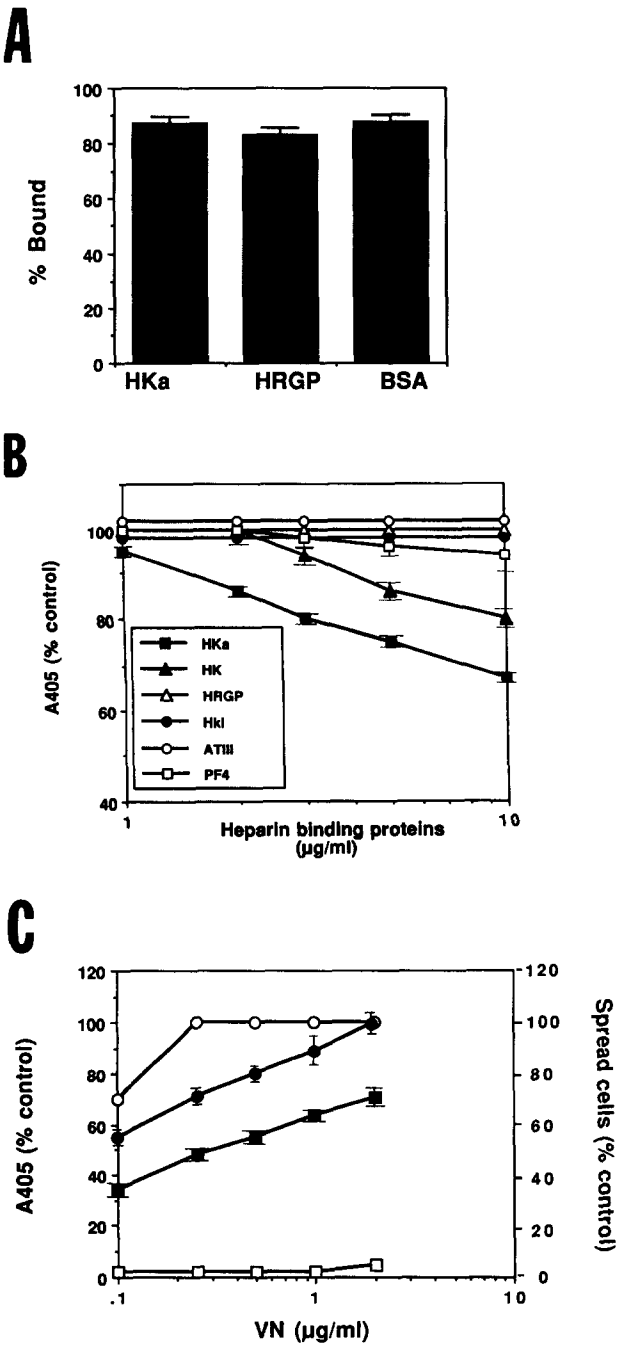


Figure 12. Displacement of vitronectin by HKa, histidine-rich glycoprotein, and other heparin-binding proteins. (*A*) Measurement of ^{125}I-radiolabeled vitronectin remaining in the well after incubation with HKa or histidine-rich glycoprotein (*HRGP*) as described in Materials and Methods. Data are presented as the mean ± SD of six determinations. (*B*) Measurement of vitronectin antigenicity by an ELISA. HKa, HK, HKi, histidine-rich glycoprotein, platelet factor 4 (*PF4*), or antithrombin III (*AT III*), 1–10 μg/ml, were incubated with vitronectin-coated wells. The antigenicity of vitronectin was measured by ELISA using a polyclonal antibody against vitronectin as described in Materials and Methods. Control A^{405} was that found when incubation with a second protein was not done. Data presented are the means of quadruplicate determinations. (*C*) Comparisons of loss of vitronectin antigenicity and cell spreading activity in the presence of HKa. Indicated concentrations of vitronectin were coated in 24- or 96-well tissue culture plates at 4°C overnight. After blocking with 1% BSA in TBS, 10 μg/ml of HKa were added for 30 min at 37°C. MG-63 cells were added to the 24-well plates for 1 h at 37°C. Spread cells were counted and expressed as percentage of control in which wells were coated with vitronectin (2 μg/ml) and not incubated with HKa. ○, Without HKa; □, with HKa. Antigenicity of vitronectin in the absence or presence of HKa was detected under the same conditions in 96-well tissue culture plates as described in Fig. *B*. ●, Without HKa; ■, with HKa. The 24- and 96-well plates were purchased from the same manufacturer (Costar Corp.).

