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Apoptotic Effect of Cleaved High Molecular Weight Kininogen Is Regulated by Extracellular Matrix Proteins

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Abstract We previously reported that cleaved high molecular weight kininogen (HKa) and its domain 5 (D5) inhibit critical steps required for angiogenesis and in vivo neovascularization (Colman et al. [2000]: Blood 95:543–550). We have further shown that D5 is able to induce apoptosis of endothelial cells, which may represent a critical part of the anti-angiogenic activity of HKa and D5 (Guo et al. [2001]: Arterioscler Thromb Vasc Biol 21:1427–1433). In this study, we demonstrate that HKa- and D5-induced apoptosis is closely correlated with their anti-adhesive effect. An important new finding is that the apoptotic activity of HKa and D5 is highly regulated by their interactions with different extracellular matrix (ECM) proteins. HKa inhibited cell adhesion to vitronectin (Vn, 90%) and gelatin (Gel) (40%), but it had no apparent effect on cell adhesion to fibronectin (Fn). D5 showed a similar pattern on cell adhesion but was less potent than HKa. HKa induced apoptosis of endothelial cells grown on Vn and Gel but not cells grown on Fn which closely parallels with its anti-adhesive potency. Further results revealed that the anti-adhesive effect and the apoptotic effect of HKa are associated with its ability to inhibit phosphorylation of focal adhesion kinase (FAK) and paxillin, two important signal molecules required for cell adhesion and cell viability. We conclude that the anti-adhesive activity of HKa and D5 is responsible for their apoptotic effect and that Vn is likely an ECM component that mediates the effect of HKa and D5. J. Cell. Biochem. 89: 622–632, 2003. © 2003 Wiley-Liss, Inc.

Key words: apoptosis; angiogenesis; cell adhesion; focal adhesion kinase; endothelial cells

High molecular weight kininogen (HK) is a plasma protein that was first identified as a precursor of the bioactive peptide bradykinin, a potent vasodilator that regulates local blood pressure, microvessel permeability, and pain sensation. We now recognize that HK is a multifunctional protein that plays important roles in many pathophysiological processes, such as fibrinolysis, thrombosis, and inflammation [Colman, 1999]. HK is a 120 kDa single-chain glycoprotein consisting of six domains (designated as D1–D6, respectively) with each having distinct functions [Colman and Schmaier, 1997]. After proteolytic cleavage of HK by kallikrein and release of bradykinin contained within D4, the remaining portion of the molecule, HKa, consists of a heavy chain containing D1, D2, and D3, and a light chain containing D5 and D6. The heavy and light chains are linked together by a single disulfide bond. The transition from HK to HKa involves major conformational changes and leads to a greater surface exposure of the D5 region. As a result, HKa acquires new properties. In comparison with HK, HKa shows increased anti-adhesive effect due to domain rearrangements [Asakura et al., 1992; Weisel et al., 1994]. HK can specifically and reversibly bind to endothelial cells through D3 and D5 in a Zn^{2+}-dependent manner. Thus, the endothelial cell surface is an important site for the generation of both bradykinin and HKa, each of which affects the physiology of endothelial cells. While the involvement of bradykinin in the regulation of many cardiovascular processes has been intensively studied, the physiological implications of the generation of HKa are not clear. Recent studies from our laboratory and other investigators indicate that HKa may

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act as a naturally occurring angiogenic inhibitor [Colman et al., 2000; Zhang et al., 2000; Guo et al., 2001, 2002]. Interestingly, it has recently been shown that bradykinin stimulates angiogenesis [Parenti et al., 2001; Colman et al., 2003]. Therefore, HK is a precursor of both an angiogenic stimulator (bradykinin) and an angiogenic inhibitor (HKa). Because of their vastly different half-lives and their distinct receptors, HKa and bradykinin may play divergent roles in the regulation of angiogenesis.

Angiogenesis is the process of formation of new capillaries from existing blood vessels. It involves several steps beginning with localized degradation of the basement membrane of the existing vessels by proteases bound to the endothelial cell membrane. This process is followed by the detachment of endothelial cells from adhesion proteins in the extracellular matrix (ECM) and migration into the perivascular space where endothelial cells proliferate rapidly. The new endothelial cells then form tube structures that eventually join and form new capillaries [Folkman and Shing, 1992]. This process is highly regulated by both positive and negative effectors [Beckner, 1999; Browder et al., 2000]. Many growth factors and cytokines stimulate angiogenesis. Basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) are among the well-characterized angiogenic factors [Beckner, 1999]. The recent identification of several endogenous peptides with anti-angiogenic activity provides important insight into how angiogenesis is negatively regulated. An emerging paradigm has been developed that certain proteolytic fragments of plasma or ECM proteins are potent inhibitors of angiogenesis [Browder et al., 2000]. Angiostatin derived from plasminogen and endostatin, a fragment of collagen XVIII, are prototypes of this group of polypeptides [Cao et al., 1997; O'Reilly et al., 1997]. Generation of HKa from HK through proteolytic cleavage follows a similar model to the formation of angiostatin and endostatin. D5 resembles the anti-angiogenic effect of HKa; thus, it has been named kininostatin [Colman et al., 2000]. These endogenous angiogenic inhibitors interfere with one or more of the steps of angiogenesis; however, they may utilize distinctive mechanisms because they are derived from different molecules. In addition to angiogenic stimulators and angiogenic inhibitors, the ECM plays an important role in the regulation of angiogenesis.

