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Opposing Effects of Low and High Molecular Weight Kininogens on Cell Adhesion[1]

Shinji Asakura,*,†,2 Wei Yang,† Jane Sottile,* Qinghong Zhang,* Yong-ming Jin,† Iwao Ohkubo,‡ Makoto Sasaki,§ Michio Matsuda,† Hajime Hirata,‖ and Deane F. Mosher*

*Departments of Medicine and Biomolecular Chemistry, University of Wisconsin, Madison, WI 53706 USA; †Department of Hematology, Division of Thrombosis and Hemostasis, Jichi Medical School, Tochigi 329-0498; ‡Department of Biochemistry, Shiga University of Medical Science, Tsukinowa Seta, Ohtsu, Shiga 520-2129; §Department of Biochemistry, Nagoya City University Medical School, Nagoya 467-0001; and ‖Laboratory of Biosignaling, Department of Life Science, Faculty of Science, Himeji Institute of Technology, Harima Science Garden City, Hyogo 678-1201

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High molecular weight kininogen (HK) blocks cell spreading but not cell attachment to surfaces coated with vitronectin and other ligands of $\beta3$ integrins. We sought to learn the structural basis of this phenomenon. Monoclonal antibodies against the histidine-rich D5 domain in the light chain of 2-chain HK abolished the inhibitory effect of 2-chain HK on spreading of MG-63 osteosarcoma cells on vitronectin-coated tissue-culture plastic. The antibodies were effective only if incubated with 2-chain HK in solution and did not abolish the anti-cell-spreading effect of 2-chain HK that was pre-adsorbed to tissue-culture plastic. Exposure of an epitope in the histidine-rich domain was less when HK was adsorbed to tissue-culture plastic (oxidized polystyrene) than when it was adsorbed to ELISA plastic (untreated polystyrene). Loss of the epitope correlated with increased anti-cell-spreading activity of HK on tissue-culture plastic. The light chain of 2-chain HK containing D5 and that containing recombinant D5 both had anti-cell-spreading activity, but only when present in solution during adhesion assays. Pre-adsorption of recombinant D5 to tissue-culture plastic resulted in a surface on which adsorbed 2-chain HK had little anti-cell-spreading activity. Binding study revealed that HKa bound to immobilized vitronectin. The histidine-rich D5 domain of light chain of HK was identified as one of the binding sites of vitronectin, suggesting that the masking of the RGD cell-binding site of immobilized vitronectin is the molecular mechanism of anti-cell-spreading effect of HKa. In contrast, low molecular weight kininogen (LK), which lacks D5, augmented cell spreading on vitronectin-coated tissue-culture plastic. Thus, HK and LK have opposing effects on VN-dependent cell adhesion. The augmenting effect of LK was greater if LK was preincubated with cells or adsorbed to the surface at pH>7.0. Analysis of fragments of LK and antibody inhibition studies localized the cell-adhesion activity to the D3 domain that is common to LK and HK. These findings indicate that the D5 domain mediates the adsorption of HK or 2-chain HK to vitronectin substratum in anti-adhesive conformations, *i.e.*, masking of the RGD cell-binding site of vitronectin. Such conformers inhibit cell spreading on vitronectin even though a cell-adhesion site is present in D3.

Key words: anti-cell-adhesion, high molecular weight kininogen, histidine rich domain, low molecular weight kininogen, Vitronectin.

Specific cellular attachment and spreading on an extracellular matrix are central events in embryogenesis, tumor cell metastasis, wound healing, and thrombus formation. Such events can be replicated by coating adhesive proteins onto polystyrene tissue-culture plates. Many adhesive proteins are present in extracellular matrices and blood. These proteins have specific adhesive recognition sequences, often an Arg-Gly-Asp (RGD). RGD and other adhesive sequences of different adhesive proteins are recognized by integrin cell surface adhesion receptors (*1*). Cells must also detach from the substrate. For example, cell division and cell movement require the making and breaking of interactions with the extracellular matrix. Therefore, increasing attention is being paid to the molecu-

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[2] Present address and to whom correspondence should be addressed at: Department of Hematology, Division of Thrombosis and Hemostasis, Jichi Medical School, Tochigi, 329-0498.

Abbreviations: HK, native, single form of high molecular weight kininogen; HKa, two-chain form of kinin-free high molecular weight kininogen, cleaved by kallikrein, and having blood coagulation-promoting activity; HKi, two-chain form of high molecular weight kininogen, cleaved by factor XIa, and lacking blood coagulation-promoting activity; LK, low molecular weight kininogen; VN, vitronectin; r-HRD, bacterially expressed histidine-rich domain of HK; TBS, Tris-buffered saline; PBS, phosphate-buffered saline; SDS-PAGE, polyacrylamide gel electrophoresis in sodium dodecyl sulfate.

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lar basis of anti-adhesion (2). SPARC or osteonectin (3, 4), hevin (5), tenascin (6-8), and thrombospondin (9, 10) are cell surface and matrix-associated molecules that have anti-adhesive activity when studied *in vitro*.

One of the most potent anti-cell-spreading molecules in blood plasma is high molecular weight kininogen (HK), which inhibits $\alpha v\beta 3$-mediated spreading on vitronectin (VN) and other adhesive substrates but not $\alpha 5\beta 1$-mediated adhesion to fibronectin (11). HK is cleaved by kallikrein during contact activation of blood coagulation *in vitro*. This cleavage releases bradykinin and creates a 2-chain molecule (HKa) with a heavy chain containing the cystatin-like domains D1, D2, and D3, and a light chain containing the cationic, histidine-rich D5 domain and the D6 domain, which binds prekallikrein and factor XI (12, 13). Uncleaved HK is less active than HKa in anti-cell-spreading assays as described previously (11). HKa also binds more strongly than HK to surfaces that activate blood coagulation and has enhanced coagulation activity (14). HK plays an important role in the "Vroman effect," whereby fibrinogen absorbed on artificial surfaces is replaced by other proteins; HKa causes a stronger "Vroman effect" than HK (14, 15). HKa can be cleaved further to release the histidine-rich D5 domain and yield HKi, which lacks anti-cell-spreading activity and coagulation-promoting activity (11, 14).

The strong adsorptivity of HK and HKa to surfaces and their anti-cell-spreading activity when coated on polystyrene tissue-culture plates make these molecules good candidates for structure-function studies of how an adsorbed protein blocks cell adhesion. Therefore, we undertook additional experiments to learn the basis of HK's anti-cell-spreading activity and to relate this activity to recently described cell-binding sequences of HK (16, 17). In this paper, we show that interaction of D5 of HK or HKa with the surface is important for expression of anti-cell-spreading activity in the adsorbed protein. In contrast, LK augments cell-spreading activity. Thus, LK and HK have opposing effects on VN-dependent cell adhesion. We describe a site common to LK and HK in D3 that causes cell attachment of MG-63 osteosarcoma cells. We report that recombinant D5 in solution blocks cell spreading but when adsorbed to a substrate opposes the anti-cell-spreading activity of HKa. Binding study revealed that HKa bound to immobilized VN *via* D5 in the light chain of HKa. Thus, the masking of the cell-binding site of VN may be the molecular mechanism of the anti-cell-spreading effect of HKa on VN-dependent cell spreading.

MATERIALS AND METHODS

Materials—Polystyrene plates treated for tissue culture (tissue-culture plates), which had undergone a proprietary vacuum plasma treatment to modify the surface permanently, making it more hydrophilic and negatively charged, were purchased in 12-, 24-, and 96-well formats from Costar (Cambridge, MA) and Falcon (Becton Dickinson, Lincoln Park, NJ). Microtiter polystyrene plates for ELISA (ELISA plates), made of unmodified polystyrene, were purchased from Falcon, Corning (Corning, NY), and Dynatec (Chantilly, VA). MG-63 human osteosarcoma cells were obtained from the American Type Culture Collection. These cells were cultured in Dulbecco's modified Eagle's essential medium (DMEM) with 5% fetal calf serum (Gibco, New York, NY). The cells do not contain HK as assessed by immunoblotting of cell extracts. Alkaline phosphatase-conjugated goat anti-mouse IgG and anti-rabbit IgG were from Bio-Rad (Richmond, CA).

