ADAM10-mediated Release of Complement Membrane Cofactor Protein during Apoptosis of Epithelial Cells*

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Membrane cofactor protein CD46 controls complement activation on cells, is a receptor for several pathogens, and modulates immune responses by affecting CD8⁺ T cells. Cells can release CD46 in an intact form on membrane vesicles and in a truncated form by a metalloproteolytic cleavage. The mechanism of shedding and its relationship to cell physiology has remained unclear. We have found using RNA interference analysis that a disintegrin and metalloproteinase (ADAM) 10 is responsible for the regulated shedding of the ectodomain of CD46 in apoptotic cells. The shedding of CD46 was initiated with staurosporine and UVB. Exposure of cell cultures to either UVB or staurosporine resulted in changes of cell morphology and detachment of cells from their matrices within 8–24 h. During this process CD46 was released both in apoptotic vesicles (vCD46) and proteolytically (sCD46) into the medium. Both the metalloproteinase inhibitor GM6001 and RNA interference of ADAM10 completely prevented the release of sCD46 and increased the expression of vCD46 on HaCaT cell vesicles, suggesting that ADAM10 releases sCD46 from the apoptotic vesicles. To explore whether the release of sCD46 is associated with apoptosis we analyzed the effects of caspase inhibitors. As expected, the inhibition of caspase activity attenuated the characteristic features of apoptosis and also decreased the release of sCD46. Our results reveal ADAM10 as an important regulator of CD46 expression during apoptosis. The ADAM10-mediated release of CD46 from apoptotic vesicles may represent a form of strategy to allow restricted complement activation to deal with modified self.

Almost ubiquitously expressed membrane cofactor protein CD46 is a complement regulator that protects cells and tissues from complement damage (1). CD46 can also function as a receptor for several pathogens (2). Cross-linking of CD46 initiates signaling events in a number of cell types (3–6). Recently it was found that CD46 can modulate immune responses by affecting CD8⁺ T cell cytotoxicity, CD4⁺ T cell proliferation, and the production of interleukin-2, -10, and transforming growth factor-β (7). Furthermore, CD46 induces T-regulatory cell 1 phenotype (8). Normally membrane-bound CD46 has been detected in a soluble form in the cerebrospinal fluids of multiple sclerosis patients with a human herpesvirus 6 infection, for which CD46 has been reported to be a receptor (9). The sera from both normal individuals and cancer patients contain three forms of CD46 with the molecular masses of 56, 47, and 29 kDa. The levels of the 56- and 42-kDa forms are increased in the sera of cancer patients (10). Elevated levels of CD46 have been observed in the sera of patients with systemic lupus erythematosus (11); the urine of patients with glomerular diseases contains three molecular mass forms of CD46 (52, 46, and 35 kDa) (12).

Matrix metalloproteinases and the closely related a disintegrin and metalloproteinase (ADAM)² family of membrane-bound proteases have the capacity to convert transmembrane proteins into soluble forms to generate and regulate their biological functions (13). The shedding is stimulated in most experimental systems by the phorbol ester PMA (13). In addition, reactive oxygen species (ROS), sphingomyelinase P1, and detachment of cells from the pericellular matrix, but unexpectedly not PMA, enhance the proteolytic release of sCD46 (14, 15). The biologically active constitutively secreted 55–60-kDa form sCD46 lacks the hydrophobic transmembrane sequence and is produced by constitutive proteolytic cleavage of the intact form on the tumor cell membranes (14). However, the metalloproteinase responsible for the shedding has not been identified yet.

ROS can activate latent proforms of metalloproteinases as well as induce apoptosis (16, 17). These findings, together with our earlier observations of reactive oxygen species and detachment of cells from the pericellular matrix enhancing the proteolytic release of sCD46, formed the basis for our hypotheses that sCD46 is released during apoptosis beyond the constitutive release. UVB is a potent inducer of ROS-generating oxidative stress and apoptosis in cells (18). We have therefore investigated the effects of UVB radiation and staurosporine-induced apoptosis on the metalloproteolytic shedding of sCD46.

