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cMet and Fas Receptor Interaction Inhibits Death-Inducing Signaling Complex Formation in Endothelial Cells

Lesley Ann Smyth, Hugh J.M. Brady

Abstract—Fas receptor is constitutively expressed on endothelial cells; however, these cells are highly resistant to Fas-mediated apoptosis. In this study, we examined death-inducing signaling complex (DISC) formation in endothelial cells after Fas receptor stimulation. Nonfunctional DISC formation was observed in human umbilical vein endothelial cells (HUVECs). Fas-associated death domain (FADD) and large amounts of FADD-like interleukin-1–converting enzyme–inhibitory protein-L were recruited to the receptor; however, no caspase 8 recruitment was observed. A role for the cell surface molecule cMet in controlling Fas sensitivity in endothelial cells was observed. Here, we report that Fas is associated with cMet in HUVECs. Such an interaction may inhibit self-association of Fas in these cells, as suggested by the fact that monomeric Fas is expressed in these cells. Endothelial cells undergoing cell matrix detachment, anoikis, are sensitive to Fas-mediated apoptosis. Despite upregulating the level of Fas receptor, endothelial cells undergoing anoikis have reduced cMet/Fas interaction, in part because of cMet being cleaved in these cells. Dimeric Fas was observed on anoikis cells. These data suggest that cMet/Fas interaction may inhibit self-association of Fas receptor such that reduced DISC formation occurs in these cells after Fas receptor ligation. cMet/Fas interaction may help explain why endothelial cells are resistant to Fas-mediated apoptosis. (*Hypertension*. 2005;46:100-106.)

Key Words: apoptosis ■ endothelium

Endothelial cells lining the blood vessels are constantly exposed to a variety of stimuli capable of inducing death through receptor-mediated apoptosis. Fas (APO-1/CD95) is a type I membrane receptor belonging to a member of the tumor necrosis factor receptor superfamily. This receptor is expressed on many cells, including vascular endothelial cells.¹ Despite expressing the Fas receptor, primary vascular endothelial cells are resistant to Fas-mediated cell death.¹⁻³ Protection from Fas-mediated apoptosis has been shown to require matrix attachment, and under anoikis conditions (matrix detachment) endothelial cells become sensitive to Fas-mediated cell death.⁴ Endothelial cells also become sensitive to Fas-mediated apoptosis in response to certain atherogenic factors, such as oxidized LDLs (OxLDLs) and homocysteine.⁵⁻¹¹ Because many atherogenic factors can induce apoptosis in cultured endothelial cells, Fas-mediated apoptosis leading to endothelial cell loss may be a major factor contributing to the pathogenesis of endothelial dysfunction and atherosclerosis.⁵

Although Fas has been recognized predominantly as an apoptosis inducer, evidence for additional apoptosis-independent functions of Fas signaling has been described.^{12,13} In endothelial cells, Fas signaling may have a role in angiogenesis,¹⁴ the maintenance of endothelial function, and the regulation of blood pressure in the cardiovascular system.¹⁵

The aim of this study was to characterize the molecular basis for endothelial cell resistance to Fas-mediated cell death through analyzing the molecules recruited to the death-inducing signaling complex (DISC) after receptor ligation. We were also interested in determining whether the cell surface molecule cMet, which is highly expressed in endothelial cells, plays a role in Fas resistance in these cells. Recently, resistance to Fas-mediated cell death in a mouse hepatocellular carcinoma cell line as well as a human hepatoblastoma cell line has been attributed to the strong interaction of Fas receptor with cMet.¹⁶ This interaction prevents self-association of Fas receptors as well as Fas ligand (FasL)–receptor interaction.

Our results demonstrate that Fas receptors associate with cMet, with such interactions inhibiting the self-association of Fas in endothelial cells. Despite this interaction, we observed that after Fas aggregation, a nonactive DISC containing FADD-like interleukin-1–converting enzyme–inhibitory protein-L (FLIP_L) is formed. These observations suggest 2 additional stages during which resistance to Fas-mediated apoptosis in endothelial cells is regulated.

