Urokinase-Type Plasminogen Activator Receptor Is Involved in Mediating the Apoptotic Effect of Cleaved High Molecular Weight Kininogen in Human Endothelial Cells

Dian J. Cao, Yan-Lin Guo, Robert W. Colman

Abstract—Cleaved high molecular weight kininogen (HKa) has been shown to inhibit in vivo neovascularization and induce apoptosis of endothelial cells. We have shown that HKa-induced apoptosis correlated with its antiadhesive effect and was regulated by extracellular matrix (ECM) proteins. In this study, we identified the urokinase-type plasminogen activator receptor (uPAR) as a target of HKa activity at the endothelial cell surface. Anti-uPAR antibodies blocked the apoptotic effect of HKa. Further studies revealed that uPAR formed a signaling complex containing integrin α3β1, or αvβ3, caveolin, and Src kinase Yes in endothelial cells. HKa physically disrupted the formation of this complex in a manner that paralleled its apoptotic effect. For the first time, our results provide a mechanistic explanation for the previous observation that HKa selectively induces apoptosis of endothelial cells grown on vitronectin, but not cells grown on fibronectin. These data also resolve the controversial role of uPAR in mediating the apoptotic and antiadhesive activities of HKa. (Circ Res. 2004;94:1227-1234.)

Key Words: urokinase-type plasminogen activator receptor ■ high molecular weight kininogen ■ endothelial cells ■ apoptosis ■ angiogenesis

Angiogenesis, the formation of new capillaries from existing blood vessels, is a cellular event crucial for both physiological and pathological processes such as embryogenesis and cancer development,1 respectively. A positive balance in favor of angiogenic factors leads to new blood vessel formation, whereas the predominance of angiogenic inhibitors will switch the equilibrium to vessel quiescence or vessel regression.2-5

High molecular weight kininogen (HK) is a plasma protein that was first identified as a precursor of the bioactive peptide bradykinin. We now recognize that HK is a multifunctional protein that plays important roles in many pathophysiological processes, such as fibrinolysis, thrombosis, and inflammation.6 HK is a 120-kDa single-chain glycoprotein consisting of six domains (designated as D1 to D6, respectively) with each having distinct functions.7 After proteolytic cleavage of HK by kallikrein and release of bradykinin contained within D4, the remaining portion of the molecule, HKa, undergoes major conformational changes that lead to a greater surface exposure of the D5 region. As a result, HKa acquires new properties. In comparison with HK, HKa shows an increased antiadhesive effect due to domain rearrangements.8,9 HK specifically and reversibly binds to endothelial cells in a Zn2+-dependent manner. Thus, the endothelial cell surface is an important site for the generation of both bradykinin and HKa, each of which regulates the functions of endothelial cells. Although the involvement of bradykinin in the regulation of many physiological and pathophysiological processes has been intensively studied, the functional implications of the generation of HKa are incompletely understood. Recent studies from our laboratory and other investigators indicate that HKa may act as a naturally occurring angiogenic inhibitor.10,11 Interestingly, it has recently been shown that bradykinin stimulates angiogenesis.12,13 Therefore, HK is a precursor of both an angiogenic stimulator (bradykinin) and an angiogenic inhibitor (HKa).

After our initial discovery of HKa and its domain 5 as angiogenic inhibitors,10 we have further shown that HKa inhibits human umbilical vein endothelial cell (HUVEC) proliferation and induces apoptosis,14,15 which together may represent a critical step of their antiangiogenic activity. In our most recent study,16 we demonstrated that the apoptotic effect of HKa is related to its antiadhesive activity and its ability to disrupt signaling pathways required for cell adhesion. Furthermore, the apoptotic effect of HKa is highly regulated by interactions with different extracellular matrix proteins; it strongly induces apoptosis of endothelial cells grown on vitronectin (Vn) but has no significant effect on the viability of cells grown on fibronectin (Fn).16,17 However, a fundamentally impor-
tant unanswered question is how the effects of HKa and D5 are mediated at the endothelial cell membrane.