Proliferation of endothelial cells and maintenance of their viability is vital not only for angiogenic process but also is critical for preserving the integrity of newly formed blood vessels. Consequently, inhibition of proliferation and/or induction of apoptosis of endothelial cells will effectively disrupt angiogenesis. Following the initial study demonstrating that HKa and D5 inhibited angiogenesis, we further reported that HKa and D5 were able to inhibit proliferation and induce apoptosis of endothelial cells, which together may underlie their anti-angiogenic activity [Guo et al., 2001]. However, the mechanisms of HKa and D5 remain to be determined. In this study, we present substantial evidence to support the hypothesis that HKa- and D5-induced apoptosis of endothelial cells is associated with their anti-adhesive activity, which is specifically regulated by ECM proteins. We further demonstrate that HKa inhibits cell signaling essential for both cell adhesion and cell viability, corresponding to its anti-adhesive effect and apoptotic effect.

MATERIALS AND METHODS

Materials

HKa was purchased from Enzyme Research Laboratories (South Bend, IN). Low molecular weight kininogen (LK) was from Calbiochem (San Diego, CA). Glutathione S-transferase (GST) and recombinant domain 5 (GST-D5, simplified as D5 in the text) were prepared as previously described [Colman et al., 2000]. Endotoxin levels in the preparations of GST and D5 were determined with the chromogenic limulus amebocyte lysate assay using an endotoxin testing kit (BioWhittaker, Walkersville, MD) as described in detail in our previous study [Guo et al., 2001]. Endotoxin contributed from GST and D5 preparations was less that 0.5 U/ml in the cell culture medium, which did not affect proliferation or viability of endothelial cells. Fibronectin (Fn) and bFGF were purchased from Life Technologies (Grand Island, NY). Gelatin (Gel) was from Sigma Chemical Co. (St Louis, MO). Vitronectin (Vn) was kindly provided by Dr. K. Preissner (Max-Planck-institute, Bad Nauheim, Germany).

Cell Culture

Human umbilical vein endothelial cells (HUVEC) were purchased from Clonetics (Walkersville, MD). They were maintained in an endothelial cell growth medium (EGM,

containing growth factors and 10% fetal calf serum) at 37°C in a humidified incubator (5% CO_2, 95% air). Cells from three to six passages were used. Zn^{2+} is required for HKa and D5 binding to endothelial cells; therefore, the cell culture medium contained 15 μM $ZnCl_2$, which did not affect cell viability.

Cell Adhesion and Spreading Analysis

The 96-well cell culture plates were coated with 0.2% Gel, 2 μg/ml Vn, or 10 μg/ml Fn for 12 h at 4°C. The coated dishes were blocked with 0.1% BSA and then washed with phosphate buffered saline (PBS, pH 7.4). To test the effect of HKa or D5, they were included in the coating solutions at the concentrations as indicated in individual experiments. Endothelial cells (2×10^4 cells per well, counted with a hemocytometer) were plated in EGM medium. After incubation for 1 h at 37°C, unattached cells were washed away with PBS. The attached cells were quantified by a Cell Titer AQueous analysis kit from Promega (Madison, WI) according to the manufacturer's instructions.

The effect of HKa and D5 on endothelial cell cytoskeleton structure and cell spreading were examined microscopically. In this assay, glass coverslips were coated with Gel, Vn, or Fn using the same method as for 96-well culture plates. After incubation, the cells adhered to coverslips were fixed with 4% paraformaldehyde in PBS for 20 min at room temperature. Cell morphology was analyzed under a microscope with a phase contrast lens. To analyze the cytoskeleton structure, fixed cells were permeabilized with 0.1% Triton X-100 in PBS for 5 min at room temperature and stained with 5 μg/ml fluorescein-labeled phalloidin (in PBS, Sigma Chemical Co.) to detect F-actin. After washing with PBS, cells were examined under a fluorescence microscope. The images were captured with a CCD digital camera.

Cell Viability and Apoptosis Analysis

Cell viability and apoptosis were assessed by a combination of morphological and metabolic changes by the methods previously described [Guo et al., 1999] with some modifications. Viable cells were quantified by a Cell Titer AQueous analysis kit (Promega). Briefly, cells were plated in 96-well dishes coated with Vn, Gel, or Fn (5×10^3 cells per well) and incubated in EGM medium for 3 h to allow cells for attachment. Cells were then treated with or without HKa or D5 in EBM medium (EGM minus serum and growth factors) in the presence of bFGF for 48 h. At the end of cell treatment, Cell Titer AQueous analysis solution was added to the cell culture medium and incubated for 90 min at 37°C. The absorbencies, which correlate with the amount of live cells, were determined at 490 nm. Cell morphological changes were examined under a microscope periodically during and after cell treatment. Apoptotic cell death was further assessed by nuclear fragmentation/condensation analysis with Hoechst 33285 cell staining as described in our previous studies [Guo et al., 2001].

Cell Lysate Preparation and Western-Blot Analysis

After treatment, cells were collected and resuspended in the lysis buffer [10 mM Tris-Cl (pH 7.5), 150 mM NaCl, 1 mM Na_3VO_4, 1% SDS, 0.5 mM EDTA, 1 μM leupeptin, and 1 μg/ml aprotinin]. Cells were lysed by sonication at 4°C (eight pulses, output control 3) using a Branson sonicator. The solution was centrifuged at 15,000*g* for 15 min. Clarified supernatant was designated as whole cell lysate and was used for Western-blot analysis. Protein concentration was determined using a protein assay kit (Pierce, Rockford, IL). Protein samples were subjected to SDS–PAGE, and separated proteins were then transferred onto nitrocellulose membranes. The membranes were blocked with 5% nonfat dry milk in Tris-buffered-saline containing 0.05% Tween-20, and probed with the following primary antibodies: anti-phospho-focal adhesion kinase (FAK) antibodies (Tyr-397, Upstate Biotechnology, Inc., Lake Placid, NY), anti-FAK (c-903, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-phospho-paxillin antibodies (Tyr-118, Biosource International, Inc., Camarillo, CA), or anti-paxillin (P49620, BD Biosciences, Palo Alto, CA). The membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies. The immunoblots were visualized by an enhanced chemiluminescence kit (Pierce).