Materials and Methods

Cells

Human umbilical vein endothelial cells (HUVECs) were purchased from Cascade Biological and maintained in Media 200, purchased

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from the supplier. Experiments were performed using HUVECs between passages 2 and 4.

Jurkat T cells were maintained in RPMI medium 1640 (Gibco) supplemented with 10% heat-activated FCS (Globe Farm), 2 $\mu\text{mol/L}$ L-glutamine, and antibiotics.

Antibodies

CH11, a monoclonal antibody (mAb) recognizing the human cell surface antigen Fas, was purchased from Upstate Biotechnology. Monoclonal anti-human Fas-associated death domain (FADD) antibody (A66-2) was obtained from BD Pharmingen. Monoclonal anti-caspase 8 antibody (Ab-3) was purchased from Oncogene/CN Biosciences. Monoclonal mouse anti-poly(ADP-ribose)polymerase (PARP) antibody (clone C2-10) was from R & D Systems. Anti-receptor interacting protein antibody was obtained from Transduction Laboratories. Rabbit anti-cMet polyclonal antibody was purchased from Santa Cruz Biotechnology. Monoclonal anti-FLIP antibody (NF-6) was a kind gift from Marcus Peter (The Ben May Institute for Cancer Research, Chicago, Ill). Second-step horseradish peroxidase (HRP)-conjugated antibodies anti-IgG1 and Protein A were from BD Pharmingen and Amersham Pharmacia Biotech, respectively. Anti-mouse IgM (μ -chain specific) biotin-conjugated antibody was purchased from Sigma-Aldrich. Streptavidin HRP was from Amersham Pharmacia Biotech.

Immunoprecipitations and Immunoblotting

DISC formation was studied in 1 to 2×10^7 Jurkat and HUVECs adapting a method described previously.¹⁷ In brief, cells were stimulated with the anti-Fas mAb CH11 for 15 minutes, washed in ice-cold PBS, and lysed in a Nonidet P-40-containing DISC lysis buffer. Components of the DISC were immunoprecipitated using anti-mouse IgM-agarose beads (Sigma-Aldrich). In some experiments, HUVECs were stimulated with a purified mouse IgM (MOPC-104E) (Sigma-Aldrich).

For total cell lysates, 1×10^6 unstimulated or CH11-stimulated HUVECs and Jurkat T cells were lysed in DISC buffer and resolved on 12.5% SDS-PAGE gels. After SDS-PAGE and Western Blotting, expression of Fas, FADD, pro-caspase 8, FLIP, RIP, and cMet were analyzed via immunoblotting using the antibodies described above. The protocol for CH11 immunoblotting was as described by the manufacturer, except that the second-step reagents used were an anti-mouse IgM (μ -chain specific) biotin-conjugated antibody and streptavidin HRP.

Anoikis Experiments

Anoikis was induced by plating HUVECs into 10-cm culture dishes treated with Poly-Heme (Sigma-Aldrich). Cells were kept at 37°C for 16 hours in the presence or absence of 1 $\mu\text{g/mL}$ of CH11 before cell viability was determined using trypan blue exclusion. For immunoprecipitations, cells were kept under anoikis conditions for 16 hours before being treated as described above.

Statistical Analysis

Statistical analysis was performed using an unpaired Student *t* test, and results are expressed as mean \pm SD ($n=3$). A value of $P < 0.05$ was considered significant.