In the present study, we provide substantial evidence to support the hypothesis that urokinase-type plasminogen activator receptor (uPAR), a glycosylphosphatidylinositol (GPI)-anchored cell surface protein that plays an important role in cell adhesion and signal transduction, is a target of HKa action at the endothelial cell surface. We have previously shown that HKa physically binds to uPAR in endothelial cells. We now demonstrate the specific downstream events: HKa disrupts the signaling complex by decreasing uPAR-integrin and/or uPAR-caveolin association. Thus, this study constitutes the first report that provides a mechanistic explanation for the previous observation that HKa selectively induces apoptosis of endothelial cells grown on Vn but not cells grown on Fn.

Materials and Methods

Apoptosis Analysis

Apoptotic cell death induced by HKa has been characterized by a combination of DNA and nuclear fragmentation and annexin V staining, which have been described in our previous studies. The present study used nuclear fragmentation analysis with Hoechst 33285 staining to identify apoptotic cells at the cellular level. HUVECs were seeded on Vn- (0.053 mmol/L) or Fn- (0.023 mmol/L) coated coverslips in EGM. After the cells attached to the coverslips, the medium was changed to EBM (EGM minus growth factors and FBS) plus 0.59 μmol/L bFGF in the presence or absence of 100 nmol/L HKa. P-D2D3 (the anti-serum against the D2 and D3 domains of uPAR) or the same amount of control preimmune serum (PIS) was added to the medium at a concentration of 30 μL/mL. After incubation for 48 hours, cells were analyzed for apoptosis according to methods described previously.

Apoptosis was quantified by a Cell Titer Aqueous kit (Promega) as described previously. The cells were incubated with bFGF (0.59 μmol/L) in the presence or absence of 100 nmol/L HKa. Monoclonal anti-uPAR antibodies (E33, E174, and E180) or control mouse IgG, P-D2D3, or rabbit PIS, were added to the medium at different concentrations as indicated. Apoptosis assay was also performed using anti-cytokeratin-1 and anti-gC1qR (clone 74.5.2) antibodies at concentrations indicated.

Immunoprecipitation and Immunoblot Analysis

HUVECs were plated on cell culture dishes coated with Vn or Fn in EGM. After the cells attached to the dishes, the culture medium was changed to EBM plus 0.59 μmol/L bFGF. Cells were treated with or without 100 nmol/L HKa for 25 to 30 hours at 37°C. After treatment, the cells attached to the dishes and those from the medium were collected and lysed with modified M-per mammalian cell protein extraction buffer (Pierce) containing 5 mmol/L Ca2+, 2 mmol/L Mg2+, and protease inhibitor cocktail (Chemicon International). After incubation for 10 minutes at room temperature, cell lysates were collected by centrifugation at 21 000g for 15 minutes at 4°C.

The complex formation of uPAR with other signaling molecules was determined by immunoprecipitation (IP) according to the methods described by Wei et al with some modifications. Cell lysate was incubated with antibodies to αβ2, αβ3, uPAR, or PECAM-1 followed by incubation of protein A/G beads. The immunoprecipitates were subjected to SDS-PAGE under reduced conditions, and immunoblot analysis was performed as described.

For a description of other methods, see the expanded Materials and Methods available in the online data supplement at CircRes.ahajournals.org.

Results

Anti-uPAR Antibodies Block Apoptotic Effect of HKa in HUVECs

A previous investigation from this laboratory has shown that HKa directly binds to uPAR because an anti-uPAR D2D3 (domains 2 and 3) antibody blocked the binding of HKa to endothelial cells. We postulated that uPAR may be involved in mediating the apoptotic effect of HKa. To test this hypothesis, we used P-D2D3, an antibody raised against the D2D3 region of uPAR, to examine if this antibody can block the apoptotic effect of HKa. HUVECs were cultured on Vn or Fn and incubated with HKa in the presence or absence of P-D2D3 (30 μL/mL) (bFGF+HKa, bFGF+HKa+P-D2D3, respectively). After 48 hours of treatment, cells were stained with Hoechst 33258. Typical nuclear morphology of apoptotic cells is indicated with white arrows.