ADAMs are plasma membrane-bound proteases, some of
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which are associated with vesicular structures (19). Interestingly, ADAM10, 12, and 17 are activated in response to ROS (20). Furthermore, calcium ionophores stimulate ADAM10-mediated ectodomain shedding (21). In the present work we have identified ADAM10 as the enzyme responsible for the solubilization of the ectodomain of CD46 (sCD46) during apoptosis. The identification of the sCD46-releasing protease opens new views for studies on pathological processes involving overactivation of complement that are related to deficiencies of CD46.

EXPERIMENTAL PROCEDURES

Reagents—The metalloproteinase inhibitor GM6001 was from Calbiochem. The pan-caspase inhibitor IV (Z-VAD-fmk) and staurosporine were obtained from Calbiochem. N-Acetyl-l-cysteine (NAC) was obtained from Sigma. The murine monoclonal antibody (mAb) J4.48 against human CD46 was purchased from Immunotech (Marseille, France). Rabbit antibodies against ADAM10 and ADAM17 (TACE) were obtained from Abcam plc (Cambridge, UK). Peroxidase-conjugated immunoglobulins to rabbit or mouse IgG were from Jackson ImmunoResearch Laboratories (Burlingame, CA). Goat anti-mouse IgG conjugated to Alexa Fluor 594 or Alexa Fluor 488 were from Molecular Probes (Leiden, the Netherlands). Fluorescein isothiocyanate-conjugated Annexin V was from Sigma.

Cell Culture—Human keratinocyte HaCaT (22) and ovarian adenocarcinoma Caov-3 (23) cells were obtained from American Type Culture Collection (ATCC, Manassas, VA). The cells were grown in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% heat-inactivated fetal calf serum (Invitrogen), 2 mM L-glutamine (BioWhittaker, Walkersville, MD), and antibiotics (10 units/ml of penicillin and 10 μg/ml of streptomycin) at 37 °C in a humidified atmosphere containing 5% CO₂. The cells were subcultured twice a week.

UVB and Staurosporine Treatment of the Cells—Cells were cultivated in 6-well culture plates or in individual 35-mm diameter plates until 90–95% confluent. For the assays the complete medium was replaced with serum-free Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% heat-inactivated fetal calf serum (Invitrogen), 2 mM L-glutamine (BioWhittaker, Walkersville, MD), and antibiotics (10 units/ml of penicillin and 10 μg/ml of streptomycin) at 37 °C in a humidified atmosphere containing 5% CO₂. The cells were harvested twice a week.

UVB and Staurosporine Treatment of the Cells—Cells were treated with UVB or staurosporine exposure and harvested twice a week.

Flow Cytometric Analysis of CD46 Release from Staurosporine-treated Cells—Some cells are spontaneously detached from the growth substratum during culture, resulting in the form of apoptotic cell death called anoikis. Significant C3 and C4 deposition can be detected on the apoptotic cells only in the late phase of apoptosis and is mainly focused on small cells and subcellular fragments (26). To understand the interrelationships between these events we compared the expression of CD46 between substratum-bound and detached cells. Subconfluent cultures of Caov-3 cells were treated with staurosporine together with the metalloproteinase inhibitor (10 μM GM6001) as indicated. After 8 h of incubation the spontaneously detached floating cells were isolated from their culture medium by low-speed centrifugation. The substratum-bound cells were then detached with 0.02% EDTA in phosphate-buffered saline. The cells were kept on ice during the processing for the flow cytometric analysis. After washing, the cells were incubated with a monoclonal antibody to CD46. In the controls the primary antibody was replaced with an irrelevant antibody of the same subclass as the specific mAb. After washing with TBS/Tween the bound antibodies were visualized using peroxidase-conjugated anti-mouse IgG and an electrochemiluminescence substrate (SuperSignal® ULTRA Substrate; Pierce).