Results

Endothelial Cells Are Insensitive to CD95-Mediated Cell Death

Endothelial cells are resistant to Fas-mediated cell death despite expressing the Fas receptor.¹ It has been published previously that ligation of Fas using recombinant FasL induced apoptosis in Jurkat T cells but not in endothelial cells.^{15,18} To confirm Fas insensitivity in our study, we stimulated HUVECs for 16 hours with increasing concentrations of the anti-Fas antibody CH11 and assessed

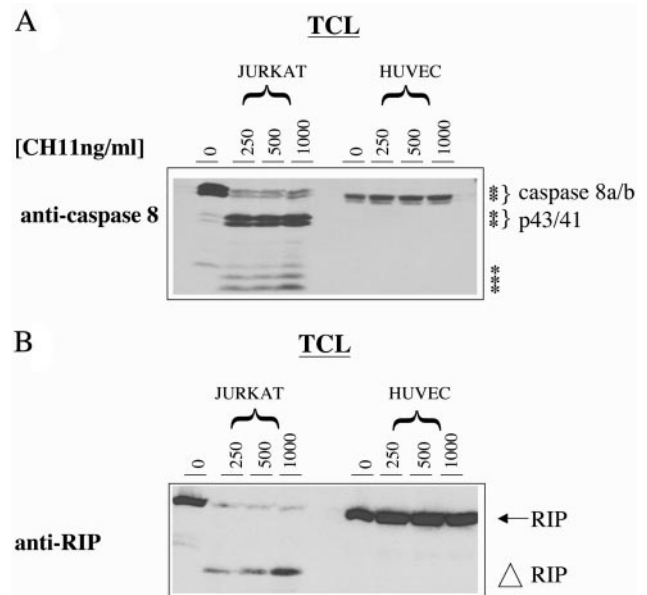


Figure 1. Endothelial cells are resistant to Fas-mediated apoptosis. Jurkat T cells and HUVECs were stimulated for 16 hours in the presence of 250, 500, and 1000 ng/mL of CH11. Unstimulated controls were cultured in the absence of CH11. Cells were then lysed in and the total cell lysate (TCL) run on a 12% SDS-PAGE gel. A, Caspase 8 cleavage was analyzed after Western blotting using anti-caspase 8 antibodies. Asterisks indicate cleaved products of caspase 8. B, RIP cleavage was analyzed after Western blotting using an anti-RIP antibody. Full-length 74-kDa RIP is indicated with an arrow, and the 38-kDa cleaved RIP fragment is indicated by Δ . Data are representative of 3 experiments.

whether cleavage of pro-caspase 8 to its active components occurred. Activation of caspase 8 has been shown in cells sensitive to Fas after stimulation. Despite expressing pro-caspase 8a/b¹⁹ at high levels, no cleavage of this molecule to the active p43/41-kDa subunits was observed in HUVECs, even after stimulation with high concentrations of CH11 (Figure 1A). In contrast, the p43/41-kDa and what may be the p18, 12-, and 10-kDa subunits of cleaved caspase 8²⁰ were observed in Jurkat T cell lysates after CH11 stimulation (Figure 1A).

RIP is cleaved by activated caspase 8 after stimulation through the Fas receptor.^{21,22} To further confirm a lack of pro-caspase 8 cleavage in HUVECs, we analyzed the status of RIP after Fas receptor ligation. No cleavage of RIP was observed in HUVECs; however, cleaved RIP was observed in Jurkat T cells, confirming previous reports (Figure 1B).²¹ We conclude, unlike Jurkat T cells, HUVECs are resistant to Fas-mediated cell death such that no cleavage of pro-caspase 8a/b occurs. This observation is in agreement with other studies in which the sensitivity of Jurkat T cells and endothelial cells was compared.^{15,18}

"Nonactive" DISC Formation in HUVECs

To date, biochemical analysis of DISC formation in endothelial cells has not been studied. Comparing components of the DISC formed in Jurkat T cells and HUVECs may shed light on the mechanisms of Fas resistance in