VN purified from fresh frozen human plasma by the method of Dahlbäck and Podack and the 8E6 monoclonal antibody to VN have been described previously (18). Human VN preparations consisted of 75- and 65-kDa polypeptides as assessed by polyacrylamide gel electrophoresis in SDS (SDS-PAGE) (19). HKi, HKa, and HK were purified and characterized as described previously (11). LK and the light chain of reduced and carboxyamidomethylated HKa were purified as described (20-22). LK preparations that consisted of a 60-kDa on SDS-PAGE band did not contain VN, fibronectin, fibrinogen, or von Willebrand factor when tested by immunoblotting (data not shown). D1 (residues 1-160 disulfide-linked to residues 372-401), D2 (residues 160-244), and D3 (residues 245-362) of LK were obtained by cleavage with cyanogen bromide or with trypsin and chymotrypsin and purified and characterized as described (23). Protein concentrations were determined by the method of Lowry *et al.* (24).

Cell Attachment and Spreading Assays—Cell spreading in 12-, 24-, or 96-well plates was assessed microscopically (11). The number of cells adherent to 96-well tissue-culture plates was quantified in a plate reader after washing, fixation, and staining with bromophenol blue (25). Spread cells and attached cells were defined according to their appearance on phase-contract microscopy: the former were polygonal and dark and the latter were round and white. Adherent cells were defined as the sum of spread cells and attached cells. Thus, anti-cell-spreading is defined as inhibitory activity of cell spreading; and corresponding definitions apply for anti-cell adhesion and anti-cell attachment.

Iodination of HK and VN—VN and HKa were radioiodinated by the iodogen (chloramide-1,3,4,6-tetrachloro-3a-6a-diphenyl-glycouril, Pierce Chemical, Rockford, IL) method (26). The specific activities were 0.5 and 0.6 mCi/mg, respectively. Preparations of HKa and VN were 96%, and 94% insoluble in 10% TCA, respectively. Labeled HKa migrated as a 115-kDa band without reduction, and under reducing conditions as 64- and 45-kDa bands when analyzed by SDS-PAGE followed by autoradiography. Radiolabeled VN migrated as a 75-kDa band under non-reducing conditions as analyzed by SDS-PAGE followed by autoradiography.

Analysis of VN and HKa on Surfaces Treated with Various Proteins—Tissue-culture plates were coated with ^{125}I-VN (2 μg/ml) or ^{125}I-HKa (10 μg/ml) in TBS for 12 h at 4°C, then blocked with TBS containing 3% albumin. The wells were rinsed three times with TBS, various proteins were added, *i.e.*, r-HRD (10 μg/ml) or VN (10 μg/ml) to ^{125}I-HKa-coated plates, and r-HRD (10 μg/ml) or HKa (10 μg/ml) to ^{125}I-VN-coated plates, and the plates were incubated for 1 h at room temperature. The wells were then rinsed three times with TBS, and bound proteins were solubilized by incubation with 2% SDS and 1 M sodium hydroxide for 24 h at room temperature. Radioactivity was quantitated using a gamma counter. Data represent the average of six determinations.

Binding of VN and HKa or r-HRD—Tissue-culture plates were coated with r-HRD (10 μg/ml), HKa (10 μg/

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ml), or albumin (1 μg/ml) in TBS for 12 h at 4°C, then blocked with TBS containing 3% albumin for 6 h at room temperature. The wells were rinsed three times with TBS, then ^{125}I-VN (2 μg/ml, 2.51×10^{6} cpm/well) was added, and the plates were incubated for 3 h at room temperature. After extensive washing with TBS, bound proteins were quantitated using a gamma counter. Data represent the average of six determinations. Also tissue-culture plates were coated with VN (2 μg/ml) or albumin (1 mg/ml) in TBS for 12 h at 4°C, then blocked with TBS containing 3% albumin for 6 h at room temperature. After washing with TBS, ^{125}I-HKa (5 μg/ml, 3.89×10^{6} cpm/well) was added, and the plates were incubated for 3 h at room temperature. After extensive washing with TBS, bound proteins were quantitated as described above.

Monoclonal Antibodies against HK and LK—H3, H12, L3, L5, L6, L7, and L8 monoclonal antibodies to LK and/or HK were isolated from mouse ascites using protein-A cellulofine columns and characterized as shown in Fig. 1. H202, H218, H219, and H226 to D1 of HK and LK were produced and characterized by similar methods. SDS-PAGE and immunoblotting to further localize the epitope of H12 were performed as described previously (*27*). Immunoblotting analysis revealed that H12 recognized D3 of the heavy chain of kininogen (Fig. 2).

Production of the Recombinant Histidine Rich Domain of HK (Recombinant $HK_{411\text{-}501}$, r-HRD)—PCR primers were synthesized to amplify HK cDNA encoding amino acids from His-411 to Trp-501 (antisense primer, 5′-CCGGCATATGTCTAGACCCAACCATTGTGCTTTCC-3′; sense primer, 5′-CCGGCATATGCATGACTGGGGCCATG-3′). Underlined sequences represent bases introduced at the 5′ ends of the DNA to create an *Nde*I site for cloning into pET3a (*28*). The ATG start site for expression of $HK_{411\text{-}501}$ is provided by the ATG in the *Nde*I cloning site. The antisense primer contains an *Xba*I restriction enzyme site that provides an in-frame stop codon. Such a construct adds the initiating methionine and a valine following Trp-501 to the HK sequence. Amino acids are numbered from the N-terminal residue after removal of the signal peptide of HK (*29, 30*). PCR was performed according to established procedures (*31*) using Taq polymerase. Clone phKG6 [*29*, a gift from Dr. S. Nakanishi, Kyoto University] was used as a template for amplification. Amplified DNA was gel purified, digested with *Nde*I, and cloned into pET3a. Clones in the correct orientation were selected, sequenced, and transformed into the bacterial strain BL21(DE3) (*32*).

Overnight cultures of $HK_{411\text{-}501}$ pET3a were grown in LB broth containing 100 mg/ml ampicillin and 30 mg/ml chloramphenicol. Cultures were diluted 1:80 with fresh medium containing antibiotics, and isopropyl-β-D-thiogalactopyranoside (IPTG) was added at midlog phase. Extracts of induced bacteria reacted with monoclonal antibody to the histidine-rich domain by Western blot analysis (Fig. 3, A and B). Western blotting was used to design and follow the purification. Bacteria were pelleted by low-speed centrifugation, then resuspended in a volume of buffer (50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 100 mM sodium chloride containing 2 mM phenylmethylsulfonylfluoride) equal to the original volume of the bacterial culture. Cells were flash frozen in a dry ice/methanol bath and stored overnight at −20°C. Bacteria were thawed, spun at 10,000 rpm to remove insoluble material, then the cell extract was separated by chromatography on CM-sepharose. r-HRD was eluted with a gradient of 0.2-1.0 M NaCl (50 ml) in 50 mM phosphate buffer, pH 6.5 (PBS), and fractions of 1.0 ml were collected. Peak fractions (r-HRD) were pooled (fraction numbers 40-55) and stored at −80°C until use.

Purified r-HRD showed a single band and was 99% pure by SDS-PAGE as shown in Fig. 3C. Protein concentration of r-HRD was determined by the method of Lowry, and also by staining with Coomassie Brilliant Blue and densitometry against a hemoglobin standard after SDS-PAGE. Amino acid sequence analysis showed that the amino terminal of purified r-HRD was HDWGHEKQRK (data not shown).