Figure 1. The anti-uPAR D2D3 antibody blocks apoptosis induced by HKa. HUVECs grown on Vn (A) or Fn (B) were treated with 100 nmol/L HKa in the presence or absence of P-D2D3 (30 μL/mL) (bFGF+HKa, bFGF+HKa+P-D2D3, respectively). After 48 hours of treatment, cells were stained with Hoechst 33258. Typical nuclear morphology of apoptotic cells is indicated with white arrows.
bFGF). HKa caused condensation/fragmentation of nuclei (apoptotic nuclei) in many cells grown on Vn (Figure 1A, bFGF + HKa). The effect of HKa was reversed by P-D2D3 (Figure 1A, bFGF + HKa + P-D2D3), whereas preimmune serum (PIS) had no effect (Figure 1A, bFGF + HKa + PIS). Consistent with our previous observation that HKa did not induce apoptosis of HUVECs grown on Fn,16 neither HKa nor P-D2D3 and PIS exhibited an apparent effect on cell viability when cells were grown on Fn (Figure 1B). P-D2D3 alone did not show apparent effect on the viability of cells grown on Vn and Fn (data not shown).

We have previously shown that apoptosis of endothelial cells induced by HKa was largely due to its antiadhesive effect.16 As a result, most of late apoptotic cells are released into the medium. The number of viable cells still attached to the culture plate after treatment, which is inversely related to the number of apoptotic cells in the medium, was quantified to reflect HKa-induced apoptosis. When cells were grown on Vn, viable cells left in the HKa-treated cells was 30% of the control (in the absence of HKa), representing 70% of the cells death from apoptosis. The apoptotic effect of HKa under this condition was defined as 100%. As shown in Figure 2A, the apoptotic effect of HKa was significantly inhibited by antibody P-D2D3 in a concentration-dependent manner, whereas PIS had no effect (Figure 2A). The same experiments were performed using three monoclonal anti-uPAR antibodies (E33, E174, and E180) at a concentration of 10 \( \mu \text{g/mL} \). All of these antibodies significantly blocked the apoptotic effect of HKa but with different potency (Figure 2B). Because both the polyclonal and monoclonal antibodies were made against D2D3 domains of uPAR, it is likely the D2D3 domains of uPAR are the targeted regions by HKa. A nonrelevant mouse IgG (m-IgG) as a control for the monoclonal antibodies did not affect the effect of HKa (Figure 2B). Similar experiments were also performed using anti-cytokeratin-1 and anti-gC1qR antibodies (Figure 2A, Ab1 and Ab2, respectively). The results showed that the apoptotic effect of HKa was blocked by anti-cytokeratin-1, but not by anti-gC1qR antibodies (Figure 2A). Consistent with the results presented in Figure 1B, HKa did not induce apoptosis of endothelial cells grown on Fn (data not shown).

**uPAR Forms Complexes With Integrins and Caveolin**

Because uPAR is a GPI-anchored cell surface protein, the signal received by uPAR must be transduced through its interaction with other signaling molecules. uPAR has been shown to interact with integrins and caveolin in other cell types.21–24 We then tested whether this is the case in endothelial cells. Cell lysates from HUVECs were incubated with anti-\( \alpha_v\beta_3 \) or anti-\( \alpha_v\beta_1 \) antibodies. As shown in Figures 3A and 3B, both uPAR and caveolin were detected in the samples precipitated either by anti-\( \alpha_v\beta_1 \) or by anti-\( \alpha_v\beta_3 \) antibodies, indicating that uPAR, \( \alpha_v\beta_3 \), or \( \alpha_v\beta_1 \), and caveolin form a complex in HUVECs grown on gelatin (Figure 3A, Gel), Vn (Figure 3B, Vn), or Fn (Figure 3B, Fn). The presence of integrin \( \alpha_v\beta_1 \) and \( \alpha_v\beta_3 \) was confirmed by probing the immunoprecipitates with anti-integrin \( \alpha_v \) or \( \beta_3 \) subunits, respectively. Further results showed that the complex precipitated with anti-\( \alpha_v\beta_1 \) antibody contained \( \alpha_v \) subunit (Figure 3C), whereas the complex precipitated with anti-uPAR antibody contained both \( \alpha_v \) and \( \beta_1 \) subunits (Figure 3D). In a control experiment, cell lysate was incubated with anti-PECAM-1 antibodies. The immunoprecipitate contained abundant PECAM-1, as expected, but not uPAR (Figure 3E). These results indicate that uPAR form a complex that contains specific signaling components.

