Different mechanisms define the antiadhesive function of high molecular weight kininogen in integrin- and urokinase receptor–dependent interactions

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Proteolytic cleavage of single-chain high molecular weight kininogen (HK) by kallikrein releases the short-lived vasodilator bradykinin and leaves behind 2-chain high molecular weight kininogen (HKa) that has been previously reported to exert antiadhesive properties as well as to bind to the urokinase receptor (uPAR) on endothelial cells. In this study we defined the molecular mechanisms for the antiadhesive effects of HKa related to disruption of integrin- and uPAR-mediated cellular interactions. Vitronectin (VN) but not fibrinogen or fibronectin-dependent αvβ3 integrin–mediated adhesion of endothelial cells was blocked by HKa or its isolated domain 5. In a purified system, HKa but not HK competed for the interaction of VN with αvβ3 integrin, because HKa and the isolated domain 5 but not HK bound to both multimeric and native VN in a Zn²⁺-dependent manner. The interaction between HKa or domain 5 with VN was prevented by heparin, plasminogen activator inhibitor-1, and a recombinant glutathione-S-transferase (GST)-fusion peptide GST-VN (1-77) consisting of the amino terminal portion of VN (amino acids 1-77), but not by a cyclic arginyl-glycyl-aspartyl peptide, indicating that HKa interacts with the amino terminal portion of VN (“somatomedin B region”). Furthermore, we have confirmed that HKa but not HK bound to uPAR and to the truncated 2-domain form of uPAR lacking domain 1 in a Zn²⁺-dependent manner. Through these interactions, HKa or its recombinant His-Gly-Lys–rich domain 5 completely inhibited the uPAR-dependent adhesion of myelomonocytic U937 cells and uPAR-transfected BAF-3 cells to VN and thereby promoted cell detachment. By immunogold electron microscopy, both VN and HK/HKa were found to be colocalized in sections from human atherosclerotic coronary artery, indicating that the described interactions are likely to take place in vivo. Taken together, HK and HKa inhibit different VN-responsive adhesion receptor systems and may thereby influence endothelial cell– or leukocyte-related interactions in the vasculature, particularly under inflammatory conditions. (Blood. 2000;96: 514-522)

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Introduction

Cell-to-cell and cell-to-extracellular matrix interactions determine morphogenetic processes during development, vascular remodeling, or inflammation. The dynamics of cellular contacts require the presence of pro- and anti- or counter-adhesive components as well as different proteolytic systems that need to be regulated in a spatiotemporal manner. In particular, the pattern of expression of adhesion receptors such as selectins or integrins in the vasculature as well as changes in the composition of the extracellular matrix are directly related to alterations of vascular cell interactions and relate to the pathophysiology of cardiovascular diseases. The multifunctional proteins fibrinogen (FBG)/fibrin and vitronectin (VN) accumulate at extracellular matrix sites associated with wound healing, malignant tumors, or angiogenesis. These and other adhesive glycoproteins are recognized by integrins of the αv subfamily, which are prominently expressed on migrating and proliferating vascular cells and whose functions can effectively be blocked by synthetic antagonists such as cyclic arginyl-glycyl-aspartyl (cRGD) peptides. Moreover, the plasminogen activation system contributes to cell invasion by mediating not only pericellular proteolysis but also by participating in the modulation of cell adhesion in a nonproteolytic fashion. Through direct interactions particularly expressed at vitronectin-rich matrix sites, urokinase (uPA) acts as a proadhesive factor, whereas plasminogen activator inhibitor-1 (PAI-1) abrogates both integrin- and urokinase receptor (uPAR)-dependent cell adhesion. In the latter system, uPAR also serves as a high-affinity cell surface–associated binding protein for VN. A third ligand for uPAR is high molecular weight kininogen (HK); however, the functional relationships among these components in cellular contacts remain to be defined.

HK is composed of 6 domains and is present in plasma at a concentration of 0.67 µmol/L. It serves a nonenzymatic cofactor role in the initiation of the contact phase, associated with vascular injury, inflammation, or activation of complement in humoral immune defense. In particular, kallikrein can liberate the short-lived vasodilator peptide bradykinin from HK, thereby generating HKa (2-chain kinin-free HK), which lacks most of its domain 4. Domain 5 in HKa is rich in His, Gly, and Lys, which enables HKa to bind to anionic surfaces, zinc, or heparin.