Detection of Caspase Activation in UVB- and Staurosporine-treated Cells—The Caspase-Glo™ 3/7 luminometric assay (Promega, Madison, WI) was used according to the manufacturer’s instructions to measure the activities of caspases 3 and 7. Briefly, after UVB or staurosporine exposure the cells were harvested from the culture plates into the culture medium with a cell scraper. Aliquots of the medium (25 μl) in triplicate were mixed with equal amount of Caspase-Glo™ 3/7 assay reagent and measured with a luminometer. Unexposed cells served as a negative control. The caspase activation data are expressed as a-fold over negative control.

SDS-PAGE and Immunoblotting—The trichloroacetic acid precipitates were washed with ice-cold acetone and dissolved in Laemmli sample buffer. The precipitated polypeptides were then separated by SDS-PAGE (10% acrylamide monomer concentration). Subsequently, the proteins were transferred onto nitrocellulose. After the saturation of the nonspecific protein binding sites of the membranes with 5% (w/v) nonfat milk powder in Tris-buffered saline (TBS), pH 7.5, containing 0.05% Tween 20 (TBS/Tween) the filters were incubated overnight at 4 °C with pretested dilution of mAb to CD46 in TBS/Tween supplemented with 5% nonfat milk powder. In the controls the primary antibodies were omitted or replaced with an irrelevant antibody of the same subclass as the specific mAb. After washing with TBS/Tween the bound antibodies were visualized using peroxidase-conjugated anti-mouse IgG and an electrochemiluminescence substrate (SuperSignal® ULTRA Substrate; Pierce).

Flow Cytometric Analysis of CD46 Release from Staurosporine-treated Adherent and Floating Cells—Some cells are spontaneously detached from the growth substratum during culture, resulting in the form of apoptotic cell death called anoikis. Significant C3 and C4 deposition can be detected on the apoptotic cells only in the late phase of apoptosis and is mainly focused on small cells and subcellular fragments (26). To understand the interrelationships between these events we compared the expression of CD46 between substratum-bound and detached cells. Subconfluent cultures of Caov-3 cells were treated with staurosporine together with the metalloproteinase inhibitor (10 μM GM6001) as indicated. After 8 h of incubation the spontaneously detached floating cells were isolated from their culture medium by low-speed centrifugation. The substratum-bound cells were then detached with 0.02% EDTA in phosphate-buffered saline. The cells were kept on ice during the processing for the flow cytometric analysis. After washing, the cells were incubated with a monoclonal antibody to CD46. In the controls the primary antibody was replaced with an irrelevant antibody of the same subclass. The bound antibodies were detected with anti-mouse antibodies labeled with a fluorescent probe. Finally the cells were analyzed by a BD Biosciences FACScan 440 flow cytometer. From similarly treated cultures, equal numbers (1.6 × 10⁶) of adherent or spontaneously detached cells, isolated vesicles, and supernatants were analyzed for the expression of CD46 by SDS-PAGE and immunoblotting. For immunofluorescence microscopy analysis the cells were stained with a monoclonal antibody to visualize CD46 and fluorescein isothiocyanate-conjugated Annexin V to stain apoptotic cells. The bound antibodies were detected with Alexa Fluor 594.

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Small Interfering RNA (siRNA) Transfection—ADAM10 siRNA (5′-AGACAUUAUGAAGGAUAU-3′) was synthesized by Qiagen (West Sussex, UK) as described (27). HaCaT cells were transfected with annealed siRNAs using Lipo-fectamineTM2000 (Invitrogen). Control cells were transfected with nonsilencing scrambled siRNA. After 48 h, the cells were exposed to 0.1 μM staurosporine and analyzed for sCD46, vCD46, and cellular CD46 from total cell lysates as described above. The expression level of ADAM10 in total cell lysates and in vesicles was assessed by immunoblotting as described above. ADAM17 was detected as a nonsilenced control protein in the immunoblots.