Analyses of HKa on Surfaces Treated with r-HRD—

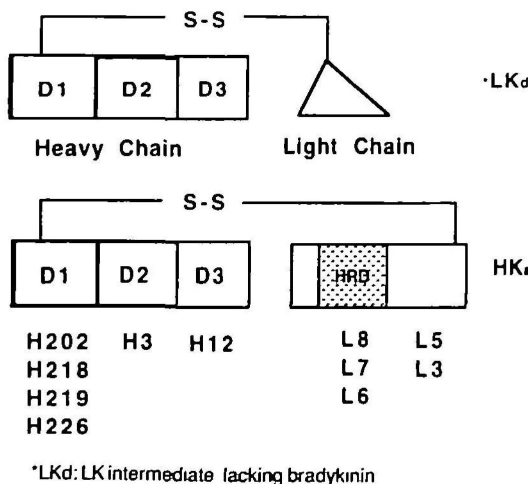

Fig. 1. **Models of HK and LK localizing the epitopes recognized by the monoclonal antibodies, adapted from Ishiguro *et al.* (*23*).** Antibodies designated "H" recognize the common heavy chain of HK and LK after cleavage to remove bradykinin. Antibodies designated "L" recognize the light chain of 2-chain HK but not the non-identical light chain of 2-chain LK.

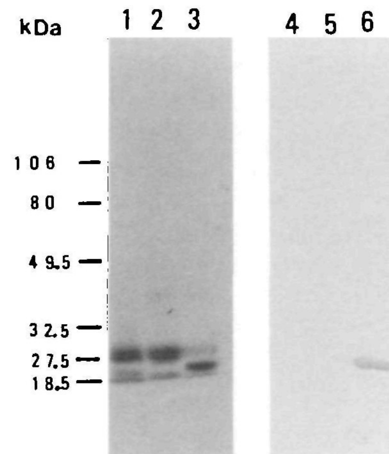

Fig. 2. **Immunoblotting of D1, D2, and D3 of LK with monoclonal antibody H12.** Left: SDS-PAGE of purified D1, D2, and D3 using 5-20% gradient gels in the presence of reducing agent and stained with Amido black. Right: Immunoblotting by H12 of replicate lanes. Lanes 1 and 4, D1; lanes 2 and 5, D2; lanes 3 and 6, D3. The positions of size markers are indicated on the left.

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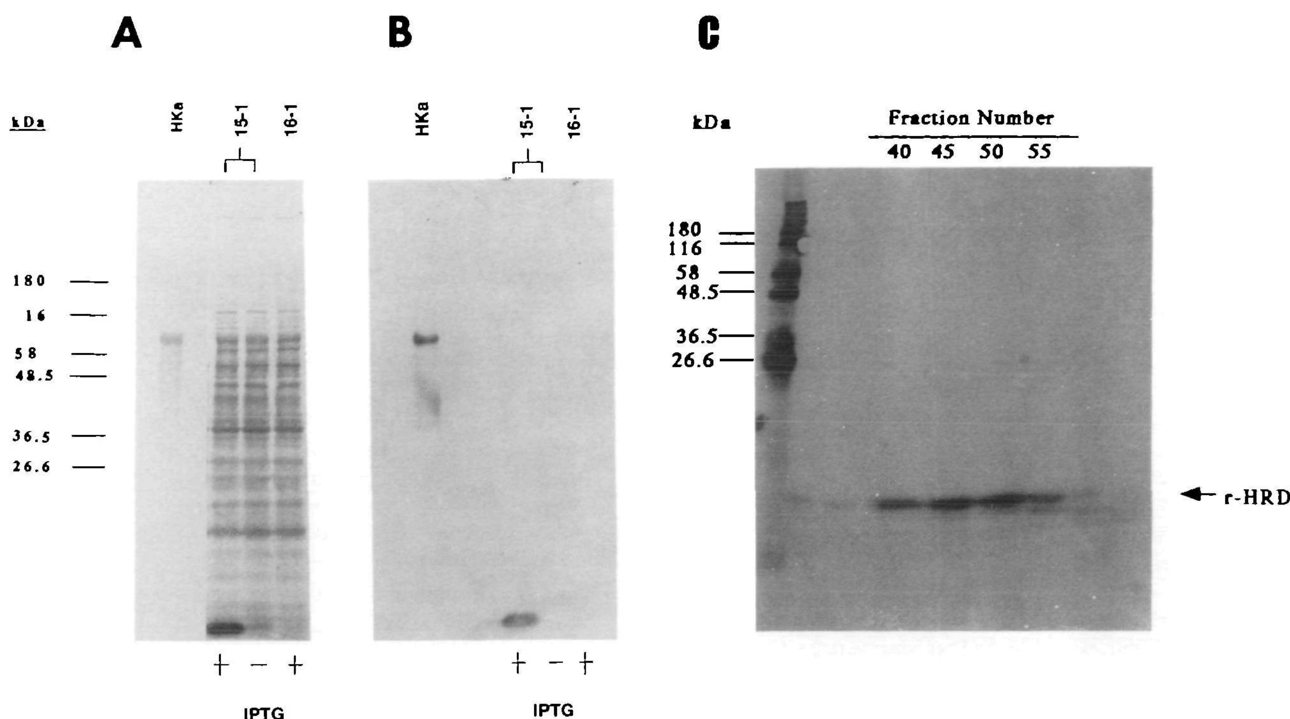

Fig. 3. **Expression of r-HRD of HK.** (A) SDS-PAGE of lysates of *E. coli* carrying the coding sequence for amino acids 411-501 of HK (r-HRD) in pET3a vector correctly oriented (clone 15-1) or, as a control, in the incorrect orientation (clone 16-1) were resolved in 15% acrylamide gels as described in "MATERIALS AND METHODS." Bacteria were (+) or were not (−) induced with IPTG. Purified HKa was also analyzed. Molecular mass markers are shown on the left. (B) Immunoblotting of r-HRD with L8 monoclonal antibody against the histidine-rich domain. After SDS-PAGE of bacterial lysates and transfer to nitrocellulose, immunoblotting was performed as described in "MATERIALS AND METHODS." (C) SDS-PAGE analysis of r-HRD purified by CM-Sepharose column chromatography. Purified r-HRD as described in "MATERIALS AND METHODS" was applied to SDS-PAGE (15% polyacrylamide) under non-reducing conditions. Molecular mass markers are indicated on the left.

Tissue-culture plates (12-well) were coated with VN (2 μg/ml) for 12 h at 4°C, then blocked with Tris-buffered saline (TBS; 10 mM Tris, 150 mM sodium chloride, pH 7.4) containing 1% albumin. After rinsing three times with TBS, ^{125}I-HKa (2×10^5 cpm/well) and unlabeled HKa (10 μg/ml) with or without r-HRD (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 in 1 M sodium hydroxide for 24 h at room temperature. Radioactivity was quantitated using a gamma counter.

RESULTS

Neutralization of the Anti-Cell-Spreading Effect of HKa by Monoclonal Antibodies Directed against HK—We previously found that preincubation of HKa with rabbit anti-HKa IgGs neutralizes its anti-cell-spreading activity (*11*). We used monoclonal antibodies (*23*) in order to identify regions of HKa important for anti-cell-spreading activity. Three antibodies (L6, L7, and L8) preincubated with HKa for 30 min prior to adsorption of HKa to plates each partially blocked the anti-cell-spreading activity of HKa (Fig. 4A). These antibodies recognize the histidine-rich D5 domain of the light chain in HKa (*23*). Other antibodies that recognize the D1, D2, and D3 domains of the heavy chain of HKa or the D6 domain of the light chain did not block HKa's anti-cell-spreading activity on cells. In other experiments, antibodies H219, H226, and H12 described in Fig. 1 were found to be equivalent to H218, H202, H3, L3, and L5 in their lack of activity.