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Moreover, HK/HKa binding to cells is mediated by domains 3 and 5, contributing to the regulation of pericellular plasmin generation by modulating plasma kallikrein-dependent formation of uPA, a reaction dependent on the binding of plasma prekallikrein to domain 6. On granulocytes, HK and FBG compete for binding to the integrin α5β2 whereas on endothelial cells the binding proteins for globular C1q (denoted gC1qR) as well as cytokeratin-134-36 were identified as binding proteins for HK. This role for gC1qR appears to be a mitochondrial protein. On endothelial cells, HKa is recognized by uPAR, and this Zn2+-dependent binding can be inhibited by VN.

These diverse observations, together with a recent report on antiadhesive properties of HKa, prompted us to investigate the underlying mechanisms for the contribution of kinogenins in adhesive interactions involving different adhesion receptors on blood and vessel wall cells. Our results indicate that direct binding of HKa to matrix-associated VN competes for integrin- and uPAR-dependent cell adhesion uncovering a plausible mechanism for the antiadhesive properties of HKa in tissue remodeling.

### Materials and methods

#### Reagents

Recombinant Gly158scuPA (noncleavable mutant of high molecular weight uPA) was produced in Chinese hamster ovary cells and was provided by Dr H. Roger Lijnen (Leuven, Belgium). uPA was from Medac (Hamburg, Germany); VN was purified from human plasma and converted to the multimeric form as previously described41-42. FBG was purchased from Kabivitrum (Munich, Germany); fibronectin was from Sigma (Munich, Germany); αvβ3-containing peptide (cRGDfV) was from Bachem (Heidelberg, Germany); and recombinant αvβ3 integrin was kindly provided by Dr Simon Goodman (Merck KGaA, Darmstadt, Germany). Recombinant soluble uPAR (suPAR) was obtained from Dr Niels Behrendt (Finsen Laboratory, Copenhagen, Denmark). Single- and 2-chain high molecular weight kinogenins (HK and HKa) were purchased from Enzyme Research Laboratory, Copenhagen, Denmark. Single- and 2-chain high molecular weight (HK and HKa) were purchased from Enzyme Research Laboratories (South Bend, IN). The purified HK and HKa (more than 95%) appeared as a major band of 140 kD and 110 kD, respectively, on nonreduced sodium dodecyl sulfate gels. HK had been digested with plasma kallikrein (HK to kallikrein = 100:1, mol/mol) for 20 minutes at 37°C. The resulting HKa was composed of 2 bands of 62 and 46 kDa when analyzed by reduced sodium dodecyl sulfate gel electrophoresis. Glutathione-S-transferase (GST) fused to domains 3, 5, and 6 of HK were produced as previously described.43-45 GST was N-terminally attached to the following sequences of HK: G235 to M357 (domain 3), K420 to S513 (domain 5), and T503 to S626 (domain 6). Likewise in GST-VN (1-177), GST was fused to the amino terminal portion (amino acids 1-77) of VN and produced as previously described.44 Active PAI-1 was from Astra Hassle AB (Mölndal, Sweden). Murine monoclonal antibody (mAb) 131H against human VN was kindly provided by Dr Paul Declerck (Leuven, Belgium); αvβ3 integrin (each at 5 µg/mL) was provided by Dr Paul Declerck (Leuven, Belgium); or 3 integrin (each at 5 µg/mL) was provided by Dr Paul Declerck (Leuven, Belgium). Binding was quantitated at 405 nm in a Thermomax Reader (Molecular Devices, Menlo Park, CA). The identical protocol was used when the binding of HK/HKa in the presence or absence of 50 µmol/L NaCl37 to immobilized multimeric or native VN, uPAR, or αvβ3 integrin (each at 5 µg/mL) was tested, except that mAb HKH13 or HKH18 was used for the detection of bound kinogenins. Nonspecific binding to BSA-coated wells was used as an additional control in all experiments and was subtracted in calculating the specific binding.