RESULTS

UVB and Staurosporine Induce the Shedding of sCD46 in a Metalloproteinase-dependent Manner—The origin and the mechanisms of the metalloproteolytically released sCD46 have remained unclear. Recent observations of sCD46 release from injured or dying cells (14, 15) and the down-regulation of complement regulatory proteins (CD35, CD46, CD55, and CD59) during apoptosis (28–30) suggested to us that sCD46 could be released during apoptosis. To study this we induced apoptosis in HaCaT and Caov-3 cells by increasing doses of UVB (500–4000 J/m²) or of staurosporine (0.001–1 μM). GM6001 was used to inhibit metalloproteinase activity where indicated. Trichloroacetic acid-precipitated sCD46 was detected by immunoblotting from the serum-free supernatant fluids that were clarified by centrifugation after harvesting, first at 2000 × g and then at 200,000 × g to separate vCD46 and sCD46. The two shed CD46 species differ in their molecular masses; sCD46 is slightly smaller (55–60 kDa) than the membrane-associated vCD46 detected in the vesicles (60–65 kDa) (14). Treatment of the cells with UVB (Fig. 1A) resulted in the shrinkage and retraction and finally detachment of some of the cells from the matrix. The keratinocyte cell line HaCaT required higher doses of UVB (4000 J/m²) than Caov-3 carcinoma cells (1000 J/m²) to induce the shedding of sCD46 (Fig. 1B). The untreated cells did not release detectable amounts of sCD46 during the 24-h incubation. Human skin fibroblasts, used as a control, did not shed sCD46 at any of the UV doses tested (data not shown). The metalloproteinase inhibitor GM6001 (10 μM) completely prevented the cleavage of CD46 in UVB-treated cells (Fig. 1B).

Staurosporine is a broad spectrum inhibitor of protein kinases that induces apoptosis in a number of normal and malignant cell lines (31). Treatment of both HaCaT and Caov-3 cell cultures by staurosporine resulted in changes of cell morphology resembling those of UVB treatment (Fig. 1A). We found that, in parallel, sCD46 was released into the medium in a concentration-dependent manner (Fig. 1B). Accordingly, the metalloproteinase inhibitor GM6001 also completely prevented the staurosporine-induced release of sCD46.

Both UVB and Staurosporine Result in Increased Caspase Activity and sCD46 Release in Caov-3 and HaCaT Cells—Caspases are major players in the initiation of the apoptotic program leading to cell death. Caspases 3 and 7 belong to the group of effector or executioner caspases that act downstream of the point of no return in the apoptotic cascade to cleave the cells into apoptotic bodies (32). The increase in caspase activities correlated with the increase of the shedding of sCD46 both in UVB- and staurosporine-treated HaCaT and Caov-3 cells (Fig. 1, B and C). Over 5-fold increase in the caspase activity was required to initiate the shedding of sCD46 as judged from the immunoblot. A plateau in caspase activity was reached at 1000 J/m² exposure of UVB for Caov-3 cells. For HaCaT cells 2000
J/m² was required to induce approximately the same level of caspase activity. As expected, GM6001 (10 μM) did not have any effect on the caspase activity (data not shown). Accordingly, staurosporine (0.1 μM) stimulated the caspase activities 5-fold in Caov-3 and 14-fold in HaCaT cells (Fig. 1C). To measure the time lag for the initiation of the sCD46 release we exposed the cells to UVB and monitored the solubilization of CD46 within 4–24 h. We found that sCD46 became gradually visible in the immunoblots after 8 h (Fig. 2).