12 *C*). This level of vitronectin, in the absence of HKa, supported 100% cell spreading (Fig. 12 *C*). However, no spreading was observed when HKa was present (Fig. 12 *C*).

Discussion

We purified an anti-adhesive protein from dextran sulfate-treated human plasma and found that this protein is the cleaved form of HK (HKa). We considered the possibility that low density lipoprotein, previously shown to have anti-adhesive activity (Myllylä et al., 1966), was present in our preparation. However, our experiments indicate that HKa is the active moiety and is responsible for the major anti-cell adhesive activity in the globulin fraction made with 30–60% saturated ammonium sulfate. First, anti-cell adhesive activity was found in the same fractions as HKa after gel filtration chromatography (Fig. 1 *D*). Low density lipoprotein is a high molecular weight particle (~1 million) and should elute in void volume. Second, there was no anti-cell adhesive activity in the globulin fraction of normal plasma immunodepleted by passage over an immobilized monoclonal anti-HK antibody column or of kininogen-deficient plasma (Table II).

HK (Fig. 2) is a well-characterized component of blood plasma, circulating at a concentration of 30–90 μg/ml (Mandle et al., 1976; Thompson et al, 1977; Proud et al., 1980; Schmaier et al., 1983*a*) in complex with prekallikrein or blood coagulation Factor XI (Mandle et al., 1976; Thompson et al., 1977). During the contact activation of blood coagulation, HK binds to the activating surface alongside Factor XII; three zymogens—Factor XII, Factor XI, and prekallikrein—are thus concentrated in a way that allows activation via reciprocal cleavages (Heimark et al., 1980). HK can be cleaved several times during contact activation, by kallikrein to release bradykinin and create the two-chain molecule (HKa) and by activated Factor XI (XI_a) to release the cationic histidine-rich domain (Han et al., 1978; Scott et al., 1985) and create HKi. HKa binds more strongly to activating surfaces than HK (Scott et al., 1984; Brash et al., 1988).

The primary structure of HK, starting at the amino terminus, contains (Fig. 2): (*i*) a signal sequence, (*ii*) one incomplete and two complete copies of the cystatin homology, (*iii*) the bradykinin moiety, (*iv*) the cationic, histidine-rich domain, and (*v*) the anionic domain that binds prekallikrein and Factor XI (Takagaki et al., 1985; Kellermann et al., 1986; Tait and Fujikawa, 1986). Thus, HK inhibits cysteine proteases (Ohkubo et al., 1984, 1988; Sueyoshi et al., 1985; Higashiyama et al., 1986); is one source of bradykinin, a potent vasodilating (Rocha e Silva et al., 1949; Bönner and Schunk, 1984) and growth-promoting (Owen and Villereal, 1983) peptide; and facilitates activation of blood coagulation pathways on negatively charged surfaces (Schiffman and

Lee, 1975). The cystatin sequences are defined by characteristic sets of disulfides (Kellermann et al., 1989). HK is synthesized by endothelial cells (Schmaier et al., 1988) and is present in α granules of platelets (Schmaier et al., 1983*a*). There are four kininogen genes in rodents (Kageyama et al., 1985; Kato et al., 1985), and a homologue of HK is the principal acute phase reactant of rats (Anderson and Heath, 1985; Kageyama et al., 1985).