#### ELISA for ligand-receptor interactions

Maxisorp plates (high binding capacity; Nunc, Roskilde, Denmark) were coated with uPAR (5 µg/mL) or different forms and fragments of HK (HKa, D3, D5, D6; each at 5 µg/mL), respectively. After incubation for 2 hours at 22°C, mAb VN-71 against VN at a concentration of 125 ng/mL was added, followed by addition of a secondary goat antimouse immunoglobulin G (Dako, Hamburg, Germany) and the substrate ABTS, and the reaction was quantitated at 405 nm in a Thermomax Reader (Molecular Devices, Menlo Park, CA). The identical protocol was used when the binding of HK/HKa in the presence or absence of 50 µmol/L ZnCl2 to immobilized multimeric or native VN, uPAR, or αvβ3 integrin (each at 5 µg/mL) was tested, except that mAb HKH13 or HKH18 was used for the detection of bound kinogenins. Nonspecific binding to BSA-coated wells was used as an additional control and was subtracted to calculate the specific binding. In case of binding of HK/HKa to immobilized VN, uPAR, or αvβ3 integrin, binding to noncoated wells and to BSA-coated wells in the absence or presence of ZnCl2 was also estimated to calculate nonspecific binding. These values were also subtracted to estimate the specific binding of HK/HKa.
Cell adhesion assays

Cell adhesion to VN-, FBG-, or fibronectin-coated plates (and to BSA-coated wells as control) was tested according to a previously described protocol. Briefly, multwell plates were coated with 2 µg/mL native or multimeric VN or 10 µg/mL FBG or fibronectin (dissolved in bicarbonate buffer, pH 9.6), respectively, and blocked with 3% (wt/vol) BSA. BAF-3 or U937 cells, which had been differentiated for 24 hours with vitamin D3 (100 nmol/L) and transforming growth factor-β (2 ng/mL), were washed in serum-free RPMI and plated onto the precoated wells for 60 to 90 minutes at 37°C in the absence or presence of competitors in serum-free RPMI. Proteolytic conversion of HK to HKa in the absence and the presence of monocytic cells as described in the same adhesion assay was performed and, after the incubation period, the supernatant was collected and analyzed for HK or HKa in a Western blot using the antibody HKH18.7

Confluent BREC, BAEC, or MG63 human osteosarcoma cells were detached with trypsin, which was subsequently neutralized with soybean trypsin inhibitor (Sigma), washed, and plated onto precoated wells as described above. After the incubation period for the adhesion assay in serum-free DMEM, the wells were washed and the number of adherent cells were quantified by crystal violet staining at 590 nm.

Electron microscopy

Secondary antibodies conjugated to 15 nm colloidal gold were from BioCell Research Labs (Boston, MA), and protein A coupled to 10 nm gold was from the Department of Cell Biology, University of Utrecht, The Netherlands. All other reagents used for electron microscopy were from TAAB Laboratory Equipment Ltd, Reading, England.

Sections of human atherosclerotic coronary arteries were obtained within 2 hours of surgery, cut into approximately 1-µm pieces, and fixed in 4% (wt/vol) paraformaldehyde/phosphate-buffered saline (PBS) on ice for 30 minutes. Specimens were dehydrated through a graded series of ethanol solutions with progressive lowering of temperature for 1 hour each (30% at 0°C, 50% at −20°C, 70% at −30°C, 90% at −30°C, 100% ethanol at −30°C) and then infiltrated with 50% (vol/vol) K4M Lowicryl (TAAB) in ethanol at −30°C for 16 hours followed by 100% K4M Lowicryl at −30°C for 2 × 12 hours. Finally, tissues were embedded in K4M Lowicryl at −30°C for 16 hours under UV light, and polymerization was completed at room temperature under UV light. Blocks were cut on an Ultracut microtome, and 80-nm–thick sections were taken onto 400 mesh copper/formvar film, and sections were contrasted using uranyl acetate and lead citrate. Sections were analyzed using a Philips 201 transmission electron microscope.