Addition of L8 after adsorption of HKa did not result in neutralization of the anti-cell-spreading activity of HKa (Fig. 4B). The requirement that L8 be present during adsorption is not due to failure of the HKa-L8 complex to adsorb, as ascertained by the following experiment. When HKa was preincubated with L8 at a molar ratio of 0.5, 1.0, or 2.0, the adsorption of HKa to VN-coated dishes was the same (there was no statistical difference between these three data) as evaluated by quantification of surface antigenicity using the H202 antibody, which recognizes the heavy chain of HKa (Fig. 4C). Similar results were obtained with radiolabeled HKa. That is, when ^{125}I-HKa was preincubated with L8 at a molar ratio of 0.5, 1.0, or 10, the quantities of adsorbed radiolabeled HKa to VN-coated dishes were the same (there was no statistical difference between them), as shown in Fig. 4D.

To probe further the effect of the conformation of adsorbed HK, we investigated cell attachment and spreading activity on two different surfaces: tissue-culture plates and ELISA plates. These experiments were modeled after published studies in which cell-adhesive activity of fibronectin was demonstrated to be better on tissue-culture plates than on ELISA plates and to correlate with expression of epitopes for anti-fibronectin antibodies that block adhesive activity (*33*). In contrast, VN is equally effective

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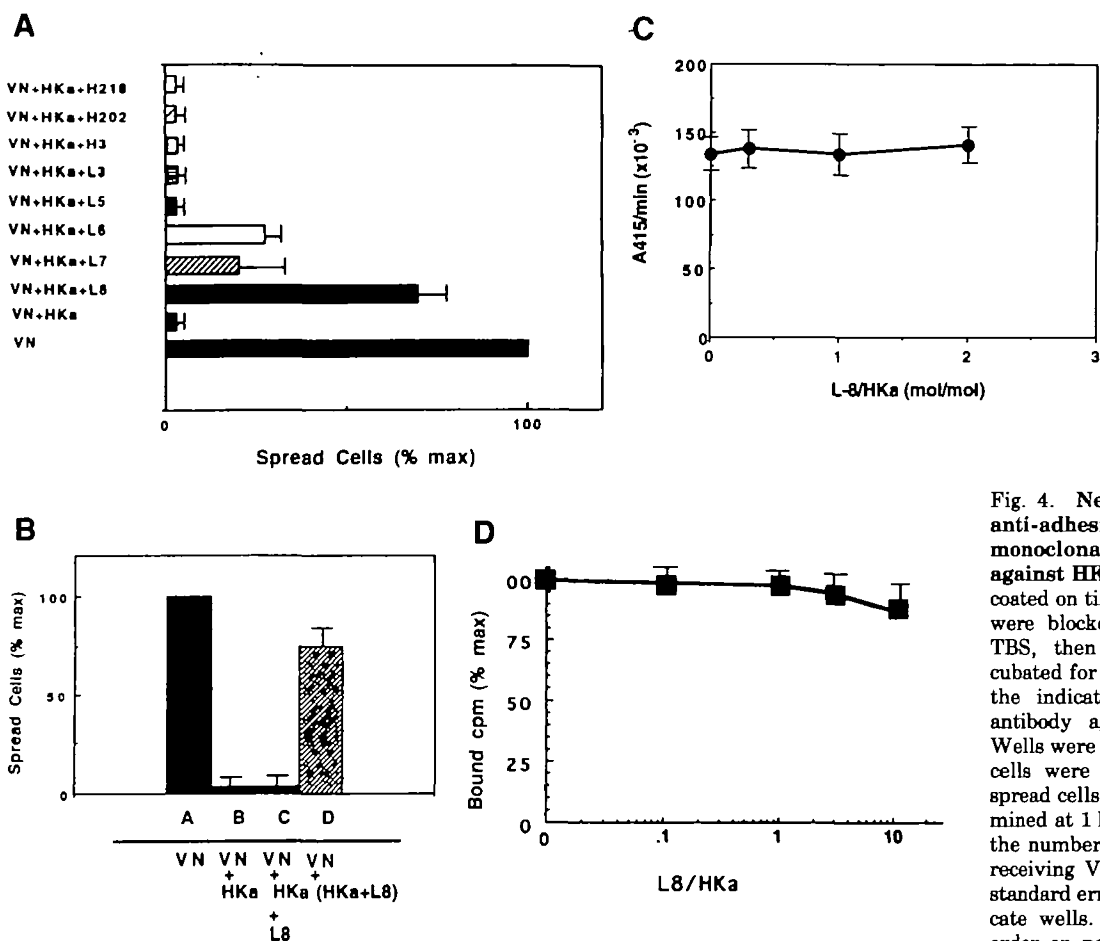

Fig. 4. **Neutralization of the anti-adhesive effect of HKa by monoclonal antibodies directed against HK.** (A) VN (2 μg/ml) was coated on tissue-culture plates, wells were blocked with 1% albumin in TBS, then 10 μg/ml HKa preincubated for 30 min with 30 μg/ml of the indicated purified monoclonal antibody against HK was added. Wells were then washed, and MG-63 cells were added. The number of spread cells per unit area was determined at 1 h and normalized against the number of cells spread in wells receiving VN alone. Bars represent standard errors of the mean of triplicate wells. (B) Effects of addition order on neutralization. Bars A, B, and D represent, respectively, cell spreading in tissue-culture wells treated with VN alone, VN followed by HKa, and VN followed by HKa preincubated with L8. Bar C represents wells in which VN, HKa, and L8 were added sequentially after washing between each addition. Treatment of the wells, concentrations of proteins, and normalization of results were as described in Fig. 2A. (C) HKa was preincubated with L8 at a molar ratio of 0.5, 1.0, or 2.0, then added to the wells and incubated for 3 h at room temperature. After washing plates with TBS three times, the antigenicity of HKa was measured by ELISA using the monoclonal H202 antibody (3 μg/ml), which recognizes the heavy chain of HKa, as described in "MATERIALS AND METHODS." (D) ^{125}I-HKa was preincubated with L8 at molar ratios of 0.1, 1.0, or 10. The adsorptions of HKa to VN-coated dishes were quantitated by use of gamma counter as described in "MATERIALS AND METHODS."

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in supporting cell adhesion when adsorbed on tissue-culture or ELISA plates (*33*). For these studies, we used uncleaved HK, which has less anti-cell-spreading activity than the 2-chain form and presumably less conformational lability. HK had little anti-cell-spreading activity when adsorbed to ELISA plates, whereas HK adsorbed to tissue-culture wells had substantial anti-cell-spreading activity [spread cells (mean±SEM, $n=6$): tissue-culture plates, 8%±1%, ELISA plates, 45±5% of control]. We compared the antigenicity of immobilized HK on the two surfaces. Monoclonal antibody L8 to D5 reacted more with HK immobilized on ELISA wells than HK immobilized on tissue-culture wells (Table I). In contrast, L5 monoclonal antibody to D6 in the light chain of HK and the H202 antibody to the heavy chain in HK recognized HK immobilized on tissue-culture wells better than HK adsorbed on ELISA wells. The L6 antibody to D5 recognized HK equally well on the two forms of polystyrene.

Effect of Isolated Light Chain and r-HRD on VN-Dependent Cell Spreading—Previous studies (*11*) and those described above suggest that D5 is critical for anti-adhesive

TABLE I. **Effects of different surfaces on HK's antigenicity of HK.** Tissue-culture or ELISA 96-well microtiter plates were coated with 2 μg/ml VN, blocked with 0.2% albumin and postcoated with 10 μg/ml HK, and 0.2% albumin (experimental) or with 0.2% albumin alone (control). Parallel wells were assayed by ELISA. Visual inspection of wells confirmed that osteosarcoma cells were spread on ELISA plates coated with HKa and VN but not on tissue-culture plates coated with the same proteins. The OD_{405} values shown have been corrected for background color developed in wells not receiving HKa (0.15-0.18 for tissue-culture plates and 0.09-0.11 for ELISA plates). Note that when adsorbed on ELISA plates, on which HK was relatively inactive in blockage of adhesion, the L8 epitope of HK was preferentially expressed. Results using Costar tissue-culture plates and Corning ELISA plates are shown in the table. Similar substrates from different distributors, *i.e.*, tissue-culture type from Falcon and ELISA type from Dynatec and Falcon, gave similar results.