RESULTS

Anti-Adhesive Effect of HKa and D5 on Endothelial Cells Is Regulated by Different ECM Proteins

An increased anti-adhesion effect of HKa after its conversion from HK has long been

recognized [Asakura et al., 1992], but the physiological implications of this phenomenon are not clear. To test how HKa and D5 affect adhesion of primary endothelial cells, we examined their effect on the adhesion of HUVEC to Vn, Fn, or Gel, three ECM proteins known to promote endothelial cell adhesion. In this assay, the culture dishes were coated with Gel, Vn, or Fn in the absence (control) or presence of HKa or D5. After incubation for 1 h, more than 90% of seeded cells fully attached to the dishes in the control experiments. As shown in Figure 1, HKa (200 nM) almost completely blocked cell adhesion to Vn. It also partially inhibited cell adhesion to Gel (~40% inhibition) but had no apparent effect on cell adhesion to Fn. D5 (300 nM) displayed a similar pattern on cell adhesion to the three proteins but was less potent than HKa (Fig. 1). In all cases, LK or GST (as a control for D5), had no effect on cell adhesion (Fig. 1). LK has identical D1, 2, 3, and 4 of HK, but its D5 is totally different from that of HK. LK does not have D6 [Colman et al., 1997]. Because LK did not show any anti-adhesive effect (Fig. 1), the effect displayed by HKa is most likely due to its unique amino acid sequence of D5. The anti-adhesion activity of HKa and D5 was further characterized on cells seeded on Vn. As shown in Figure 2, HKa and D5 inhibited cell adhesion in a concentration-dependent manner, with D5 showing a weaker effect than that of HKa at all concentrations tested.

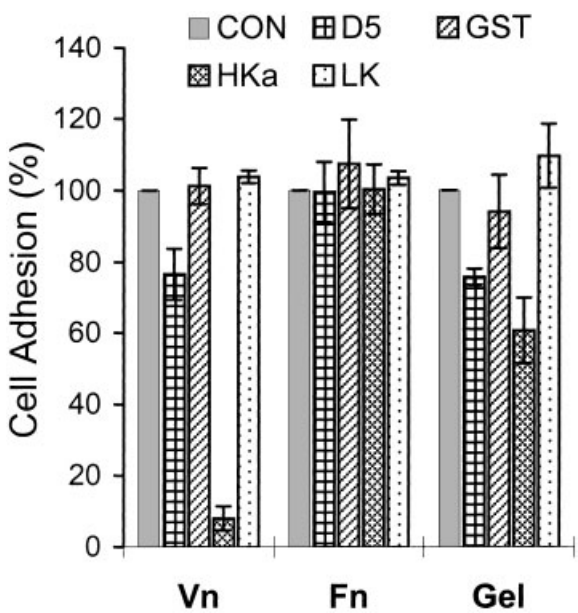

Fig. 1. Cleaved high molecular weight kininogen (HKa) and domain 5 (D5) inhibit adhesion of human umbilical vein endothelial cells (HUVEC) to vitronectin (Vn) and gelatin (Gel) but not fibronectin (Fn). The 96-well cell culture dishes were coated with Vn (2 μg/ml), Fn (10 μg/ml), or Gel (0.2%) in the presence of D5 (300 nM), GST (300 nM), HKa (200 nM), or LK (200 nM). Cells seeded on the coated dishes in the absence of the above agents were used as controls (CON). HUVEC (2×10^4 cells) were seeded on each well in endothelial cell growth medium (EGM) medium. After incubation at 37°C for 1 h, the number of attached cells was determined using the method described in experimental procedures. Controls were taken as 100%. Results are means ± SEM of three experiments.

Effect of HKa and D5 on the Spreading of Endothelial Cells Plated on Different ECM Proteins

Cell adhesion and spreading are closely related but distinctive cellular events. To test how HKa and D5 affect endothelial cell spreading on different ECM proteins, cells grown on Vn, Gel, or Fn were fixed and examined under a phase contrast microscope. Figure 3 illustrates the morphology of HUVEC attached to Vn. In the control experiment, cells showed a fully spread morphology (CON). HKa or D5 significantly inhibited cell spreading (HKa, D5, respectively) while LK did not have any effect.

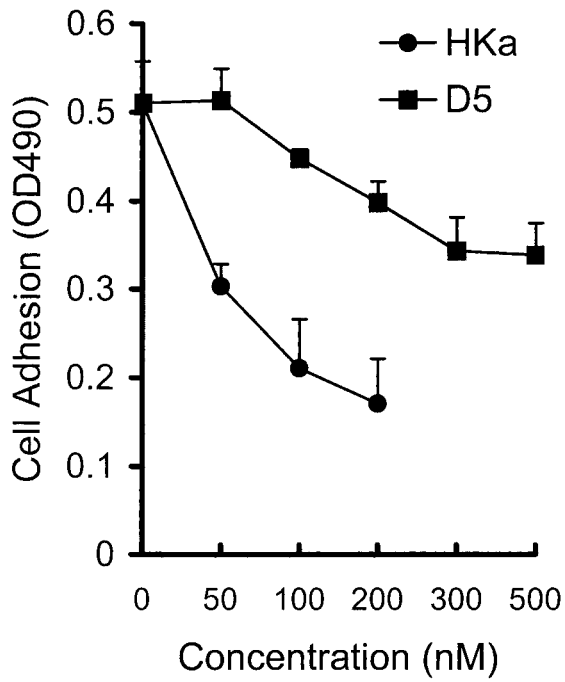

Fig. 2. Inhibition of HUVEC adhesion to Vn by different concentrations of HKa and D5. The 96-well cell culture dishes were coated with Vn (2 μg/ml) in the presence of D5 or HKa at the concentrations as indicated. HUVEC (2×10^4 cells) were seeded on each well in EGM medium. After incubation at 37°C for 1 h, the number of attached cells was determined. Controls were taken as 100%. Results are means ± SEM of three experiments.