Inhibition of Apoptosis with the Pan-caspase Inhibitor Z-VAD-fmk Reduces the Shedding of sCD46—To further characterize whether the UVB- and staurosporine-stimulated release of sCD46 resulted from the induction of apoptosis, we carried out analyses with the pan-caspase inhibitor Z-VAD-fmk. The inhibitor was added into the serum-free medium of the cells 60 min prior to the treatments. We found that the inhibition of caspase activity in the cells was accompanied by decreased release of sCD46 into the medium in a dose-dependent manner, both in the UVB- (Fig. 3B) and staurosporine- (Fig. 3C) treated cultures. Z-VAD-fmk (100 μM) completely prevented the release of sCD46 from HaCaT cells (Fig. 3B). Accordingly, Z-VAD-fmk reduced the release of sCD46 from UVB-treated Caov-3 cell cultures in a concentration-dependent manner (Fig. 3B).

The Radical Scavenger NAC Inhibits the Shedding of sCD46—UVB is a potent inducer of ROS-generating oxidative stress in cells (18). NAC can increase the cellular synthesis of endogenous antioxidant glutathione or protect the cells by directly scavenging ROS. To assess this, we found that the stimulatory effect of UVB irradiation on sCD46 shedding was attenuated dose-dependently by NAC in both cell models (Fig. 4), which suggested the involvement of ROS in the signal transduction of staurosporine- and UVB irradiation-enhanced sCD46 shedding.

FIGURE 3. Pan-caspase inhibitor Z-VAD-fmk decreases UVB-induced shedding of soluble sCD46. Caov-3 and HaCaT cells were grown to 85–95% confluency. The pan-caspase inhibitor Z-VAD-fmk was added into the medium at the indicated concentrations 60 min before exposure of the cells to UVB (4000 J/m²). After the exposure of HaCaT cells to UVB (4000 J/m²) resulted in detachment and shrinkage of cells during 24 h of incubation. Untreated control cells and cells exposed to Z-VAD-fmk remained firmly attached to the matrix (phase contrast ×40). After 24 h of incubation the cell culture medium was harvested and clarified by centrifugation. Immunoblot analysis was used to detect sCD46 from the supernatants after precipitation with trichloroacetic acid. Z-VAD-fmk decreased the UVB-(B) and staurosporine-(C) induced release of sCD46 in a concentration-dependent manner.

FIGURE 4. NAC inhibits the release of soluble sCD46 from UVB- and staurosporine-treated cells. Caov-3 and HaCaT cells were grown to 85–95% confluency. NAC was added at the indicated concentrations 60 min before exposure of the cells to UVB (4000 J/m²) or staurosporine (0.1 μM). After 24 h of incubation the cell culture medium was harvested (see “Experimental Procedures”). The trichloroacetic acid-precipitated sCD46 was detected by immunoblotting.

Inhibition of Apoptosis with the Pan-caspase Inhibitor Z-VAD-fmk Reduces the Shedding of sCD46—To further characterize whether the UVB- and staurosporine-stimulated release of sCD46 resulted from the induction of apoptosis, we carried out analyses with the pan-caspase inhibitor Z-VAD-fmk. The inhibitor was added into the serum-free medium of the cells 60 min prior to the treatments. We found that the inhibitor clearly reduced the rounding and other morphological changes both in the staurosporine- and UVB-treated HaCaT (Fig. 3A) and Caov-3 cells (data not shown). The inhibition of caspase activity in the cells was accompanied by decreased release of sCD46 into the medium in a dose-dependent manner, both in the UVB- (Fig. 3B) and staurosporine- (Fig. 3C) treated cultures. Z-VAD-fmk (100 μM) completely prevented the release of sCD46 from HaCaT cells (Fig. 3B). Accordingly, Z-VAD-fmk reduced the release of sCD46 from UVB-treated Caov-3 cell cultures in a concentration-dependent manner (Fig. 3B).
Metalloproteolytic and Nonproteolytic Release of CD46 during Apoptosis—Some of the apoptotic cells are spontaneously detached from the culture substratum during culture. Using immunofluorescence microscopy we did not detect any changes in the CD46 expression on the cells firmly attached to the culture substratum with or without GM6001 (data not shown). We therefore hypothesized that the sCD46 could become shed from the detached apoptotic cells. To explore this possibility we used flow cytometry to analyze the expression of CD46 on floating cells and on cells attached on culture matrix. Caov-3 cells were treated with 1 μM staurosporine and the metalloproteinase inhibitor GM6001. Because sCD46 was detected by immunoblotting at 8 h (data not shown), we isolated the spontaneously detached floating cells from the medium by low speed centrifugation at this time point. The substratum-bound cells were detached with 0.02% EDTA in phosphate-buffered saline. After washing the cells with ice-cold phosphate-buffered saline containing 1% bovine serum albumin, the cells were incubated with a monoclonal antibody to CD46 on ice, and the bound antibodies were detected with anti-mouse antibodies labeled with Alexa Fluor 488.