Results

Inhibition of αvβ3 integrin–dependent adhesion to VN by kinogen

Endothelial cells adhere via different integrins to matrix proteins such as VN, FBG, or fibronectin. The effect of HK or HKa on the adhesion of BREC, BAEC, and MG63 human osteosarcoma cells to these proteins was tested. Adhesion to VN was αvβ3 integrin–mediated, as it was abolished by cRGDfV. Similar to PAI-1, which can block this αvβ3 integrin–VN interaction, HKa reduced endothelial cell adhesion to VN by 70% to 80%, whereas HK had a much weaker effect. Neither HK nor HKa affected the adhesion of these cells to FBG or fibronectin (Figure 1A). To further define the specificity of the antiadhesive properties of HKa for the VN substrate, 2 adhesion protocols were compared: (1) cells and competitors were added simultaneously to the VN-coated wells, or (2) prior to the adhesion assay, the VN-coated wells were incubated with the competitors for 1 hour, followed by extensive washing. In both cases, HKa inhibited cell adhesion in a pattern as described above.
similar to PAI-1 or mAb 13H1 directed against VN, which both block VN-dependent adhesion due to direct binding to VN. In contrast, the cRGDfV inhibited adhesion when added simultaneously to the cells, but it had no effect when it was preincubated on the VN substrate. The latter was expected, because cRGDfV abolishes adhesion by binding specifically to the \( \alpha v \beta_3 \) integrin but not by binding to VN (Figure 1B). Adhesion to FBG was not blocked even by high concentrations of kininogens, when added simultaneously to the seeded cells, whereas partial inhibition of cell adhesion was observed after preincubation of HKa with immobilized FBG prior to the cell adhesion assay. This reduction in cell adhesion was due to the Vroman effect, because HKa displaced FBG from the adhesion plate (data not shown), while VN was resistant to the Vroman effect (data not shown). When endothelial cell adhesion was compared with both multimeric VN or native VN, there was hardly any difference between both substrates. HKa but not HK could block cell adhesion to multimeric VN and native VN to the same extent (not shown).

In subsequent experiments, the effect of different forms of kininogen on adhesion of endothelial cells to VN was investigated (Figure 2A). HKa was by far the most effective component (inhibitory concentration of 50% \( = 25 \text{ nmol/L} \)) and could completely block cell adhesion at a concentration of 10 \( \mu \text{g/mL} \) (about 85 nmol/L). While domain 5 but not 3 could also reproduce most of the HKa effect, higher concentrations of HK were needed to provide a maximal inhibition of 50%. In contrast to cRGDfV, which was able to reverse the adhesion process partially (Figure 2B), HK or HKa did not promote detachment of endothelial cells that had adhered onto the VN substrate for 1 hour. Similar results as with BRECs and BAEC were also obtained with MG63 human osteosarcoma cells (not shown).

**Binding of kininogen to VN**

The specificity of the antiadhesive effect of HKa in the \( \alpha v \beta_3 \) integrin–VN interaction can be explained by direct blockade of either the integrin \( \alpha v \beta_3 \) or the substrate VN by HKa. The latter was demonstrated by the fact that preincubation of the VN substrate with HKa but not with HK could inhibit cell adhesion. HKa blocked the binding of \( ^{125}\text{I}-VN \) to isolated \( \alpha v \beta_3 \) integrin (Table 1), whereas HK had minimal inhibitory activity. While HKa at 20 \( \mu \text{g/mL} \) (170 nmol/L) reduced specific binding by 60%, PAI-1 at 200 nmol/L diminished binding by 85%; however, these concentrations of HKa and PAI-1 induced a similar inhibition of endothelial cell adhesion to VN. Because both HKa and VN are \( \text{Zn}^{2+} \)-binding proteins, the binding of different concentrations of VN to immobilized HK or HKa was tested in the absence or presence of \( \text{ZnCl}_2 \) in an ELISA for direct ligand-receptor interactions. \( \text{Zn}^{2+} \)-dependent specific binding of VN to only HKa but not to HK was observed. In addition to HKa, VN could specifically interact with immobilized domain 5 in a \( \text{Zn}^{2+} \)-dependent manner, whereas no binding to domains 3 or 6 was found (Figure 3A). In the reverse situation, specific binding of soluble HKa and domain 5 but not HK to immobilized multimeric or native VN was noted, where more efficient binding was seen with immobilized multimeric VN (Table 2). Also, multimeric VN bound more efficiently to immobilized HKa or domain 5 than did native VN (Table 3). Heparin almost completely abolished the binding of multimeric VN to HKa or domain 5 but only partially interfered with the binding of native VN (Table 3). Because the interaction between VN and HKa or domain 5 was prevented by PAI-1 or by the fusion product GST-VN (1-77) (Figure 3B), kininogen forms can compete with PAI-1 for an overlapping binding site on VN, which