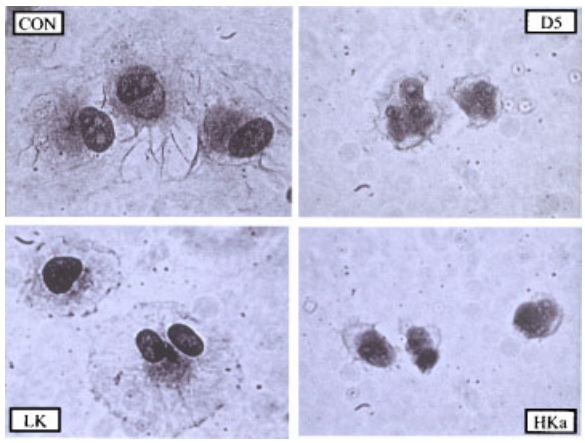

Fig. 3. HKa and D5 inhibit spreading of endothelial cells attached to Vn. HUVEC were plated on coverslips coated with Vn (2 μg/ml) in the presence of 200 nM HKa or LK, or 300 nM D5. The control experiment (CON) represents cells plated on a Vn coated coverslip in the absence of HKa or D5, or LK. After incubation in EGM medium for 1.5 h, cells were fixed and examined under a microscope (400× magnification) and photographed.

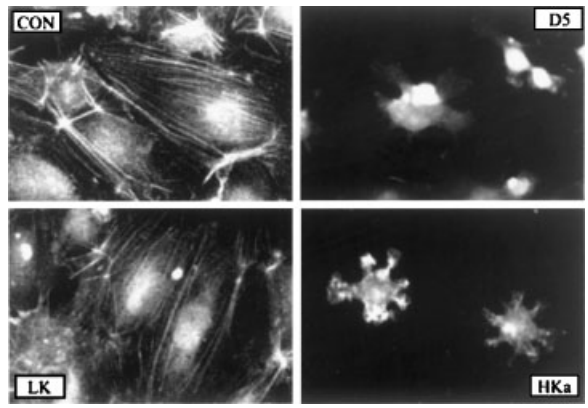

Fig. 4. HKa and D5 inhibit F-actin formation of endothelial cells attached to Vn. HUVEC were plated on coverslips coated with Vn under the same conditions as described in Figure 3. After incubation in EGM medium for 1.5 h, cells were fixed and stained with fluorescein-labeled phalloidin for F-actin. Stained cells were examined under a fluorescence microscope (400× magnification) and photographed.

Similar results were observed when HUVEC were grown on Gel, but HKa and D5 did not inhibit spreading of cells grown on Fn (data not shown).

To examine the effect of HKa and D5 on the cytoskeleton structures of endothelial cells, HUVEC treated under the same conditions described in Figure 3 were stained with fluorescein-labeled phalloidin for actin-filament (F-actin). The fully spread cells adhered to Vn, as seen in the control and LK treated cells in Figure 3 (CON and LK), displayed well-organized F-actin fibers with an ordered orientation (Fig. 4, CON and LK). In contrast, HKa and D5 treated cells showed condensed fluorescence staining with no F-actin fibers detectable (Fig. 4, HKa and D5), demonstrating the typical architecture of nonspreading cells.

HUVEC have been widely used in in vitro experiments to study the steps involved in angiogenesis, but HUVEC are derived from large vein vessels. It is necessary to verify the results obtained from HUVEC in human microvascular endothelial cells (HMEC). These endothelial cells are probably more relevant to in vivo angiogenesis. In our previous study, we found that HKa and D5 showed similar effects on cell proliferation and cell viability in HUVEC and HMEC [Guo et al., 2001]. In the present study, we also observed that HKa and D5 inhibited adhesion and spreading of HMEC on Vn and Gel but not on Fn (data not shown), the same patterns as seen in HUVEC. Taken together, these results suggest that HUVEC are reasonable cells for our in vitro studies.

Effect of HKa and D5 on the Phosphorylation of FAK and Paxillin, the Signaling Molecules Essential for Cell Adhesion and Cell Spreading

Cell adhesion and spreading involve a series of complex signaling events generated from the interaction between cell surface proteins and ECM components. To test whether HKa and D5 interfered with signal transduction, we first examined the activation of FAK, a nonreceptor tyrosine protein kinase that plays critical roles in cell motility and reorganization of cytoskeleton [Rosales et al., 1995]. Activation of FAK requires phosphorylation on its tyrosine residues. Therefore, tyrosine phosphorylation of FAK, which can be detected by Western-blot analysis using antibodies that specifically recognize the phosphorylated forms of FAK, has often been used to indicate its activation. We tested the effect of HKa on the phosphorylation state of FAK. HUVEC were plated on the culture dishes coated with Vn, Gel, or Fn under the conditions as described in Figure 1. Figure 5A illustrates the results obtained from cells that were incubated for 60 min, a time point when more than 90% of seeded cells attached to the dishes in the absence of HKa (controls). Phosphorylated FAK (p-FAK, Tyr-397) was readily detectable in all three control