**HKa Disrupts the Complex Formation Among uPAR, Integrin, and Caveolin**

We have shown that HKa-induced apoptosis in endothelial cells is ECM protein dependent.16 HKa exhibited a maximum apoptotic effect on HUVECs grown on Vn, whereas its effect on cells grown on Fn was minimal. We further analyzed the effect of HKa on complexes formed by uPAR, \( \alpha_v\beta_3/\alpha_v\beta_1 \), and caveolin in HUVECs grown on Vn and Fn. As shown in Figure 4A, HKa treatment reduced the amount of uPAR associated with \( \alpha_v\beta_3 \) to a similar extent in cells grown on Vn and Fn; however, the amount of caveolin in the complex was markedly reduced in cells grown on Vn compared with cells grown on Fn (Figure 4A). Figures 4B and 4C quantified the relative amount of uPAR and caveolin associated with \( \alpha_v\beta_3 \) determined by densitometry from three independent experiments. In Figure 4G, total cell lysates from control and HKa-treated groups before IP were probed with anti-caveolin.
and anti-uPAR antibodies. No significant differences in total amount of these proteins were detected among groups.

In parallel experiments, cell lysates were incubated with anti-\(\alpha_v/\beta_3\) antibodies as shown in Figure 4D. Interestingly, in cells cultured on Vn, HKa almost completely disassociated uPAR and caveolin from the complex precipitated with anti-\(\alpha_v/\beta_3\) antibody. On the other hand, when cells were grown on Fn, although HKa also disassociates uPAR from \(\alpha_v/\beta_3\) to some extent, it had no apparent effect on the association of caveolin with \(\alpha_v/\beta_3\) (Figures 4D through 4F). Cell lysates before IP were also analyzed for actin. As shown in Figure 4A and 4D, the amount of actin was similar among groups, indicating that the same amount of proteins was used for IP experiments. HKa disassociates uPAR with a higher potency from the complex formed by \(\alpha_v/\beta_3\) than it does from the complex formed with \(\alpha_5/\beta_1\). HKa almost completely dissociates caveolin from both complexes in cells grown on Vn, indicating that disassociation of caveolin from the complex may play an important role in mediating the apoptotic and antiadhesive effects of HKa.

HKa Disrupts the Association of Src Kinase Yes From Integrin \(\alpha_v/\beta_3\)-Assembled Complex

Src family tyrosine kinases are nonreceptor tyrosine kinases that have been reported to interact with caveolin and integrins.\(^{23}\) Therefore, Src kinases are downstream effectors of integrin pathways in some cells.\(^{24,25}\) To test whether this is the case in endothelial cells, we chose to examine the presence of Yes as a representative of this kinase family. The immunocomplex precipitated by anti-\(\alpha_v/\beta_3\) antibodies was probed with an anti-Yes antibody. Results shown in Figure 5A demonstrate that Yes was associated with \(\alpha_v/\beta_3\) in endothelial cells, and that HKa caused almost complete loss of Yes from the \(\alpha_v/\beta_3\) complex in cells grown on Vn, and to a lesser extent, in cells grown on Fn. In Figure 5B, a dose-response was performed regarding HKa-induced dissociation of Yes from the \(\alpha_v/\beta_3\) complex in cells grown on Vn. These data suggest that the HKa not only disrupts the uPAR-integrin-caveolin signal complex formation at the cell membrane, it also effectively blocks signal transduction at downstream uPAR-integrin signaling pathways.

uPAR Mediates HUVEC Adhesion to Vn

Given the fact that Vn is a well-known adhesion molecule for many types of cells including endothelial cells and that uPAR is well-recognized adhesion receptor for Vn in epithelial cells\(^{26}\) and leukocytes,\(^{27}\) the contribution of uPAR-mediated cell adhesion in endothelial cells is not clear. A report by Kanse et al.\(^{28}\) indicated that uPAR is a major Vn binding protein. Surprisingly, uPAR-mediated cell adhesion was not detected in that study, an observation made from the indirect experiments using uPA as an uPAR ligand. However, it has been reported that uPAR mediates several cellular processes in a ligand (uPA)-independent manner.\(^{29}\) In an effort to resolve this uncertainty in endothelial cells, we treated cells with PI-PLC to cleave uPAR from the cell surface. The result, as shown in Figure 6, demonstrated that uPAR is responsible for 50% of HUVEC binding to Vn under the conditions tested. PI-PLC treatment, however, was not able to significantly affect the cell adhesion on Fn (Figure 6).
Discussion