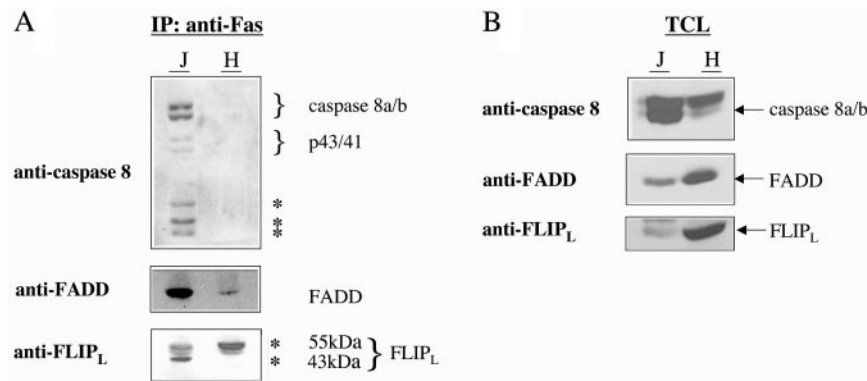


Figure 2. “Nonfunctional” DISC formation in endothelial cells. A, Components of the DISC were immunoprecipitated (IP) from 1 to 2×10^7 Jurkat T (J) cells or HUVECs (H) stimulated for 15 minutes with CH11. Proteins were resolved on a 12% SDS-PAGE gel, and after Western blotting, the membrane was immunoblotted with anti-FADD, anti-caspase 8, or anti-FLIP_L antibodies. The asterisk indicates cleaved products of caspase 8 and FLIP_L. B, Pre-IP lysate from Jurkat and HUVECs cells were run on a 12% SDS-PAGE gel. Pro-caspase 8, FADD, and FLIP_L were detected after Western blotting using anti-caspase 8, -FADD, and -FLIP_L antibodies, respectively. TCL indicates total cell lysate. Data are representative of 6 experiments.

endothelial cells. Previous reports have shown that Fas oligomerization results in FADD recruitment to the death domain of the receptor.²³ In turn, this results in the recruitment of pro-caspase 8, as well as FLIP_L to the death effector domains of FADD.²³ It has been shown previously that the presence of full-length FLIP_L is necessary for the initial cleavage of pro-caspase 8 molecule. Active caspase 8 then cleaves FLIP_L.^{24–29}

Components of the DISC were coprecipitated from HUVECs and Jurkat T cells after stimulation with the anti-Fas antibody CH11. Recruitment of FADD to the Fas receptor death domain occurred in Jurkat T cells and HUVECs, albeit at lower levels in the latter (Figure 2A). Low-level recruitment of FADD was not attributable to a lack of FADD protein in HUVECs (Figure 2B). Pro-caspase 8 was recruited and cleaved to the p43/41-kDa subunits as well as to the active p18, 12-, 10-kDa subunits only in Jurkat T cells after CH11 stimulation (Figure 2A). In contrast, despite recruiting a small quantity of FADD, no recruitment of pro-caspase 8 was observed in HUVECs (even after an overnight exposure of the blot [data not shown]; Figure 2A). This was not because of a lack of pro-caspase 8 in endothelial cells (Figures 1A and 2B). No FADD or pro-caspase 8 coprecipitated with Fas receptors after stimulation with an anti-IgM antibody (data not shown). Recruitment of FADD and pro-caspase 8 required receptor ligation because no coprecipitation of these molecules with the Fas receptor occurred in unstimulated cells (data not shown).

Interestingly, FLIP_L was recruited to the DISC of Jurkat T cells and HUVECs (Figure 2A). However, 2 striking differences were apparent. The quantity of FLIP_L recruited was substantially greater in HUVECs compared with Jurkat T cells. This may reflect the differences in the total amount of FLIP_L seen in the 2 cell types. HUVECs express more FLIP_L than Jurkat T cells (Figure 2B), confirming previously published findings.³⁰ Another observation was that FLIP_L in HUVECs remained uncleaved, again confirming that very little or no recruitment of pro-caspase 8 occurred in these cells (Figure 2A). In conclusion, lack of Fas sensitivity in endothelial cells may be attributable to “nonactive” partial DISC formation after Fas aggregation. A longer exposure of HUVECs to the anti-Fas antibody

CH11 did not change the above observations (data not shown).