Antibody	OD_{405} (mean±SEM, $n=3$)	
	Tissue-culture plate	ELISA plate
L8	0.4±0.1	1.3±0.1
L6	0.7±0.1	0.9±0.1
L5	1.4±0.1	0.9±0.1
H202	1.9±0.1	0.9±0.1

activity. To analyze this domain further, we expressed the domain in *Escherichia coli* by using the pET3a vector (Fig. 3). This molecule, designated as r-HRD, was evaluated for its anti-cell-spreading activity. Figure 5A shows that the r-HRD displayed anti-cell-spreading activity in a concentration-dependent manner when present in a soluble form during adhesion of cells to limiting amounts of VN. The anti-cell-spreading activity of soluble r-HRD on MG-63 cells was not as great as that of the isolated light chain of HKa, which contains D5 plus D6, and both proteins were much less active than HKa (Fig. 5B). These results suggest that the heavy chain of HKa is also involved in the anti-cell-spreading activity of HKa. Thus, whole molecules of HKa are the best conformers for the anti-cell-spreading activity.

Anti-cell-spreading activity of HKa after adsorption of 2 μg/ml VN was abolished when HKa and r-HRD were adsorbed together on the substratum (Fig. 5C). To elucidate the mechanism underlying this result, we did a variety of studies of protein adsorption *versus* cell spreading. Previously, we found that the adsorption of ^{125}I-VN was not influenced by adsorption of HKa, although the antigenicity of adsorbed VN did decrease (*11*). To determine the quantity of adsorbed HKa in the presence of r-HRD, we incubated ^{125}I-labeled HKa and non-radiolabeled r-HRD on surfaces coated with VN, 1 μg/ml (as in bar C of Fig. 5C) and measured the bound radioactivity on plastic surfaces. The amount of HKa on the surfaces was reduced to 18+7% [(X+SD) $n=14$] of control in the presence of r-HRD as shown in Fig. 5D. These results indicate that the adsorption of HKa on the immobilized VN is necessary for it to acquire the anti-cell-spreading activity. These results suggested that HKa binds to VN, and the anti-cell-spreading activity of HKa is due to masking of the RGD cell-adhesive site of VN . Thus, a binding study was done to prove this hypothesis. As shown in Fig. 6A, radiolabeled VN bound to the HK-coated surface more strongly than to the albumin-coated surface ($p<0.001$).

Furthermore, D5 of HKa was identified as the binding domain to VN, as shown in Fig. 6A. Similar results were obtained when radiolabeled HK was substituted as a soluble ligand, as shown in Fig. 6B. Although VN bound to HK, the cell-spreading activity was not found when HK was added to the plate followed by the addition of VN. To confirm that these results were not influenced by Vroman's effect, plates were coated with radiolabeled HKa or VN, non-radiolabeled proteins was added, and after extensive washing with TBS, the residual radioactivity were counted. Figure 6C and Fig. 6D show that HKa and VN were not displaced by the exogenously added proteins.

Effect of LK on VN-Dependent Cell Adhesion—In our previous studies, cell spreading was inhibited almost

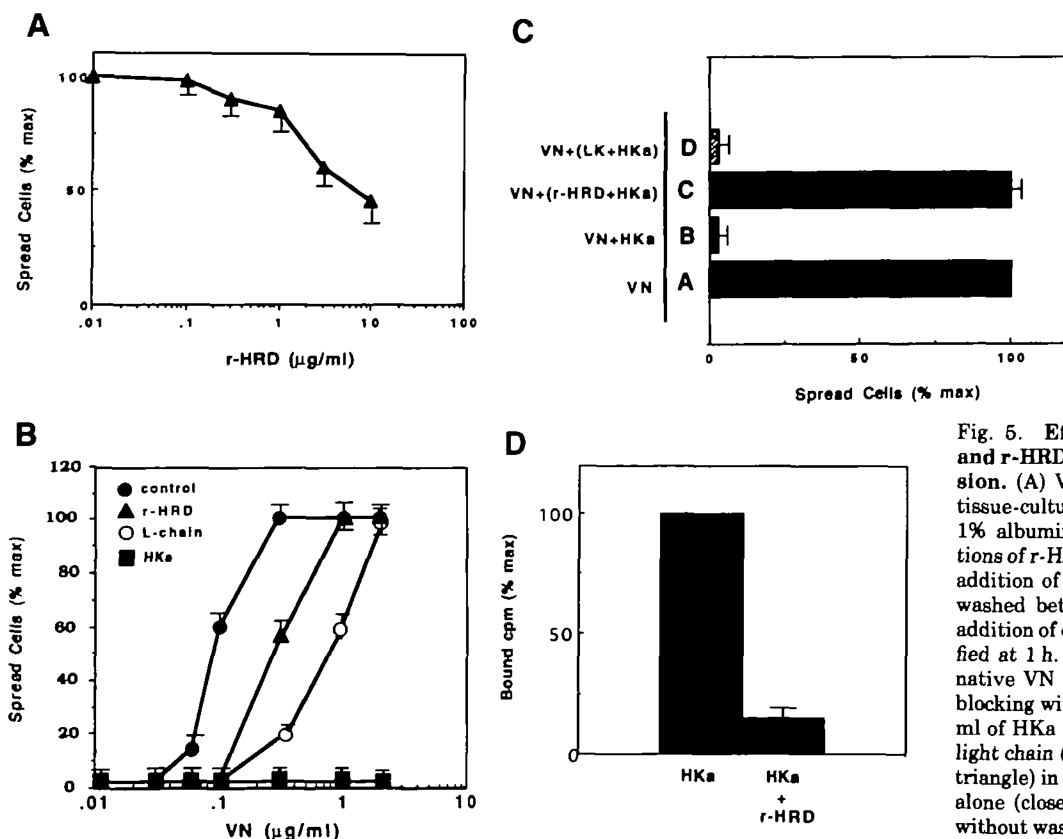

Fig. 5. **Effect of isolated light chain and r-HRD on VN-dependent cell adhesion.** (A) VN (0.1 μg/ml) was coated on tissue-culture plates. After blocking with 1% albumin in TBS, various concentrations of r-HRD were added followed by the addition of MG-63 cells. Plates were not washed between addition of r-HRD and addition of cells. Spread cells were quantified at 1 h. (B) Various concentrations of native VN were coated onto wells. After blocking with 1% albumin in TBS, 10 μg/ml of HKa (closed squares), isolated HKa light chain (open circle), or r-HRD (closed triangle) in 0.2% albumin or 0.2% albumin alone (closed circle) was added, followed without washing by the addition of MG-63 cells. Spread cells were counted at 1 h and normalized against the number of spread cells in wells treated with 1 μg/ml VN. (C) VN (2 μg/ml) was coated on tissue-culture plates. After blocking with 1% albumin in TBS, wells were treated for 1 h with 0.2% albumin alone (bar A) or containing 10 μg/ml of HKa (bar B), 10 μg/ml each of r-HRD and HKa (bar C), or 10 μg/ml each of LK and HKa (bar D). After the proteins were washed out, MG-63 cells were added, and spread cells were quantified at 1 h. Data are normalized against the wells treated only with VN and albumin. (D) VN (1 μg/ml) was coated on tissue-culture plates. After blocking with 1% albumin in TBS, wells were treated for 1 h with 1 μg/ml of ^{125}I-HKa alone or containing 10 μg/ml of unlabeled r-HRD. The proteins were washed out, and the residual radioactivity was counted.