Flow cytometric analysis showed that the level of CD46 on staurosporine-treated attached cells was comparable with that of staurosporine-treated attached cells when metalloproteinase activity was inhibited with GM6001 (Fig. 5A, upper left panel). However, the level of CD46 on floating cells was lower than that on attached cells (upper right and middle left panels). Furthermore, the floating cells isolated from staurosporine-treated cultures had lost CD46 irrespective of the inhibition of the metalloproteinases when compared with cells bound to the substratum. There was a minor increase in CD46 expression on the floating cells that were isolated from cultures treated with staurosporine and GM6001 over cultures where the inhibitor was omitted (middle panel on the right). However, GM6001 did not restore the expression of CD46 on the floating cells to the level of attached cells.

Western blotting analysis of adherent and spontaneously detached cells indicated that detached cells expressed lower amounts of CD46 than the attached cells (Fig. 5B). The metalloproteinase inhibitor (GM6001) did not have a marked effect on the CD46 expression in GM6001-treated or untreated adherent or detached cells. However, GM6001 increased vCD46 expression in vesicles and blocked the proteolytic release of sCD46 into the supernatant (Fig. 5B). For immunofluorescence microscopy we double stained the staurosporine-treated cells with Annexin V and anti-CD46. During the examination the Annexin V-stained cells (Fig. 5C, green fluorescence) expressed markedly less CD46 (red fluorescence) than the non-floating cells isolated from staurosporine- and metalloproteinase inhibitor-treated cultures (lower right panel). B, immunoblotting analysis of an assay described above. Detached cells expressed lower levels of CD46 than the attached cells. GM6001 prevented the release of sCD46 (supernatant) and increased vCD46 (vesicles). C, immunofluorescence analysis of the release of CD46 from apoptotic Caov-3 cells. Examination of the staining patterns of the cells with fluorescence microscopy revealed heterogeneous expression patterns of CD46 (red fluorescence) on individual Caov-3 cells (left panel). Staining of the apoptotic cells with fluorescein isothiocyanate-conjugated Annexin V (green fluorescence) revealed reduced expression of CD46 on apoptotic cells (right panel). The middle panel shows a merged image of CD46 and Annexin V staining. Apoptotic bleb detected on a cell is indicated (arrow).
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Immunoblotting using antibodies to ADAM10 and 17 showed that both proteins were expressed in HaCaT cells in the pro and mature form (Fig. 6). During staurosporine treatment the mature cellular ADAM10 was lost from the cells and appeared in the vesicular fraction (Fig. 6). A degraded 45-kDa immunoreactive form of ADAM10 was detected in the supernatant (data not shown). The transient transfection of cells with ADAM10 siRNA blocked the expression of both cellular ADAM10 (Fig. 6) and vesicular ADAM10. This silencing did not have any effect on expression of ADAM17, which served as a control protein. Both sCD46 and vCD46 were detected in staurosporine-treated HaCaT cell cultures. However, neither form was detectable in the cultures not exposed to staurosporine (Fig. 6). ADAM10 depletion in the cells resulted in complete inhibition of sCD46 release, whereas the level of vCD46 in vesicles was increased. This result strongly suggested that sCD46 is mainly released from the apoptotic vesicles by ADAM10. However, in total cell lysates the ADAM10 siRNA treatment slightly increased the level of cellular CD46. The negative control siRNA did not have any effect on the ADAM10 or ADAM17 expression or CD46 shedding (Fig. 6).