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**Table 1. Effect of different competitors on the binding of \( ^{125}\text{I}-VN \) to immobilized \( \alpha v \beta_3 \) integrin**

<table>
<thead>
<tr>
<th>Additives</th>
<th>Binding of ( ^{125}\text{I}-VN ) (cpm/well)</th>
<th>Specific binding (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>( 3311 \pm 122 )</td>
<td>( 100 \pm 4 )</td>
</tr>
<tr>
<td>&quot;Cold&quot; VN (nonspecific binding)</td>
<td>( 254 \pm 18 )</td>
<td>—</td>
</tr>
<tr>
<td>cRGDfV</td>
<td>( 763 \pm 31 )</td>
<td>( 17 \pm 1 )</td>
</tr>
<tr>
<td>PAI-1</td>
<td>( 621 \pm 31 )</td>
<td>( 12 \pm 1 )</td>
</tr>
<tr>
<td>HK</td>
<td>( 2883 \pm 61 )</td>
<td>( 86 \pm 2 )</td>
</tr>
<tr>
<td>HKa</td>
<td>( 1246 \pm 31 )</td>
<td>( 32 \pm 1 )</td>
</tr>
</tbody>
</table>

The binding of \( ^{125}\text{I}-VN \) to immobilized \( \alpha v \beta_3 \) integrin was carried out in the absence (no additives) or presence of cRGDfV (10 \( \mu \text{g/mL} \)), PAI-1 (200 nmol/L), and HK or HKa (170 nmol/L each) in a TBS buffer containing 50 \( \mu \text{mol/L} \text{ZnCl}_2 \). Nonspecific binding was counted in the presence of 100-fold excess "cold" VN. Data are expressed as counts per minute (cpm)/well. Moreover, specific binding was estimated (by subtracting the nonspecific binding from the total), and data are also expressed as percentage of control, which is represented by the specific binding in the absence of additives. Data are mean \( \pm \text{SEM (n = 3)} \) of a typical experiment; similar results were obtained in 3 separate experiments.
is located within the amino terminal portion of the adhesive protein. In contrast, the cRGDfV had no effect on the kininogen-VN interaction (Figure 3B). No substantial differences in the binding pattern between VN and kininogen were observed regarding

Table 2. Binding of HK or HKa to immobilized multimeric or native VN

<table>
<thead>
<tr>
<th>Additives</th>
<th>VN multimeric</th>
<th>VN native</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.024 ± 0.030</td>
<td>0.002 ± 0.001</td>
</tr>
<tr>
<td>Heparin</td>
<td>0.005 ± 0.013</td>
<td>0.001 ± 0.001</td>
</tr>
<tr>
<td>Zn2⁺</td>
<td>0.025 ± 0.012</td>
<td>0.026 ± 0.033</td>
</tr>
<tr>
<td>Zn2⁺ plus heparin</td>
<td>0.001 ± 0.016</td>
<td>0.003 ± 0.007</td>
</tr>
</tbody>
</table>

The binding of 2 μg/mL HK or HKa to immobilized multimeric or native VN (each 5 μg/mL) is shown. Binding was studied in the absence or presence of Zn2⁺ (50 μmol/L) and heparin (10 μg/mL). Binding of HK to BSA in the absence or presence of Zn2⁺ was 0.074 ± 0.001 and 0.182 ± 0.031, respectively. Binding of HKa to BSA in the absence or presence of Zn2⁺ was 0.106 ± 0.026 and 0.194 ± 0.031, respectively. This nonspecific binding was subtracted to estimate specific binding. Binding is expressed as absorbance at 405 nm. Data are mean ± SEM (n = 3) of a typical experiment, and similar results were obtained in 3 separate experiments.