HKa and its domain 5 (D5) have been shown to inhibit in vivo neovascularization in different animal models independently by this group\textsuperscript{10} and other investigators.\textsuperscript{11} However, the signaling mechanism that mediates their activity has not been elucidated. For the first time, we demonstrate that uPAR, an adhesion and signaling receptor, is a target of HKa action at the endothelial cell surface. Together with our previously published results,\textsuperscript{16} we demonstrate that HKa can disrupt the uPAR-αvβ3-caveolin-yes-FAK-paxillin signaling pathway at each level of these signaling components. The results described in this study represent major progress in our effort to characterize HKa as an angiogenic inhibitor.

Several endothelial cell membrane proteins that interact with HK or HKa have been identified, including uPAR,\textsuperscript{19} the receptor for the globular head of Clq (gClqR),\textsuperscript{30} and cytokeratin-1.\textsuperscript{30,31} It has been shown that cytokeratin-1 binds to both uPAR and gClqR, but uPAR and gClqR do not bind to each other.\textsuperscript{32} A recent study revealed that uPAR and cytokeratin-1 colocalize on endothelial cells and may form a receptor complex that mediates HK binding and the subsequent release of bradykinin.\textsuperscript{33} Among these proteins, uPAR, a GPI-anchored cell surface protein that contains three domains, is particularly relevant to cell adhesion and angiogenesis.\textsuperscript{18,34} Although initially identified as a receptor for urokinase plasminogen activator (uPA) to localize proteolytic activity on the cell surface during invasion,\textsuperscript{18,35} it is now known that uPAR also interacts with integrins at the cell membrane and binds to Vn in a uPA-dependent or independently manner.\textsuperscript{29} Vn is a major provisional ECM protein and plays an important role in angiogenesis during vascular remodeling and vessel repair.\textsuperscript{16} As a result of these interactions, uPAR acts as an adhesion receptor for Vn and transduces signals through integrin pathways.\textsuperscript{37}
The initial notion that uPAR may mediate the apoptotic effect of HKα came from our previous study, which showed that HKα binds to endothelial cells through D2 and D3 of uPAR. The binding of HKα to endothelial cells can be completely blocked by an anti-uPAR D2 and D3 antibody, or by soluble recombinant uPAR. We, therefore, postulated that HKα could be a logical target of HKα for its antiangiogenic activity. However, a recent study by Zhang et al indicated that none of the three antibodies against gC1qR, cytokeratin-1, or uPAR, which had previously been shown to inhibit HK or HKα binding to endothelial cells, affected cytokeratin-1, or uPAR, which had previously been shown to inhibit HK or HKα binding to endothelial cells.

Our examination of the other two antibodies was demonstrated in Figure 2A. We were able to show that the anti-cytokeratin-1 antibody blocked the apoptotic effect of HKα on cells grown on Vn. As demonstrated by Joseph et al, cytokeratin-1 forms a complex with uPAR. Thus, the antibody against cytokeratin-1 could interfere with the association of HKα with uPAR-cytokeratin-1 complex and block the apoptotic effect of HKα. We also performed similar experiments using the anti-gC1qR antibody. We were unable to detect any blocking effect of this antibody to HKα-induced cell apoptosis on Vn. This finding indicates that uPAR plays more significant role under our experimental condition in which Vn is used as a coating ECM protein and consistent with previous finding that gC1qR does not form a complex with uPAR.