DISCUSSION

We found here that CD46 is susceptible to cleavage by a disintegrin and a metalloproteinase 10 (ADAM10) and that the release of sCD46 is stimulated by apoptosis induced by staurosporine or UVB treatment of the cells. Our results indicate that the level of sCD46 increased in parallel with the activation of caspases. The caspase inhibitor Z-VAD-fmk, the metalloproteinase inhibitor GM6001, and siRNA to ADAM10 attenuated or completely prevented the release of sCD46. In addition, the depletion of ADAM10 expression resulted in increased expression of vCD46.

The activities of a number of cell membrane proteins are regulated by metalloproteinolytic release of the protein ectodomains from the cell surface (13). Nonetheless, the majority of the transmembrane proteins are resistant to proteolysis, suggesting that the shedding is a specific and a regulated process (13). Characteristics for the known cascades of ectodomain shedding are the stimulatory effect observed by phorbol esters (like PMA) and inhibition by selective metalloproteinase inhibitors such as BB3103, GM6001, and tumor necrosis factor-α protease inhibitor (TAPI). According to our previous observations PMA is inefficient in inducing the release of sCD46 (14). Both BB3103 and TAPI inhibited the shedding of sCD46 from a number of cell lines (14). The shedding of protein ectodomains by metalloproteinases has frequently been observed in experiments accomplished under serum-free conditions, which itself induces apoptosis in many cultured cell lines. This and the stimulatory effect of ROS on CD46 cleavage (14) suggested that the sCD46 could originate from the cells undergoing programmed cell death.

Both UVB and staurosporine are able to induce apoptosis in a number of normal and malignant cell lines. Low doses of UV radiation induce cell cycle arrest, whereas high doses bring about cell death/apoptosis. UVC induces DNA damage, whereas the longer wavelengths UVB and UVA expose the cells to ROS, lipid peroxidation, and protein damage (34). We found
here that the exposure of HaCaT and Caov-3 cells to UVB or staurosporine activated caspases 3 and 7, resulting in morphological changes and finally in the detachment of some of the cells from the growth substratum. The floating cells represent a population of cells that are in the later stages of apoptosis and cannot be rescued from death (35). We found that after \( \sim 5 \)-fold increase in the caspase activity the shedding of sCD46 was clearly detected in the immunoblots. Interestingly, lower caspase activities in Caov-3 cells resulted in the shedding of a larger amount of sCD46 than in HaCaT cells. Caov-3 cells appeared to detach more easily than HaCaT cells under serum-free conditions, probably due to apoptosis. This resulted in higher caspase activity in the untreated controls of Caov-3 cells than in HaCaT cells and is thus seen as a low “\(-fold over control\)” caspase activity in Fig. 1C. The larger amount of sCD46 may reflect the higher expression level of CD46 in Caov-3 cells than in HaCaT cells.