cMet/Fas Interaction in HUVECs May Inhibit Self-Aggregation of CD95

The initiation phase of Fas signaling can be divided into distinct steps,³¹ the first of which is ligand-independent receptor preassociation in which unstimulated Fas receptors exist as preassociated complexes.³² The next step is formation of SDS-stable Fas microaggregates after stimulation with ligand. Kinetic and inhibitor studies suggest that the third step in Fas signaling is formation of the DISC. It is feasible that Fas receptors on the surface of endothelial cells are not in this preassociated state such that no microaggregation, and hence very little DISC formation, occurs after receptor ligation. To address this possibility, we analyzed the cell surface expression of Fas on endothelial cells by Western blotting using the CH11 antibody. Fas receptors exist as 46-kDa monomers in HUVECs. In comparison, Fas receptors were present as monomers (46 kDa) and SDS-stable dimers (92 kDa) in Jurkat T cells (Figure 3A). These observations suggest a lack of Fas preassociation in endothelial cells.

It was reported recently that the interaction of the Fas receptor with the cell surface molecule cMet inhibits self-assembly of Fas.¹⁶ Because cMet is expressed in HUVECs but not Jurkat T cells (Figure 3B), it is feasible that cMet and Fas associate in endothelial cells, and that this interaction prevents self-aggregation of Fas. To test this possibility, the Fas receptor was immunoprecipitated from HUVECs, and cMet association was assessed using Western blotting. cMet was indeed associated with Fas in these cells. No association was observed after immunoprecipitation with a control IgM antibody (Figure 3C). We suggest that the interaction between cMet with Fas receptors on endothelial cells may inhibit preassociation of cell surface Fas, leading to impaired DISC formation after receptor ligation.

cMet/Fas Interaction Is Reduced in Anoikis HUVECs

To attribute further the cMet/Fas interaction to Fas resistance in endothelial cells, we analyzed the association of these molecules in HUVECs that had been rendered

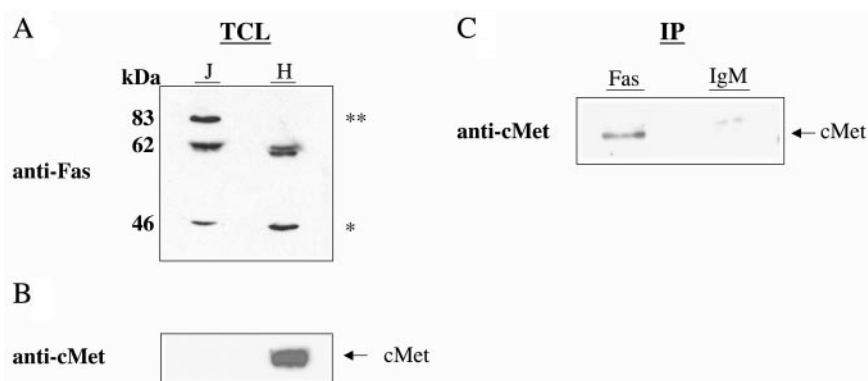


Figure 3. Fas receptors exist as monomers on HUVECs because of cMet/Fas interaction. Total cell lysate (TCL) from 1×10^6 Jurkat T cells (J) and HUVECs (H) were resolved on a 12% SDS-PAGE gel. After Western blotting, filters were immunoblotted with the anti-Fas antibody CH11 (A) and an anti-cMet antibody (B). Data represent 3 experiments. Fas receptor was immunoprecipitated (IP) from 2×10^7 HUVECs and cMet association assessed by immunoblotting. Control cells were immunoprecipitated with IgM alone (C). A single asterisk represents the 45-kDa monomeric form of Fas, whereas the double asterisk represents the 92-kDa dimeric form of Fas. Data are representative of 6 experiments.