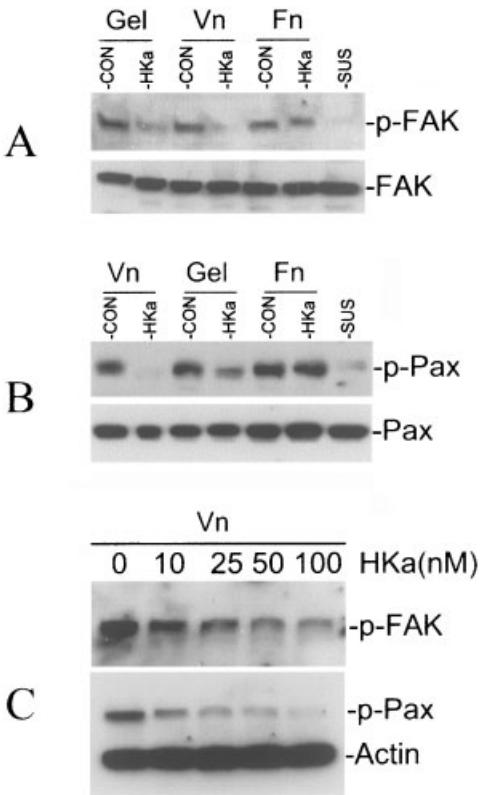

Fig. 5. HKa inhibits attachment-induced phosphorylation of focal adhesion kinase (FAK) and paxillin. HUVEC were seeded on cell culture dishes coated with Gel, Vn, or Fn in the absence of HKa (CON) or presence of HKa (HKa) as described in Figure 1. HUVEC were also kept in suspension (SUS). Cells were collected after 1-h incubation. Total FAK and phospho-FAK (**A**, FAK and p-FAK), total paxillin and phospho-paxillin (**B**, Pax and p-Pax) were detected by Western-blot analysis using their specific antibodies. **Panel C** shows the effect of HKa at different concentrations on p-FAK and p-Pax in cells seeded on Vn-coated dishes (C). The same blots were reprobed with anti-actin antibodies to show that the same amount of proteins was loaded on each lane.

experiments (Fig. 5A, CON). HKa completely abolished FAK phosphorylation in the cells seeded on Vn (Fig. 5A, Vn/HKa). p-FAK was detectable but was significantly reduced in cells seeded on Gel (Fig. 5A, Gel/HKa). HKa did not affect phosphorylation of FAK significantly when the cells were seeded on Fn (Fig. 5A, Fn/HKa). The effect of HKa on FAK phosphorylation is well correlated with its effect on cell adhesion to the three proteins, as described in Figures 1 and 3. Depriving anchorage-dependent cells from attachment by keeping them in suspension will prevent FAK phosphorylation [Yu et al., 1998]. This phenomenon has been confirmed in this study as no phosphorylation of FAK was detected in cells kept in suspension (Fig. 5A, SUS), demonstrating an essential role of FAK activity for cell adhesion.

Paxillin is a substrate of FAK whose function also depends on tyrosine phosphorylation. Together with several other cytoskeleton proteins and signaling molecules, paxillin is a critical component for the formation of focal adhesion plaques required for cell adhesion, spreading, and migration [Yu et al., 1998]. The phosphorylation state of paxillin can be determined by Western-blot using antibodies that only recognize phospho-paxillin. As shown in Figure 5B, the effect of HKa on the phosphorylation of paxillin (p-Pax, Tyr-118, a FAK phosphorylation site) parallels its effect on FAK phosphorylation (Fig. 5A). Thus, HKa inhibited phosphorylation of p-Pax completely in cells seeded on Vn, partially on Gel, but not in the cells seeded on Fn. The same samples used for detecting p-FAK and p-Pax were probed with antibodies that recognize total FAK or paxillin; the protein level of FAK (Fig. 5A) or paxillin (Fig. 5B) was not affected by HKa treatment.

The effect of HKa on the phosphorylation of FAK and paxillin was further analyzed in cells seeded on Vn. The inhibitory effect of HKa was apparent as low as 10 nM and was significant at 100 nM (Fig. 5C). Actin was used in this experiment as a control to show the equal loading of proteins in each lane (Fig. 5C).

Apoptotic Effect of HKa and D5 Is Correlated With Their Anti-Adhesive Activity

Gel, a mixture of hydrolytic products of denatured collagens, was initially chosen for our studies because it is a coating protein routinely used to improve adhesion of endothelial cells in cell culture. We have shown that HKa and D5 induced apoptosis of endothelial cells grown on Gel [Guo et al., 2001], where HKa or D5 exhibited moderate anti-adhesive effect (Fig. 1). We further examined the effect of HKa and D5 on the viability of cells grown on Vn and Fn, two naturally occurring ECM proteins on which HKa or D5 showed maximal and minimal anti-adhesive effect, respectively (Fig. 1). In this experiment, HUVEC were allowed to attach to the dishes coated with Vn or Fn prior to treatment. Cells were then incubated with or without HKa or D5 in EBM medium (EGM minus serum and growth factors) in the presence of bFGF for 48 h. Figure 6 shows the measurement of viable cells after treatment. When cells were grown on Vn, the number of