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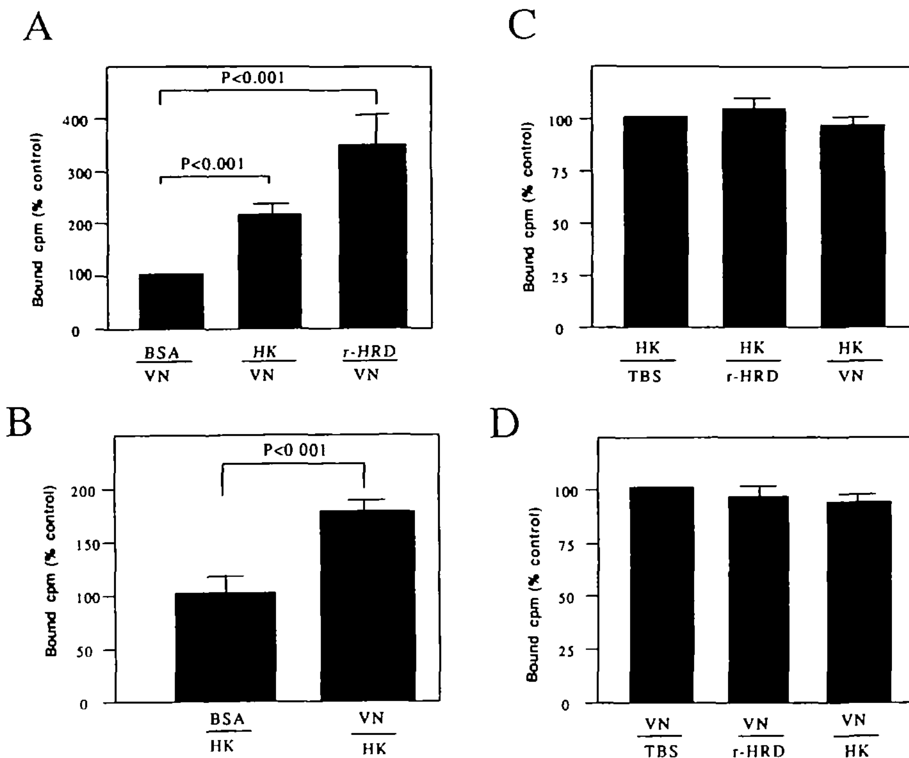

Fig. 6. **(A) Binding of VN to immobilized HKa or r-HRD.** ^{125}I-VN remaining in the well after incubation with HKa or r-HRD-coated well was measured as described in "MATERIALS AND METHODS." (B) Binding of HKa to immobilized VN. ^{125}I-HKa remaining in the well after incubation with VN-coated well was measured as described in "MATERIALS AND METHODS." (C) Displacement of HKa by r-HRD and VN. ^{125}I-HKa remaining in the well after incubation with VN, r-HRD, or TBS was measured as described in "MATERIALS AND METHODS." (D) Displacement of VN by r-HRD and HKa. ^{125}I-VN remaining in the well after incubation with HKa, r-HRD, or TBS was measured as described in "MATERIALS AND METHODS." Proteins were added sequentially to surfaces of tissue-culture plastic according to the scheme denoted X/Y. Data (A-D) are presented as the mean ± SD of six determinations.

completely in VN-coated wells that had been exposed to HKa for as little as 5 s, whereas cell attachment was inhibited by only 50% in wells that had been exposed to HKa for as long as 2 min (*11*). Figure 7 shows this phenomenon: cell attachment to VN is not abolished in the presence of HKa, despite complete inhibition of cell spreading (panels a and b). We also noted that HKi, which lacks D5, augments cell spreading on VN-coated surfaces (not shown). We reasoned that the cell-attachment activity present in HKa and HKi may also be present in LK, because LK shares the common heavy chain with HK.

We tested this hypothesis in wells coated with concentrations of VN as low as 0.1 μg/ml, in which approximately 50% fewer cells spread when compared with wells coated with 2 μg/ml VN. Cell-spreading activity after coating with the low VN concentrations was augmented in the presence of LK (Fig. 8A). Figure 8B shows that cell spreading on 0.1 μg/ml VN increased in a manner that was dependent upon coating concentration of LK up to 10 μg/ml with a maximum value of 140% of the control value (VN alone). These differences are statistically significant when evaluated by Student's t-test. The increased cell spreading on LK plus VN was not blocked by r-HRD.

When cells and various concentrations of LK were incubated together for 1 h, and the cells were then washed with PBS and added to the VN-coated wells, augmentation of cell spreading by LK was more effective when the LK was preincubated with the VN-coated substratum (Fig. 8C). This result contrasts with the anti-cell-spreading effect of HKa, which is strongest profound when HKa is preadsorbed to the substratum (*11*).

We have shown previously that the pH at which HKa is adsorbed to VN-coated wells has an effect on subsequent anti-cell-spreading activity, with pH<7.2 being the optimum for the anti-cell-spreading effect (*11*). In similar experiments, LK was incubated at different pHs conditions on VN-coated tissue-culture plastic surfaces. The cell-adhesive effect related to LK was greater when LK was adsorbed at pH>7.0 (Fig. 9A). This result was not due to a increase in adsorption of LK to the VN-coated surface at higher pH, because adsorption of LK was decreased at higher pH (Fig. 9B).

Identification of the Domain of HK and LK Which Augments Cell Spreading—The heavy chain of HK or LK contains three cystatin domains, D1, D2, and D3 (*29, 30*). D1 contains the Ca^{2+}-binding site. D2 and D3 contain the cysteine protease inhibitor modules. D3 also contains a platelet-binding site (*34*), localized to presumed hairpin loop (*16*). To determine which domain of LK accounts for the adhesion activity of adsorbed kininogens, LK was cleaved, and D1, D2, and D3 were purified (Fig. 2) and tested in adhesion assays. D3 augmented VN-dependent cell spreading (Table II).

The H12 monoclonal antibody blocked the residual attachment of cells to HKa on VN-coated surfaces (Fig. 7, panels b and c). H12 was originally mapped to D2 and D3 by ELISA (*23*), and we found that it was specific to D3 by immunoblotting (Fig. 2). This result indicates that D3 is pro-adhesive rather than anti-adhesive when present in HKa. H12 also blocked enhancement by LK of cell spreading on a limiting concentration of VN (results not shown). Attempts to demonstrate direct attachment of cells to adsorbed LK failed, however, presumably because of the fragile nature of the attachment.

DISCUSSION

HK has been shown to bind to unstimulated and thrombin-

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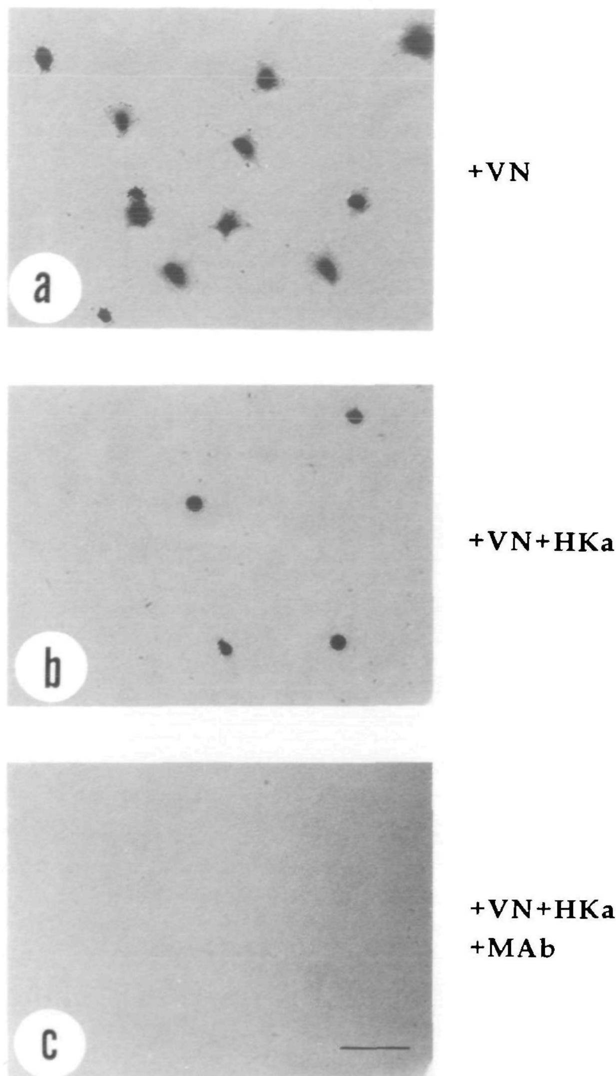