sensitive to Fas. It has been shown previously that endothelial cells undergoing matrix detachment, known as anoikis, become sensitive to Fas-mediated cell death.⁴ We confirmed that HUVECs cultured overnight under anoikis conditions, on Poly-Heme coated plates, were sensitive to Fas-mediated cell death, as shown by trypan blue staining as well as detection of PARP cleavage (Figure 4A and 4B). Under anoikis conditions, reduced cMet/Fas interaction was observed, despite the increased levels of Fas receptor on these cells (Figure 5A). Lack of interaction may have been the result of reduced cMet expression in endothelial cells under anoikis conditions (Figure 5B). cMet appeared to be cleaved under these conditions (Figure 5B). Interest-

ingly, reduced cMet association with Fas resulted in the appearance of 92-kDa SDS-stable dimeric Fas molecules in endothelial cells undergoing anoikis (Figure 5C). We suggest that reduced association with cMet and Fas contributes to Fas sensitivity in anoikis endothelial cells through allowing self-assembly of Fas.

Discussion

Vascular endothelial cells expressing the Fas receptor and FasL are normally resistant to Fas-mediated cell death. Exposure to atherogenic factors renders endothelial cells susceptible to Fas-mediated apoptosis.⁶ Because endothelial cell apoptosis represents the critical event for the initiation of atherosclerosis,³³ understanding the molecular mechanism underlying Fas resistance may allow development of therapeutic agents that will inhibit endothelial cell death induced by Fas ligation.

We show that endothelial cells are well equipped to withstand Fas stimulation at the cell surface level, through expression of cMet, and within the cell, through recruitment of FLIP_L to the DISC.

Fas receptor and cMet association has been reported previously in cell lines.¹⁶ This interaction renders cells resistant to Fas-mediated cell death through preventing self-association of Fas receptor as well as Fas/FasL interaction.¹⁶ In the present study, we show that cMet expressed on endothelial cells is associated with Fas receptors under normal conditions. However, under anoikis conditions, in which endothelial cells become sensitive to Fas, this interaction is severely impaired because of reduced cMet expression. How could the cMet/Fas interaction contribute to Fas resistance in endothelial cells? It is feasible that the cMet/Fas interaction may prevent self-association of Fas receptors in endothelial cells, as suggested by the following observations. Fas receptors exist as monomers in endothelial cells, whereas on Jurkat T cells that lack cMet, the most abundant form of the receptor is the 92-kDa SDS-stable dimer. Reducing the level of cMet resulted in 92-kDa SDS-stable Fas receptor dimers being expressed on endothelial cells, suggesting that self-association of Fas has occurred in these cells.

FLIP_L completely blocks Fas-mediated apoptosis through inhibition of caspase-8 processing at the DISC. It

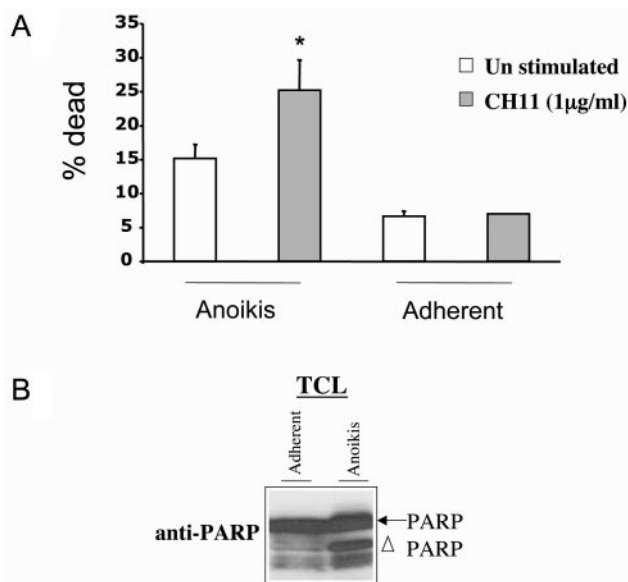


Figure 4. HUVECs undergoing anoikis are sensitive to Fas-mediated apoptosis. A, HUVECs were either cultured on Poly-Heme (anoikis) or not for 16 hours (adherent) in the presence of 1 μg/mL of CH11. Unstimulated controls were cultured in the absence of CH11. Cell viability was measured by trypan blue staining. Data are shown as the mean ± SD (n=3). Statistical significance was evaluated using an unpaired Student *t* test; **P* < 0.05 was significantly different from nonstimulated cells. B, PARP cleavage was detected by Western blotting using an anti-PARP antibody. Full-length PARP (116 kDa) is indicated with an arrow, and the 85-kDa cleaved PARP is indicated by Δ. TCL indicates total cell lysate. Data are representative of 3 experiments.