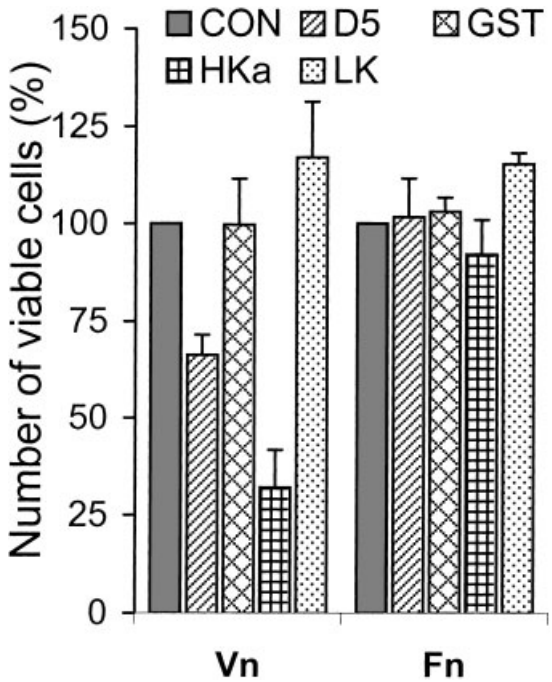

Fig. 6. Effect of HKa and D5 on the viability of endothelial cells grown on Vn and Fn. HUVEC were seeded on Vn- or Fn-coated dishes in EGM medium for 3 h to allow cells for attachment. The cells were then incubated in EBM (EGM minus serum) containing 10 ng/ml basic fibroblast growth factor (bFGF) alone as controls (CON), or in the presence of 300 nM D5 (D5) or 200 nM HKa (HKa). GST (300 nM) and LK (200 nM) were used as controls for D5 and HKa, respectively. After incubation for 48 h, the number of viable cells was determined by a Cell Titer AQueous analysis kit after a 48 h incubation. The absorbencies, which correlate with the amount of live cells, were determined at 490 nm. Absorbance determined from the controls (CON) was taken as 100%. Results are means ± SEM of three experiments performed in triplicate.

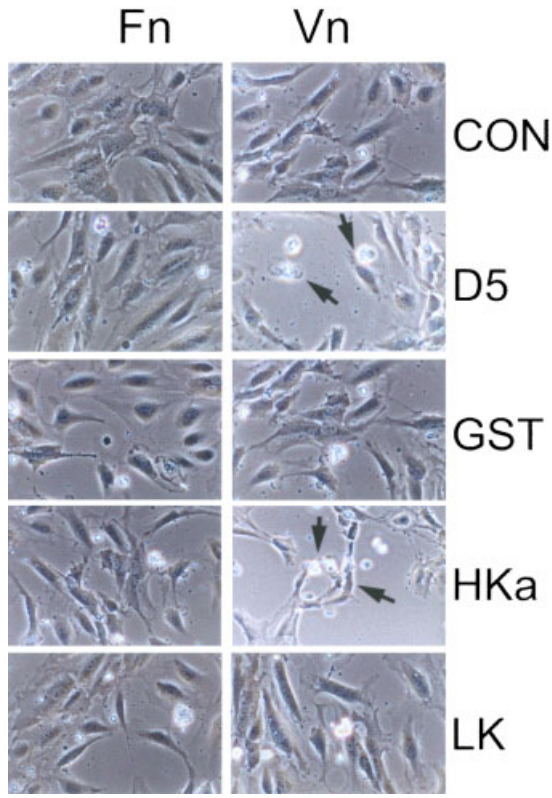

Fig. 7. HKa and D5 induce apoptosis of endothelial cells grown on Vn but not on Fn. HUVEC were treated under the same experimental conditions as described in Figure 6. After incubation for 48 h, the morphology of cells was examined under a phase microscope (200× magnification) and photographed.

viable cells left in HKa- and D5-treated cells was about 30 and 60% of the control, respectively (Fig. 6, Vn). HKa and D5 did not show significant effect on the viability of cells grown on Fn (Fig. 6, Fn). When examined under a microscope, cells in the control experiments were well attached to the dishes and showed a healthy morphology (Fig. 7; CON, GST, or LK). Neither HKa nor D5 exerted an adverse effect on the viability of cells grown on Fn (Fig. 7; Fn, D5, HKa). On the other hand, HKa and D5 significantly decreased the number of viable cells grown on Vn. Among cells that were still attached to the dishes, many showed typical morphological features of apoptosis, such as membrane blebbing and shrinkage of the cell body (Fig. 7; Vn, D5 and HKa, indicated by arrows). Apoptotic cell death induced by HKa was further confirmed by staining cells with Hoechst 33258 (Hoechst), a DNA binding dye that gives a bright blue color under a fluorescent microscope. The nuclei of viable cells are round and intact while those of apoptotic cells are condensed and fragmented. As shown in Figure 8, HKa caused nuclear fragmentation and condensation of cells grown on Vn (indicated by arrows) but not of cells grown on Fn, consistent with the morphological features of apoptosis shown in Figure 7. Apoptotic cell death caused by D5 on Gel has been previously described [Guo et al., 2001]. The apoptotic effect of HKa and D5 shown in these experiments parallels with their anti-adhesive activity described in Figures 1 and 3. Taken together, these results clearly established a close correlation between the apoptotic effect and the anti-adhesive activity of HKa and D5.