Fig. 7. **Effect of monoclonal antibody H12 on the residual cell attachment to substrata coated with VN and HKa.** Tissue-culture plates were coated with 1 μg/ml VN and blocked with 1% albumin. Then 10 μg/ml HKa in 0.2% albumin or 0.2% albumin alone was added, and plates were incubated for 1 h and washed out. This procedure was repeated with 30 μg/ml H12 in 0.2% albumin or 0.2% albumin alone. MG-63 cells adherent after 1 h on substrate coated with VN (a), VN followed by HKa (b), or VN followed by HKa followed by H12 (c) were fixed with 3% paraformaldehyde, stained with Amido black, and photographed. Note cell spreading on the surface coated with VN alone, as opposed to attached but not spread cells on the surface coated with VN and HKa.

activated platelets (*35-38*), granulocytes (*39, 40*), and endothelial cells (*41-43*). Binding of kininogen to the cell surface of platelets, endothelial cells, and neutrophils is specific, saturable and reversible (*35, 44, 45*). LK as well as HK binds to cells, indicating that the kininogens contain a cell-binding site in their common heavy chain (*45-47*). Immunoelectron microscopy revealed that HK and LK appear to be bound in clusters to cell membranes of the neutrophils (*48*). Monoclonal antibody HKH 15 blocks the interaction of kininogens with platelets and has been shown to interact with a discrete sequence in D3 (*49*). Sequences in D5, however, also contribute to the binding of HK to cells (*50-52*).

Despite the existence of multiple cell-binding sites in HK, the dominant effect of surface-bound HK or HKa is to block cell adhesion (*11*). Cell spreading was abolished almost completely in VN-coated wells that had been exposed to HKa for as little as 5 s, whereas cell attachment was inhibited by only 50% even after exposure of wells for 2 min to HKa (*11*). This result indicates that the cell-attachment activity remains even though cell spreading is inhibited. The present results indicate that D3, previously shown to mediate binding of HK and LK to suspended cells, is responsible for the residual attachment of cells to surfaces coated with VN and HKa, and for enhanced cell attachment and spreading when LK was adsorbed to surfaces alongside limiting amounts of VN. Thus, HK and LK have opposing effects on VN-dependent cell adhesion.

HKa, which degraded further to release D5 (HKi), had little anti-cell-spreading activity (*11*). These results, along with the effect of pH in the range 6-8, in which histidine side chains titrate (*11*), indicate that histidine-rich D5 is critical for anti-cell-spreading activity. In the present studies, we analyzed for anti-cell-spreading activity in parts of HK containing D5. The anti-cell-spreading effect of isolated light chain or r-HRD was much smaller than that of HKa and, unlike the activity of HKa, was only apparent when the protein was present in soluble form during the adhesion assay. r-HRD counteracted the effect of HKa on VN-dependent cell adhesion when it was preadsorbed on the surface or co-incubated with HKa during adsorption. Co-adsorption of HKa and monoclonal antibodies to D5, especially to residues 479-498, abolished the anti-spreading action of HKa. This sequence constitutes one of two subregions in D5 for interaction with anionic surfaces (*16*) and one of three subregions that mediate binding to endothelial cells (*17*). Interestingly, the gC1q receptor was identified recently as the cell surface binding site for this sequence (*53*). L8 did not block adsorption of HKa to tissue-culture plastic or the anti-adhesive activity of already adsorbed HKa. The presumed effect of L8, therefore, is to cause HKa to adsorb in conformations that do not block cell spreading. HK was less active as an anti-adhesive protein when adsorbed on ELISA plastic than when adsorbed on tissue-culture plastic; loss of activity correlated with increased expression of the L8 epitope in adsorbed HK. We conclude, therefore, that antibodies to D5 or adsorption of r-HRD prevent HKa from binding to VN-coated tissue-culture polystyrene in anti-adhesive conformations in which the L8 epitope is hidden.

Some appreciation of the power of HKa in blocking cell spreading on VN and other ligands of β3-integrins can be gained by comparison with the A18 anti-VN antibody that blocks adhesion to VN (*54*). This antibody at 50 μg/ml blocked cell adhesion to VN-coated plates by about half. When the antibody was coated on plates at 10 μg/ml and VN was then added, cell adhesion was blocked to 5% of control. This effect is thought to be due to the unfavorable orientation of VN when bound to the pre-coated antibody. HKa, in contrast to the antibody, blocks cell spreading when exposed to plates before or after adsorption of VN and is active at lower concentrations (1-10 μg/ml).

How does HKa interfere in the adhesion assays to block cell-spreading? Failure of the isolated light chain or r-HRD to mimic the anti-adhesive activity of adsorbed HK or HKa

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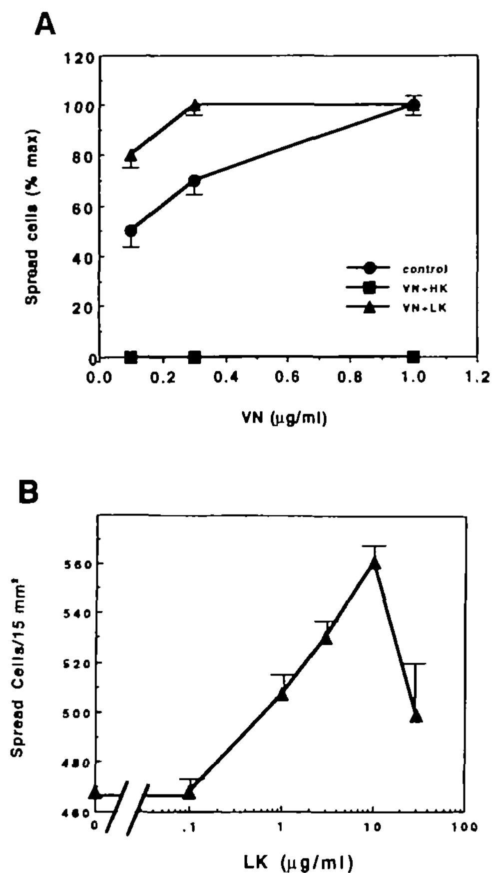

Fig. 8. **Effect of LK on VN-dependent cell adhesion.** (A) Various concentrations of VN (0.1-1 μg/ml) were coated onto tissue-culture plates. After blocking with 1% albumin, 10 μg/ml HKa (closed squares) or LK (closed triangles) in 0.2% albumin or 0.2% albumin alone (closed circles) was added, followed by the addition of MG-63 cells. Spread cells were quantitated after 1 h and normalized against the number of spread cells in wells treated with 1 μg/ml VN. (B) VN (0.1 μg/ml) was coated onto tissue-culture plates. After blocking with 1% albumin, LK (0.1-30 μg/ml) was added, followed by the addition of MG-63 cells. Spread cells were quantified after 1 h. (C) Effect of assay conditions on LK-dependent cell adhesion onto the VN substratum. Tissue-culture plates were coated with N (0.1 μg/ml) and blocked with 1% albumin. Some wells were then incubated with the indicated concentrations of LK, washed, and used in cell adhesion assays of MG-63 cells (VN+LK, closed circles). For the other wells, MG-63 cells and the indicated concentrations of LK were preincubated for 30 min. Cells were washed 2 times by centrifugation, then added to VN-coated wells (cells+LK, closed squares). Cell spreading was quantified at 1 h and normalized against controls in which cells or wells were not incubated with LK.