Although the results from Zhang’s report argue against a role of uPAR in mediating the effect of HKα, in collaboration with Chavakis et al, we were able to show that HKα or D5 completely inhibited uPAR-mediated cell adhesion to Vn, thereby promoting cell detachment in U937 and uPAR-transfected BAF-3 cells. HKα or D5 failed to elicit a similar effect when cells were plated on Fn under the same conditions. We recently demonstrated in endothelial cells that HKα and D5 exhibit dramatic apoptotic activity on the cells grown on Vn, but its effect on cells grown on Fn was minimal. These studies suggested that the effect of HKα and D5 depends on the nature of ECM proteins, and that uPAR could be a target of HKα but may require the presence of Vn. We now provide strong evidence to support this hypothesis. First, four different anti-uPAR D2/D3 antibodies blocked the apoptotic effect of HKα on cells grown on Vn but not on Fn. Secondly, we demonstrated that uPAR formed a signaling complex with α1β1/α2β1, caveolin, and Yes kinase. Although this phenomenon and the functional implication of this signaling cluster formation has been studied in other cell systems, it has not been reported in endothelial cells in previous studies. The most important finding of these experiments, however, is that HKα could disrupt the association of uPAR with these signaling components in a manner that parallels its antiadhesive and apoptotic effects. Although HKα also disrupted uPAR-α1β1/α2β1 complex formation in cells on Fn to some extent, it did not result in cell detachment or apoptosis. This result could be explained by the fact that Fn is the ligand for at least 12 integrins, whereas Vn is a ligand for only four (α1β1, α2β1, α3β1, and α5β1). The larger number of integrins binding to Fn may contribute to the resistance of cells to the antiadhesive effect of HKα on cells grown on Fn. This hypothesis may not only explain the ECM protein dependency of antiadhesive and apoptotic effect of HKα but also the failure of the anti-uPAR antibodies to block the apoptotic effect of HKα reported by Zhang et al, where the experiment was conducted in cells grown on gelatin (in the absence of Vn).
We previously demonstrated that HKa directly binds to Vn. The present study extends that report by demonstrating specific downstream events. The hypothesis that HKa binds to Vn and subsequently blocks cell adhesion to Vn does not contradict the results presented in the current study. Whether the upstream event is HKa binding to Vn and/or HKa competing with Vn for binding to uPAR, the downstream event might be the same. These two steps will finally lead to interference of uPAR/integrin interaction and subsequently endothelial cell apoptosis.

Because $\alpha_2\beta_1$ is not a ligand of Vn, we are not certain how HKa treatment also caused dissociation of uPAR and caveolin from $\alpha_2\beta_1$ complex. Because uPAR can form complexes with several integrins, including $\alpha_2\beta_1$ and $\alpha_3\beta_1$, and $\alpha_5\beta_1$, it is possible that these complexes may consist of more than one integrins. As a result of these associations, the integrins can modulate each other’s function. For example, Chapman and colleagues have shown, in 293 cells, uPAR complex with $\alpha_2\beta_1$, promoting direct (to Vn) and indirect (to Fn) cell adhesion through modulating the function of $\alpha_2\beta_1$ and $\alpha_5\beta_1$. Cross-talk between $\alpha_2\beta_1$ and $\alpha_5\beta_1$ is also implicated in the regulation of cell migration. We speculate that collaboration between $\alpha_3\beta_1$ and $\alpha_5\beta_1$ may also occur in endothelial cells. Indeed, the $\alpha_5$ subunit was detected in the uPAR-$\alpha_2\beta_1$ complex. Therefore, HKa-induced disruption of uPAR-$\alpha_2\beta_1$-caveolin may indirectly affect Vn-$\alpha_2$-interaction.