HKa-Induced Apoptosis Is Associated With the Inhibition of FAK and Paxillin Phosphorylation

FAK was initially identified as a signaling molecule that plays a central role in the

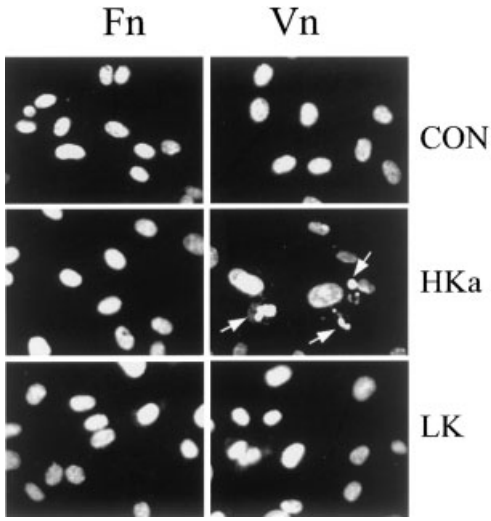

Fig. 8. Analysis of apoptotic cells by Hoechst 33258 cell staining. HUVEC were seeded on Vn- or Fn-coated dishes in EGM medium for 3 h to allow cells for attachment. The cells were then incubated in EBM (EGM minus serum) containing 10 ng/ml bFGF alone as controls (CON), or in the presence of 100 nM HKa (HKa) or LK. After incubation for 24 h, the cells were stained with 10 μmol/L Hoechst 33258 for 30 min, analyzed under a fluorescence microscope (200× magnification) with excitation at 340 nm, and photographed.

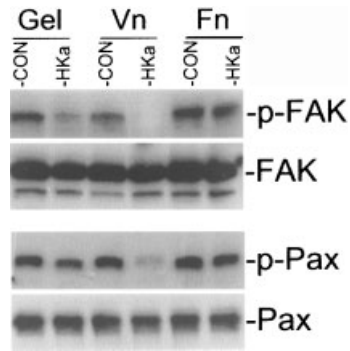

Fig. 9. HKa inhibits phosphorylation of FAK and paxillin in apoptotic cells. HUVEC were seeded on cell culture dishes coated with Gel, Vn, or Fn in EGM medium for 3 h to allow cell attachment. The cells were then incubated in EBM (EGM minus serum) containing 10 ng/ml bFGF alone as controls (CON) or in the presence 200 nM HKa (HKa). After 48 h treatment, the phospho-FAK (p-FAK)/total FAK (FAK) and phospho-paxillin (p-Pax)/total paxillin were detected by Western-blot analysis using their antibodies.

regulation of cell motility and cell morphology. Recent studies suggest that FAK is also critical for maintenance of cell viability. To test whether the apoptotic effect of HKa is related to its effect on the FAK signaling pathway, we examined the phosphorylation of FAK and paxillin in cells grown on Vn, Gel, or Fn for 48 h, a condition under which HKa induced apoptosis of cells on Vn and Gel but not those of grown on Fn. As shown in Figure 9, the amount of p-FAK and p-Pax was markedly reduced in cells grown on Vn (Fig. 9, Vn/HKa), corresponding to the strong apoptotic effect of HKa under this condition (Figs. 7 and 8, Vn/HKa). HKa also inhibited FAK and paxillin phosphorylation in cells grown on Gel (Fig. 9, Gel/HKa), where HKa also induced apoptosis of HUVEC [Zhang et al., 2000; Guo et al., 2001] and exhibited a moderate anti-adhesive effect (Fig. 1). However, the amount of p-FAK and p-Pax was not affected by HKa in cells grown on Fn (Fig. 9, Fn/HKa), where there was no apparent apoptosis (Figs. 7 and 8, Fn/HKa). In all cases, the total protein level of FAK or paxillin was not affected (Fig. 9, FAK and PAX), indicating that the reduced phosphorylation of FAK and paxillin was not due to the cleavage of their proteins.

DISCUSSION

It is well recognized that the ECM plays a critical role in the regulation of angiogenesis [Ingber, 2002]. Endothelial cell adhesion to the ECM is required for many cellular processes, such as migration, differentiation, and proliferation, all of which are essential steps of neovascularization. The contact established between the cell surface and the ECM not only serves as a mechanical support controlling tissue architecture but also initiates discrete intracellular signaling that regulates specific cellular events. Preventing anchorage-dependent cells from adhering to the ECM will disrupt the normal cellular functions and eventually lead to apoptotic cell death. This particular type of apoptosis resulted from cell detachment is known as "anoikis" [Frisch and Screaton, 2001]. Endothelial cells are among the cell types that are very sensitive to anoikis [Re et al., 1994].

A unique feature of HKa is its increased anti-adhesive activity in comparison with HK [Weisel et al., 1994]. It is now clear that D5 is the domain responsible for the anti-adhesive activity of HKa [Kunapuli et al., 1993]. The fact that D5 resembles HKa in inducing apoptosis indicates that the apoptotic effect of HKa and D5 may be related to their anti-adhesive property. The data presented in this study strongly supports this hypothesis. Furthermore, the results from the experiments using LK provided additional evidence. LK, which does not exhibit anti-adhesive activity and does not induce

apoptosis of endothelial cells, has identical D1, 2, 3, and 4 of HK, but its D5 is different from that of HK. Apparently, the anti-adhesive effect and apoptotic activity displayed by HKa is due to the unique amino acid sequence of D5. An important observation made in this study is that HKa and D5 selectively inhibit endothelial cell adhesion to different ECM proteins and cause subsequent apoptosis, suggesting that the action of HKa and D5 is not a simple biophysical effect but is highly regulated by their interactions with specific ECM components. Our results indicate that Vn, a major protein in the provisional ECM that plays an important role in angiogenesis during vascular remodeling and vessel repair [Preissner and Seiffert, 1998], is likely involved in mediating the effect of HKa in vivo. In support of this view, HK/HKa has been indeed observed to colocalize with Vn at the site of atherosclerotic plaques [Chavakis et al., 2000] where angiogenesis is a prominent event.