Table 3. Binding of multimeric or native VN to immobilized kininogen

<table>
<thead>
<tr>
<th>Additives</th>
<th>Multimeric VN binding to</th>
<th>Native VN binding to</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HK</td>
<td>HKa</td>
</tr>
<tr>
<td>None</td>
<td>0.058 ± 0.007</td>
<td>0.087 ± 0.006</td>
</tr>
<tr>
<td>Heparin</td>
<td>0.033 ± 0.001</td>
<td>0.001 ± 0.002</td>
</tr>
<tr>
<td>Zn2⁺</td>
<td>0.075 ± 0.002</td>
<td>0.701 ± 0.067</td>
</tr>
<tr>
<td>Zn2⁺ plus heparin</td>
<td>0.066 ± 0.002</td>
<td>0.021 ± 0.007</td>
</tr>
</tbody>
</table>

The binding of 2 μg/mL multimeric or native VN to immobilized HKa or D5 (each 5 μg/mL) is shown. Binding was studied in the absence or presence of Zn2⁺ (50 μmol/L) and heparin (10 μg/mL). Binding of multimeric VN to BSA in the absence or presence of Zn2⁺ was 0.163 ± 0.014 and 0.132 ± 0.006, respectively. Binding of native VN to BSA in the absence or presence of Zn2⁺ was 0.143 ± 0.005 and 0.142 ± 0.003, respectively. This nonspecific binding was subtracted to estimate specific binding. Binding is expressed as absorbance at 405 nm. Data are mean ± SEM (n = 3) of a typical experiment, and similar results were obtained in 3 separate experiments.

Inhibition of leukocyte adhesion to VN by kininogen

As previously established, the adhesion of myelomonocytic U937 cells (differentiated with transforming growth factor-β [2 ng/mL] and vitamin D₃ [100 nmol/L] for 24 hours) to immobilized VN is predominantly mediated by uPAR.¹⁸,⁵⁵ Moreover, uPA augments this adhesion by increasing the affinity of the uPAR-VN interaction.¹⁹ The same characteristics were found for uPAR-transfected BAF-3 (sense-uPAR) but not for control cells (antisense-uPAR). The addition of HK or HKa resulted in a complete inhibition of adhesion of uPAR-transfected BAF-3 cells and U937 cells also in the presence of uPA, reminiscent of the blocking effect of PAI-1 in this system.¹⁰ An identical pattern of inhibition with HKa, HK, or PAI-1 was obtained for the adhesion of human peripheral blood monocytes (data not shown). HKa but not HK was also antiadhesive when these substances were preincubated onto the VN substrate prior to the cell adhesion step (data not shown).

To characterize the involved domains of HKa responsible for antiadhesion, uPA-stimulated adhesion of U937 cells was tested in the presence of increasing concentrations of HKa, HK, and the recombinant GST-fusion domains 3, 5, or 6, respectively (Figure 4A). HKa, HK, and domain 5 but not domain 3 or 6 abolished cell adhesion, indicating that domain 5 contains most of the antiadhesive activity also for the uPAR-dependent system. To differentiate between the varying antiadhesive activities of HK and HKa, the kinetics of cell adhesion in the presence of both kininogen forms was studied. When cells were plated in the presence of uPA and HK or HKa, respectively, HKa could immediately block the augmenting effect of uPA on cell adhesion, whereas only after 1 hour could HK significantly reduce adhesion (Figure 4B). In contrast to the αv integrin-dependent endothelial cell adhesion, HKa, similar to PAI-1, promoted the immediate detachment of U937 cells that had
adhered to VN for 1.5 hours under the influence of uPA (Figure 4C), whereas about 20 minutes of lag-phase was needed for HK-induced cell dissociation. These findings indicate that HKa was directly interfering with the uPAR-VN interaction, whereas HK needed proteolytic activation by cell-derived proteases as deduced from Western blot analysis (Figure 4D).