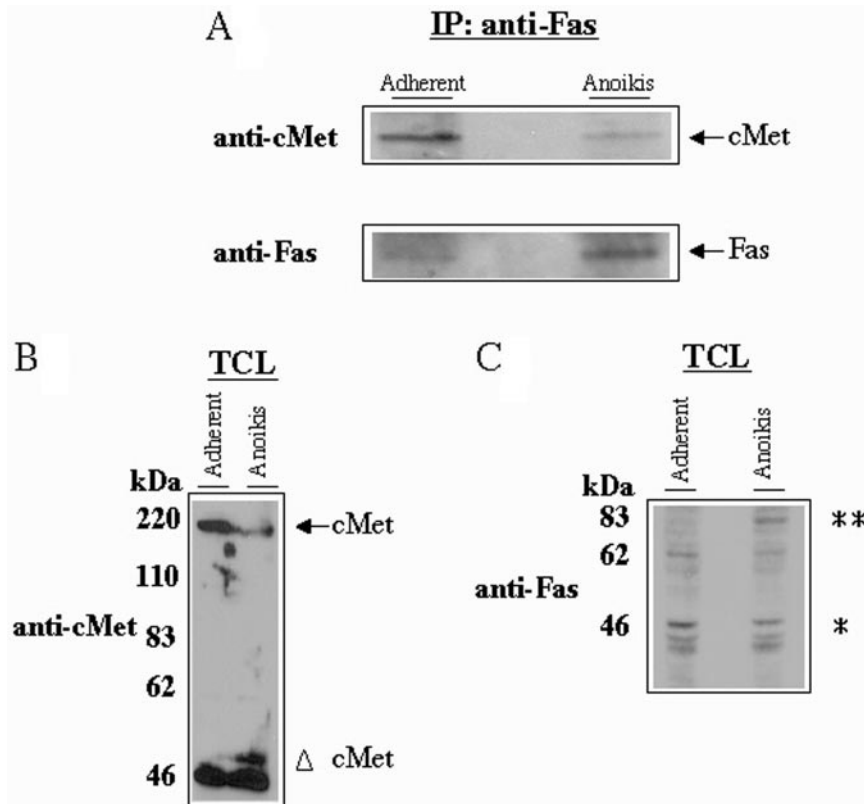


Figure 5. Reduced cMet/Fas interaction in HUVECs undergoing anoikis is associated with increased expression of SDS-stable dimers of Fas. Fas receptor was immunoprecipitated (IP) from HUVECs either cultured on Poly-Heme (anoikis) or not (adherent) for 16 hours (A). cMet and Fas were analyzed by Western blotting. Total cell lysate (TCL) from the preimmunoprecipitated samples was resolved on 12% SDS-PAGE gels, and cMet and Fas expression was analyzed by Western blotting using an anti-cMet antibody (B) or CH11 antibody (C). A single asterisk represents the 45-kDa monomeric form of Fas, whereas the double asterisk represents the 92-kDa dimeric form of Fas. Cleaved cMet is indicated by Δ . Data are representative of 4 experiments.

has been suggested that the affinity of the FADD to bind FLIP_L is higher than pro-caspase 8, which may explain why this molecule blocks recruitment of full-length caspase 8 into the complex. In endothelial cells, it is the Fas pathway inhibitor FLIP_L and not pro-caspase 8 that is preferentially recruited to DISC after stimulation. Because of the abundance of this molecule, it is possible that FLIP_L is preferentially recruited. This results in no pro-caspase 8 recruitment, processing, nor activation of downstream caspases such as caspase 3.