The roles of FAK in the regulation of cell adhesion, cell spreading, and other cellular processes related with cell motility have been well-established [Parsons et al., 2000]. We have shown that HKa inhibited the phosphorylation of FAK and paxillin in a manner paralleling its anti-adhesive potency, which explains its selective inhibition of cell adhesion to Vn and Gel but not to Fn. Recent studies have greatly extended the cellular functions of FAK far beyond those that were originally proposed. It is now known that FAK is critical for cell survival. Blocking activation or expression of FAK leads to apoptotic cell death [Hungerford et al., 1996; Xu et al., 1996] while expression of constitutively active FAK prevented cells from apoptosis [Chan et al., 1994; Frisch et al., 1996]. The importance of FAK signaling pathway for cell survival has been further demonstrated by the findings that activity of FAK, paxillin, and other focal adhesion proteins is often attenuated by proteolytic cleavage and/or by dephosphorylation during apoptosis induced by a variety of stimuli ranging from chemical agents [van de et al., 1999; Kabir et al., 2002], anticancer drugs, UV irradiation, and Fas-ligand [Widmann et al., 1998] to growth factor withdrawal [Levkau et al., 1998]. However, time courses and patterns of the FAK cleavage and dephosphorylation vary significantly depending on cell types and the nature of stimuli. For example, the cleavage of FAK in Fas ligation-induced apoptosis took place within 30 min after stimulation [Widmann et al., 1998]. However, in saturosporine [Kabir et al., 2002] and dichlorovinylcysteine [van de et al., 1999] induced apoptosis, FAK cleavage was not apparent until several hours, whereas dephosphorylation of FAK took place within minutes after the treatments. It is concluded in these studies that dephosphorylation and cleavage of FAK are two independent events. While the apoptotic effect of HKa was closely correlated with the inhibition of FAK and paxillin phosphorylation, the total protein level of FAK was not affected. These results are intriguing, but interestingly, they are similar to those described in anoikis of fibroblasts [Valentinis et al., 1998] and epithelial cells (MDCK) [Wei et al., 2002] where cell attachment was prevented by plating cells on poly-HEMA coated dishes. In both cases, FAK phosphorylation was significantly inhibited but the protein level of FAK remained unchanged [Valentinis et al., 1998; Wei et al., 2002]. We are not certain whether the resistance of FAK to proteolytic cleavage in HKa-induced apoptosis is due to this particular type of cell death (anoikis) or whether HKa somehow affects stability of FAK.

Although inhibition of phosphorylation of FAK and paxillin could be an important step for HKa action, how the effect of HKa is mediated at the cell surface is not clear. A likely explanation for our observation is that HKa may disrupt subsets of integrins that mediate cell adhesion to Vn and Gel but not those to Fn. Indeed, HKa and D5 have been shown to directly bind to the amino terminal region of Vn, where it is proximal to the RGD region (integrin binding site) [Chavakis et al., 2000]. Therefore, HKa or D5 may disrupt cell adhesion mediated by integrins that utilize Vn as a ligand. Existing evidence also favors the hypothesis that urokinase-type plasminogen activator receptor (uPAR) may be involved in mediating the effect of HKa. uPAR is a glycophosphatidylinositol (GPI)-anchored cell surface protein that was initially identified as a receptor for urokinase-type plasminogen activator (uPA) [Chapman, 1997]. It is now known that uPAR directly binds to the ECM protein, Vn, thus acting as an adhesion receptor [Wei et al., 1994]. uPAR also interacts with integrins acting as a signaling molecule and thus is considered to be a component of integrin pathways [Ossowski and Aguirre-Ghiso, 2000]. In several types of cells [Wei et al., 1999; Nguyen et al., 2000; Aguirre

Ghiso, 2002] including endothelial cells [Tang et al., 1998], it has been shown that uPAR mediates the activation of FAK. Since HKa can directly bind to D2 and 3 of uPAR through its D5 region [Colman et al., 1997], it is possible that binding of HKa to Vn and/or uPAR may disrupt Vn/uPAR-integrin-mediated cell adhesion, which may explain the anti-adhesive and apoptotic effects of HKa on the cells grown on Vn. In support of this view, we have recently observed that the distribution of uPAR on the cell surface is dramatically different between endothelial cells grown on Vn and Fn: uPAR were localized primarily in the focal adhesion areas in cells grown on Vn but were evenly distributed in cells grown on Fn (manuscript in preparation). These results may explain why HKa inhibited cell adhesion to Vn but not to Fn. It is likely that Vn–uPAR interactions, which are concentrated in the focal contact areas (the most critical part for cell adhesion), play an important role in cell adhesion to Vn. In the meantime, Vn–uPAR connections are more vulnerable targets for HKa intervention. On the other hand, uPAR may play a less important role in cell adhesion to Fn because of their uniform distribution throughout cells grown on Fn, especially in the absence of its ligand, Vn. In addition, Fn is the ligand for at least 12 integrins, while Vn and Gel (denatured collagens) are the ligands for only four ($\alpha v\beta 1$, $\alpha v\beta 3$, $\alpha v\beta 5$, $\alpha IIb\beta 3$) and three integrins ($\alpha v\beta 3$, $\alpha 5\beta 1$, $\alpha IIb\beta 3$), respectively [Plow et al., 2000]. The larger number of integrins binding to Fn likely contributes to the resistance of cells to the anti-adhesive effect of HKa. It is also noted that HKa can directly bind to Mac-1 integrin, thus blocking adhesion of HEK293 cells to fibrinogen and ICAM-1 [Sheng et al., 2000]. Therefore, HKa may potentially disrupt integrin-mediated adhesion in a similar manner through its direct association with certain integrins yet to be identified in endothelial cells. Apparently, the interaction between HKa and cell surface proteins that mediates its anti-adhesive and proapoptotic activity is a highly complex event and needs further investigation.

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