Binding of kininogen to uPAR

Subsequent experiments revealed that HKa and the isolated domain 5 inhibited the binding of 125I-VN to immobilized uPAR in a Zn\(^{2+}\)-dependent manner, whereas HK, domain 3, or domain 6 were ineffective (Figure 5). In the absence of Zn\(^{2+}\), HKa and domain 5 presented only a weak inhibition of the uPAR-VN interaction (data not shown). This property of kininogen could be explained by the direct binding of HKa and the domain 5 to VN as described above. Because uPAR has been identified as a binding site on endothelial cells for HKa but not for HK,\(^{20}\) direct binding of HKa to suPAR was tested. As shown in Figure 6A, HKa but not HK bound specifically to immobilized suPAR in a Zn\(^{2+}\)-dependent fashion. Moreover, HKa also interacted with the truncated 2-domain form of uPAR that lacks domain 1. The HKa-uPAR interaction was blocked by VN and also heparin, whereas uPA had no inhibitory effect (Figure 6B). The binding of HKa to uPAR could be attributed to domain 5, because the recombinant GST-D5 but not GST-D3 or GST-D6 inhibited the interaction (data not shown).

Colocalization of VN and HK in sections of human atherosclerotic plaques

Consequences for the above-mentioned molecular relations in (patho)physiology depend on the availability of the different
components at sites of tissue remodeling. As an example, the colocalization of VN with PAI-1 or uPAR could be demonstrated in diseased vessel sections (Lupu et al and Chavakis et al, unpublished observations), implying direct functional interactions. Double-labeling immunogold electron microscopy was performed in sections from human atherosclerotic coronary arteries obtained from cardiac surgery. VN was localized both bound to plasma membranes of cells, particularly smooth muscle cells, as well as associated with different fibrillar or amorphous structures of the extracellular matrix. HK was found in similar locations, and frequently VN and HK were localized in close proximity or direct apposition (Figure 7). These data indicate that the above-described close molecular interaction could also take place in vivo.

Discussion

Apart from its role as precursor of bradykinin that contributes important vasodilator functions in vascular homeostasis, high molecular weight kininogen exhibits antiadhesive properties especially in the 2-chain, kinin-free form (HKa). In the present study, we continued to define the underlying mechanisms of the antiadhesive function of HKa. The characterization of the binding interactions to different adhesion receptors as well as to VN-rich matrices revealed that HKa is responsible for the disruption of uPAR and αvβ3 integrin–dependent cellular contacts. HKa was shown to directly bind to VN and uPAR, to abrogate endothelial and leukocytic cell adhesion, and thereby could play a regulatory role in tissue remodeling of the vasculature. Binding of HKa to VN was blocked by recombinant uPAR and heparin as well as by active PAI-1 but not by cRGDFV, indicating that HKa does not interfere with the cell attachment sequence of VN but competes with PAI-1 or uPAR for binding to the amino terminal “somatomedin B” domain of VN, which is proximal to the integrin recognition motif.

This hypothesis is also strengthened by the fact that the kininogen-VN interaction was blocked by a GST-fusion peptide consisting of the amino terminal portion of VN (amino acids 1-77). The antiadhesive function of HKa is particularly expressed in the His-Gly-Lys–rich domain 5, which contains the major cell binding domain and resembles the activity of PAI-1 as a prominent counter-adhesive factor in VN-dependent cell adhesion mediated by both integrins and uPAR.