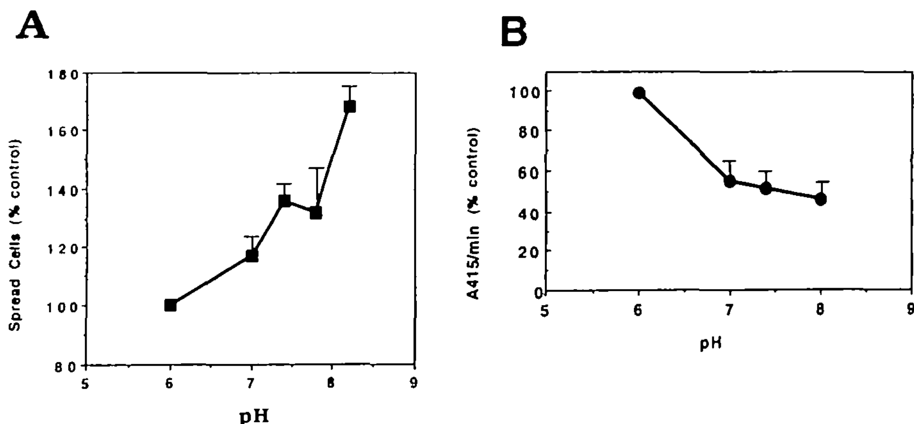

Fig. 9. **Effect of pH on LK-dependent cell attachment to VN-coated tissue-culture wells.** (A) VN (0.1 μg/ml) was coated onto tissue-culture plates. After blocking with 1% albumin, 10 μg/ml of LK at different pHs in PBS was added to the wells. After incubation for 1 h, wells were washed with PBS, MG-63 cells were added, and attached cells were counted at 1 h and normalized against controls in which cells were attached to VN-coated wells preincubated with LK at pH 6. (B) LK (10 μg/ml) at different pHs in PBS was incubated with VN-coated wells for 1 h. After washing of wells with PBS, polyclonal antibody to HK that was cross-reactive with LK (1:1,000 dilution) was added to the tissue-culture plate and incubated for 2 h at 37°C. After washing of wells with PBS, second antibody conjugated with alkaline phosphatase was added.

TABLE II. **Adhesion-promoting profiles of various kininogen heavy chain domains in the presence of vitronectin.** The adhesion of proteins (10 μg) in 1 h was assayed with tissue-culture plates coated with 0.1 μg/ml VN. Values represent means and ranges of triplicate wells.

Protein	Spread cells/15 mm²
LK	570±20
D1	410±20
D2	430±30
D3	560±10
VN alone	470±10

indicates that the modulatory effects of D5 on conformation require the presence of the heavy chain.

A likely hypothesis, described in Fig. 10, is that immobilized VN binds to soluble HKa *via* D5, resulting in changes in the conformation of the heavy chain as well as D5, and masking of the RGD cell-adhesive site of VN.

The following evidence supports our hypothesis. (i) The anti-cell-adhesive activity is strong when HKa is exposed to VN immobilized on a plastic surface. (ii) HKa binds to immobilized VN, and D5 of HKa is the pivotal domain for binding to VN, as depicted in Fig. 6. A precise binding study

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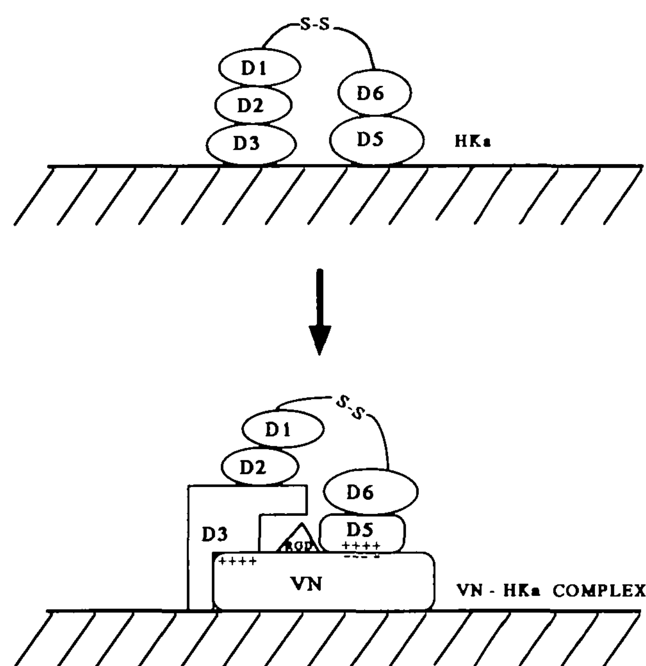

Fig. 10. **Hypothetical model of the conformational transition of surface bound-HKa to HKa complexed with immobilized VN.** The histidine-rich domain in D5 of HKa binds the negatively charged somatomedin-like domain of VN adjacent to the RGD domain. The conformational transition of D5 influences the structure of the heavy chain, especially D3, thereby masking the RGD cell-adhesive site of VN.

including scatchard analysis will be reported elsewhere. (iii) D3 as well as D5 of HKa binds to endothelial cell surface (*55*). (iv) The adsorption of ^{125}I-VN was not influenced by the adsorption of HKa, although the antigenicity of adsorbed VN did decrease (*11*). The antigenicities of VN when plates were treated with VN/r-HRD and VN/HKa were 95 and 25%, respectively, when analyzed by ELISA (precise data not shown). The antigenicities were not changed (VN/rHRD, 103%; VN/HKa, 98%) when analyzed by use of radiolabeled-VN under the same conditions. These data indicate that the heavy chain of HKa may play the pivotal role in masking of the RGD cell-binding site of VN. Further study will be necessary to analyze the interaction between the heavy chain of HKa and VN.

Interestingly, the conformational changes of HKa after binding to VN were influenced by the adsorbed conformer of VN, as depicted in Table II. Thus the masking effect of the heavy chain of HKa on immobilized VN may differ when tissue-culture plates or ELISA plates are used for cell adhesion assay. (v) There is less possibility that the heavy chain may contain the determinant that interacts with cells and blocks β3-dependent cell spreading, because monoclonal antibodies against heavy chain did not block anti-cell-spreading activities. (vi) Also there is little possibility that the heavy chain causes the light chain to adsorb in a conformation in which the light chain contains a determinant that inhibits β3-dependent cell spreading, because the anti-D5 antibodies block the anti-cell-spreading effect only when present during the adsorption step and are inactive when incubated with adsorbed HKa.

The biological importance of inhibition by HKa or HK of β3-integrin-mediated cell spreading is unclear. Humans deficient in HK do not have an obvious phenotype, despite the many interesting biological activities of HK and its derivatives (*56*). Nevertheless, these proteins may play a modulatory role in certain pathophysiological situations. We have previously speculated that HKa may be antithrombotic by virtue of its ability to block adhesion of cells to surfaces (*11*). αvβ3 has been implicated in angiogenesis, and antagonists of αvβ3 have been shown to inhibit endothelial cell growth and promote tumor regression (*57-60*). In pilot studies, we found that cell spreading of suspended MG-63 cells on a matrix elaborated by other MG-63 cells is inhibited by HKa, *i.e.*, HKa interacting with a physiological matrix acts like HKa on tissue-culture polystyrene rather than HKa on untreated polystyrene (Q. Zhang and D. F. Mosher, unpublished observations). HK and HKa, therefore, could modulate the interactions of αvβ3-bearing endothelial cells with cognate ligands in tissues. *From a therapeutic point of view, our studies* indicate that such activities of HK and HKa require the whole molecule and cannot be duplicated by a small segment.

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