HK is important for the "Vroman effect," in which the rate and extent of adsorption of fibrinogen on artificial surfaces depend on the plasma or serum concentration from which the proteins are adsorbed and on the time course of adsorption (Vroman et al., 1980). Fibrinogen, which is deposited immediately after blood contact, becomes replaced by HK, especially the two-chain or HKa form (Scott et al., 1984; Brash et al., 1988). This phenomenon is not seen with plasma samples lacking Factor XII or HK (Schmaier et al., 1983*b*; Brash et al., 1988). We found that HKa was inhibitory for adhesion to surfaces coated with a wide range of fibrinogen concentrations (Fig. 10) and did not displace radiolabeled vitronectin from polystyrene-coated surfaces (Fig. 12 *A*). The antigenicity of vitronectin was decreased in a concentration-dependent manner by HKa (Fig. 12 *B*). However, antigenicity was not decreased to a value that, in the absence of HKa, was associated with diminished cell spreading. HKa also inhibited cell adhesion to a third adhesive protein, von Willebrand factor (Table III). If HKa acts by displacement and/or modification of adhesive proteins, its actions must be different and unique for each protein.

HKa did not inhibit cell adhesion to substrates coated with fibronectin. These results contrast with results with tenascin, which counteracts the adhesive activity of fibronectin (Chiquet-Ehrismann et al., 1988). Vitronectin, fibrinogen, and von Willebrand factor are ligands of β_3 integrins, whereas fibronectin is a ligand for β_1 and β_3 integrins (Hemler, 1990). The anti-adhesive activity of HKa was overcome by Mn^{2+}, which enhances cell adhesion in a variety of situations (Evans and Jones, 1982; Grinnell, 1984; Edwards et al., 1987) and is thought to increase activity of integrins by binding to sites for divalent cations in α subunits (Gailit and Ruoslahti, 1988). Thus, we favor the hypothesis that HKa specifically interferes with the function of β_3 integrins. It could do so by direct interaction with the integrin in an antagonistic fashion or by interaction with other cell surface molecules that counteract the effects of interaction between integrins and substrate-adsorbed ligand.

HK binds to platelets (Greengard and Griffin, 1984; Gustafson et al., 1986) and neutrophils (Gustafson et al., 1989*a*,*b*) in suspension. Gustafson et al. (1989*b*) showed that fibrinogen and HK can displace each other from the surface of the neutrophil and activated platelet. They speculated that fibrinogen and HK do not share the same receptors on either neutrophil or platelet surfaces and concluded that the inhibitory effect of HK on the binding of fibrinogen to platelets and neutrophils may result from steric hindrance since both HK and fibrinogen are large asymmetric proteins. We are not sure how these findings are related to our results because there is no requirement for cleavage of HK in suspension binding assays, and the binding requires Zn^{2+}, a cation that had no activity in our experiments. In addition, low molecular weight kininogen, which has no anti-adhesive activity, has recently been shown to also bind to platelets (Meloni and Schmaier, 1991).

Adsorption of HKa to surfaces is usually thought of as a procoagulant event (Scott et al., 1984). However, because of the lack of bleeding diatheses in patients congenitally deficient in plasma Factor XII, prekallikrein, or HK, and in some patients with Factor XI deficiency, the contact system is not considered an important part of the hemostatic mechanism (Furie and Furie, 1988). Indeed, patients congenitally deficient in Factor XII probably have excess thrombosis (Lämmle et al., 1991). Our results suggest a possible mechanism whereby generation of HKa is anti-thrombogenic, inasmuch as spreading of platelets and mononuclear cells on vitronectin- and fibrinogen-coated substrates is inhibited by HKa. In addition to its roles in blood coagulation, HKa may function locally in tissues. HK is made by endothelial cells (Schmaier et al., 1988). The β-amyloid precursor protein is a specific inhibitor of Factor XIa (Smith et al., 1990), a protease that we suspect destroys the anti-adhesive activity of HKa. Further investigation, therefore, may reveal that a major role of proteases of the contact activation system and their inhibitors is to control the anti-adhesive activities of HK and its derivatives.

We thank Mark Sweet and Sarah Straight for technical assistance, and Dr. John Griffin for timely advice and gifts of reagents. We also thank Wen Sun for her help with initial experiments, and Drs. Bill Checovich, Jane Sottile, Cindy Somers, and Eliot Williams for their critical reviews of this manuscript.

Dr. Asakura thanks Mochida Pharmaceutical Company, Tokyo, Japan, for a travel grant. These studies were supported by National Institutes of Health grant HL-29586.

Received for publication 18 February 1991 and in revised form 22 October 1991.

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