Our observations further define the requirements for this function of HKa, conferred by its domain 5. HKa or domain 5 but not domains 3 or 6 inhibited the αvβ3 integrin–dependent adhesion of endothelial cells to VN but did not promote detachment of cells that had already adhered to VN. HK/HKa did not block endothelial cell adhesion to fibronectin and, in contrast to Asakura and coworkers, we could not demonstrate HKa-dependent blockade of adhesion of endothelial cells to FBG, because HKa did not directly bind to the αvβ3 integrin or this integrin ligand (data not shown). However, HKa preincubated onto FBG-coated surfaces prior to the adhesion step resulted in partial inhibition of cell adhesion. This was due to the Vroman effect—namely, the displacement of FBG from the plate by HKa. This effect did not take place when the surface was coated with VN, which is in accordance with the known “resistance” of VN to the Vroman effect. In addition, HKa or domain 5 completely abolished the uPAR-dependent adhesion to VN of differentiated U937 myelomonocytic cells as well as of uPAR-transfected BAF-3 cells. As a consequence and in contrast to the effect on integrin-mediated adhesion with endothelial cells, HKa promoted the detachment of adherent U937 cells, because HKa competed with both uPAR and the substrate VN. Thus, the inhibitory effect of HKa appears to be specific for VN-rich tissue matrices such as in provisional wounds and can be attributed to direct blockade of the substrate and of uPAR.

Instantaneously, HKa was antiadhesive when added together with the cells or when preincubated onto the VN substrate, whereas HK, which does not bind VN, was antiadhesive after a lag-phase only in the first case. These observations can be best explained by the fact that HK is converted to HKa by proteases in the cell cultures to become a potent antiadhesive factor, and data supporting this hypothesis were presented. Cell surface–dependent conversion of HK to HKa was recently described, implying that HK itself appears to be required for prekallikrein activation and thereby promotes its own conversion into HKa.

The consequences of the antiadhesive properties of kininogen are several. After tissue damage, leukocytes are recruited to the injured site, and neutrophil and monocyte adhesion to a provisional matrix is pivotal for this process. In the inflamed or injured tissue, the release of bradykinin from HK is enhanced, which also leads to a localized infiltration of additional inflammatory cells. Moreover, through inhibition of αv integrin–dependent adhesion, HKa could contribute to angiogenesis-regulating activities, because the αvβ3 integrin–VN interaction appears to be crucial during neovascularization in different tissues. Peptides derived from kininogen’s domain 5 inhibit new vessel formation in the chicken chorioallantoic membrane, and work is in progress to define the role of HKa or fragments thereof in angiogenic processes in malignancies or in diabetic retinopathy.

The binding of HKa to both uPAR and VN on the cell surface may approximate kallikrein and prourokinase, thereby leading to potent plasmin formation. In addition, HKa binds directly to plasminogen, which could further amplify plasmin generation. Indeed, we observed colocalization of VN and kininogen at sites of
tissue remodeling such as in the vasculature and, by immunogold electron microscopy, HK/VN-colocalization was demonstrated in human atherosclerotic coronary arteries. These data indicate that the described interactions between kininogen and VN may take place in vivo. Depending on its local concentration, however, HKa could also interfere with the assembly of the ternary uPA/uPAR/VN complex on the surface of vascular cells or the extracellular matrix, which augments plasmin generation.61 These interactions not only define HKa as an intrinsic regulator of the fibrinolytic system but, through its functions as antiadhesive component, HKa also may play a regulatory role in angiogenesis, arteriogenesis, or cancer metastasis.

Prior to the findings that HKa binds to VN and uPAR, other kininin binding proteins were identified that include cellular receptors such as Mac-1 on neutrophils53 or glycoprotein Ib on unactivated platelets62 as well as cell-associated thrombospondin on activated platelets.63 Moreover, cytokertatin-134 and gC1qR were identified as binding proteins for HK on endothelial cells,5,15,64 the latter also serving as binding protein for multimeric VN.64 Interestingly, VN is found on the surface of several other cell types when cultivated in serum and can possibly account for an appreciable degree of binding sites for kininogen and other cellular ligands.20 In particular, specific binding of both VN and kininogen tp various bacterial strains could play a role in the infection process and the host response toward bacterial entry.65,66

Finally, recent studies with VN knockout mice67 and with kininogen-deficient rats68 indicated a similar phenotype in both cases that was more sensitive to thrombosis. Thus, the proposed anthrombotic properties of HK and its fragments could be mediated at least in part by interactions with VN, because loss of VN in mice is expected to influence the functional status of its ligands such as PAI-1 and kininogen as well. Because several ligands of VN exhibit opposing functional activities in hemostasis and cell adhesion, a detailed dissection of transgene animal models can lead to further information on the pathophysiologic role of this multifunctional adhesive protein.

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