In our recent study, we have shown that the antiadhesive effect and the apoptotic effect of HKa are associated with the inhibition of activation of focal adhesion kinase (FAK) and paxillin, two important signal molecules required for cell adhesion and cell survival. We can now establish a signaling cascade, Vn-uPAR-$\alpha_2\beta_1$-caveolin-Src-FAK-paxillin, that mediates adhesion, proliferation, and survival of endothelial cells. HKa disrupts this signaling cascade by at least two mechanisms: first, through its direct binding to the somatomedin B (SMB) domain of Vn, HKa prevents the binding of Vn to integrin $\alpha_2\beta_1$; and secondly, HKa directly binds to the D2 and D3 domains of uPAR through its D5 region, thus preventing the binding of Vn to uPAR and its interaction with integrins as well as other signaling molecules, such as caveolin. As a result of these interactions of HKa with the cascade molecules, it is apparent that cells will not be able to adhere properly and will eventually undergo apoptosis, as we have found with cells grown on Vn in the presence of HKa. HKa can directly bind to Mac-1 integrin found in leukocytes, thus blocking adhesion of HEK293 cells to fibrinogen and ICAM-1. Therefore, HKa may potentially disrupt integrin-mediated adhesion in a similar manner through its direct association with certain integrins present in endothelial cells. It was recently reported that the apoptotic activity of HKa may be mediated through its interaction with tropomyosin. Apparently, the interaction between HKa and cell surface proteins is highly complex.

Tarui et al demonstrated that integrin $\alpha_2\beta_1$ is pivotal to the signal transduction of uPAR. Together with the evidence that uPAR binds integrin with high affinity and facilitate integrins’ function in cell adhesion and migration as discussed in this study, dissociation of uPAR from integrins diminishes these signaling events. Thus, by providing evidence that anti-uPAR antibodies blocked the apoptotic effect of HKa and HKa mediated dissociation of uPAR-integrin, the present study provides a mechanistic explanation of HKa-induced human endothelial cell apoptosis.

Acknowledgments

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Materials and Methods

Reagents and antibodies

HKa was purchased from Enzyme Research Laboratories (South Bend, IN) with more than 95 % purity. On nonreduced SDS gels, the purified HKa appeared as a major band of 110 kDa. Fn, Vn, and bFGF were purchased from Life Technologies (Grand Island, NY), and gelatin was purchased from Sigma Chemical Co. (St Louis, MO). Monoclonal antibody directed to αvβ3 (LM 609), rabbit polyclonal antibody directed to integrin αv subunit, goat polyclonal antibody against α5β1, and monoclonal antibody against integrin β1 subunit were purchased from Chemicon International (Temecula, CA). Polyclonal antibody to uPAR (399R) was obtained from American Diagnostica (Greenwich, CT). The rabbit anti-uPAR anti-serum made against uPAR domains 2 and 3 (P-D2D3) and three monoclonal antibodies against the similar region of uPAR (Clones E33, E174, and E180) were kindly provided by Drs. Andrew Mazar and Graham Parry (Attenuon, L.L.C., San Diego, CA). Antibodies to caveolin were purchased from BD Transduction Laboratories (Palo Alto, CA). Rabbit polyclonal antibodies against Yes kinase and PECAM-1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Phosphatidylinositol-specific phospholipase C (PI-PLC) was purchased from Sigma (St. Louis, MO). Anti-cytokeratin-1 and anti-gC1qR (clone 74.5.2) antibodies were purchased from Sigma (St. Louis, MO) and Upstate Biotechnology (Lake Placid, NY), respectively.

Cell culture

Human umbilical vein endothelial cells (HUVECs) were purchased from Clonetics (Walkersville, MD). They were cultured according to a protocol as described in our previous studies. We added 20 µmol/L Zn++ to the culture whenever HKa was involved, as Zn++ is essential to the binding of HKa to endothelial cells.
Cell adhesion assay

To determine the contribution of uPAR to cell adhesion to Vn and Fn, HUVEC were treated with phosphatidylinositol-phospholipase C (PI-PLC) to cleave uPAR from the cell surface, a method has been frequently used in the study of uPAR\(^3\). In this assay, Costar 96-well untreated plates were coated with 0.053 mmol/L Vn and 0.023 mmol/L Fn overnight followed by blocking with 0.1 % BSA in PBS at room temperature for 1 h. HUVEC were treated with 5 unit/ml of PI-PLC at 37\(^\circ\)C for 15 min. After the treatment, the cells were seeded on Vn- or Fn-coated plates and incubated for 30 min. Unattached cells were washed out with PBS, the adhered cells were fixed and stained with 0.5 % crystal violet. The number of cells was determined under a microscope.

Statistical analysis

Values shown represent mean ± S.E.M. where applicable. Statistical significance was calculated with student t test. Differences were considered significant for \(p < 0.05\).

Reference