HUVECs have been rendered sensitive to Fas-mediated cell death through treating the cells with atherogenic factors OxLDLs⁸ and homocysteine,⁶ or extracellular signal-regulated kinase (ERK) inhibitor PD90859.⁴ In all cases, increased sensitivity to Fas is accompanied by a reduction in c-FLIP levels. It is feasible that reducing endogenous FLIP_L may allow caspase 8 to access the few FADD molecules recruited to the DISC after aggregation. However, reducing the levels of FLIP_L may not be the only requirement. We found that FLIP_L was still recruited to the DISC of HUVECs treated with PD90859 despite this procedure, reducing FLIP_L levels by >70% (data not shown). Kataoka et al³⁴ have shown that susceptibility to Fas-mediated cell death in murine thymoma cells depends mainly on the expression level of c-FLIP versus cell surface Fas. This may also be the case in endothelial cells. Increasing the cell surface levels of Fas, as well as the accessibility to Fas receptors through reducing cMet/Fas association, may also be as important as reducing the level of FLIP_L. All these events occur in HUVECs undergoing anoikis, and interestingly, these cells are Fas sensitive.

Although the Fas receptor has been recognized predominantly as an apoptosis inducer, evidence for additional apoptosis-independent functions of Fas have been described.^{12,13} In one study, Biancone et al¹⁴ described the development of inflammatory angiogenesis in a murine model by the agonistic anti-Fas mAb Jo2. They found that subcutaneous implants of Matrigel containing mAb Jo2 were rapidly infiltrated by endothelial cells, suggesting that signaling through the Fas receptor in endothelial cells leads to proliferation rather than apoptosis. Signals through the Fas receptor that resulted in proliferation required FLIP_L.¹² It is feasible that a similar mechanism occurs in endothelial cells. The cMet receptor has also been linked to angiogenesis. Binding of hepatocyte growth factor (HGF) to the cMet receptor results in endothelial cell proliferation and migration through activation of the ERK kinase pathway.^{35–39} HGF also induces the expression of the antiapoptotic molecule Mcl-1.⁴⁰ It is feasible that the interaction of Fas with cMet may generate stronger angiogenic stimulation if both receptors encounter their cognate ligands. Therefore, not only may this interaction inhibit Fas-mediated cell death through inhibiting complete DISC formation, it may also serve as an angiogenic stimulus.

It has been shown recently that Fas signaling plays a role in regulating blood pressure. Fas ligation with either FasL or agonistic antibodies results in endothelial cell NO synthase (eNOS) upregulation in a phosphatidylinositol 3-kinase/Akt-dependent manner. NO derived from eNOS is an important regulator of blood pressure.⁴¹ Hypertension develops in mice lacking Fas.¹⁵ It is possible that the interaction of Fas and cMet could help maintain endothe-

lial cell function and blood flow through inhibiting Fas-mediated apoptosis. Recruitment of FLIP_L to the receptor, after interaction with FasL, prevents apoptosis of endothelial cells, thus allowing production of NO. However, under chronic disease conditions, endothelial cells are subjected to many factors that may abrogate the cMet/Fas interaction, thereby allowing DISC formation and cell death. Reduced expression of Fas attributable to loss of endothelial cells via apoptosis may contribute toward the development of hypertension and other circulatory diseases.

Perspectives

Recruitment of FLIP_L to Fas after impaired receptor ligation attributable to the cMet/Fas interaction may be a key mechanism underlying endothelial cell resistance to Fas. Mapping the signaling pathway downstream of FLIP_L may further elucidate the mechanisms leading to maintenance of endothelial function. Fas signaling clearly has a role in blood pressure regulation via eNOS and NO production, and defective Fas signaling is associated with hypertension. Therefore, our data highlight 2 novel mechanisms, via cMet/Fas and DISC assembly, whereby perturbation of endothelial cell function may contribute to hypertension or other circulatory disease processes.

Acknowledgments

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