The wide spectrum metalloproteinase inhibitor GM6001 completely prevented both UVB- and staurosporine-induced shedding of sCD46 during the 8–24 h incubation. The three caspase inhibitors Z-VAD-fmk, Z-DEVD-fmk, and BOC-D-fmk (data not shown) had comparable decreasing effects on the shedding of sCD46. GM6001 did not have any effect on the caspase activities. Furthermore, inhibition of caspases with Z-VAD-fmk did not have any impact on ADAM10 expression (data not shown). This further suggested that CD46 is cleaved by ADAM10 and not by caspases. Similar cleavages of E-cadherin (36), PECAM-1 (35), and latent transforming growth factor-binding proteins, LTBP2 (25), have been observed after induction of apoptosis with staurosporine or serum deprivation. UVB is a well known inducer of ROS, and staurosporine also generates ROS (37, 38). Therefore, we analyzed next the effect of a ROS scavenger (NAC) on the shedding. As expected, NAC attenuated the release of sCD46 from cells exposed to UVB. ROS can both activate and inactivate latent proforms of metalloproteinases (16, 17, 39). However, the UVB-induced shedding was inhibited by Z-VAD-fmk, indicating that ROS activated the apoptotic cascade that finally resulted in the activation of the CD46-cleaving ADAM10.

Flow cytometric analysis revealed that the expression levels of CD46 were comparable between the substratum-bound, adherent cells treated either with staurosporine or staurosporine and the metalloproteinase inhibitor GM6001 within 8 h of incubation. Loss of cell anchorage results in detachment-induced apoptosis (anoikis) in some cells (40). Unexpectedly, the floating cells isolated from the culture medium exhibited significantly reduced levels of CD46 irrespective of the inhibition of metalloproteinase activity by GM6001. This indicates that the detached cells had evidently lost the protein by mechanisms other than metalloproteolytic cleavage. The most plausible explanation is that the apoptotic cells had released vCD46 in the vesicles or apoptotic blebs. This is suggested also by the fact that staurosporine treatment resulted in the loss of mature ADAM10 from the total cell lysates and in its coinciding appearance in the vesicular fraction with vCD46. ADAM10 siRNA blocked the expression of both cellular and vesicular ADAM10. The specific blocking of ADAM10 resulted in a slight increase of cellular CD46 in HaCaT cells, suggesting that sCD46 may be partially cleaved at the cell surface.

Various lines of evidence indicate that complement contributes to the removal of dying cells. These include the C1q binding to the surface blebs on apoptotic cells (41), the activation of the alternative pathway by apoptotic umbilical vein endothelial cells (28), and the increased uptake of apoptotic cells by macrophages in the presence of serum leading to the binding of C3 (42). Significant deposition of complement components C3 and C4 on apoptotic cells can be detected only in the late phases of apoptosis and is mainly focused on small cells and subcellular fragments (26). Our results suggest that ADAM10 modifies the density of CD46 on self-tissue. The controlled increase in complement activation on apoptotic cells and vesicles may be desirable for disposal of cellular debris to facilitate repair. The reduced expression of vCD46 may result in increased complement activation on the apoptotic blebs and more efficient opsonization and clearance.

ADAMs are involved in a number of physiological and pathological processes. The expression of ADAMs can be stimulated by growth factors, cytokines, cellular stress, wounding, and the phorbol ester PMA (43). Identification of ADAM10 as an enzyme that can regulate CD46 levels on cell membranes opens new views for conditions where regulated or pathological complement activation occurs on host cells. Recent evidence suggests that limited but directed complement activation on self-tissue exists in conditions related to ischemia-reperfusion injury, autoantibodies/immune complexes, cellular toxins, apoptosis (44), facilitation of liver regeneration (45), and enhancement of reproduction (46). Controlled complement activation may facilitate wound healing and repair (44). On the other hand, complement activation on tissue relatively deficient in complement regulators may allow its pathological amplification. CD46-related deficiencies resulting in overactivation of complement have been detected in familial hemolytic uremic syndrome and rheumatoid arthritis, which also include increased expression of ADAM10 and ADAM15 (47). Down-regulation of CD46 has also been reported during certain infections (48). Recently, increased expression or stimulation of ADAM10 was reported by Helicobacter pylori or by lipoteichoic acid of Staphylococcus aureus and Streptococcus pyogenes (49, 50).

The membrane-bound complement regulators have received attention as a resistance strategy exploited by malignant cells (51). The high expression of complement regulators leads to tumors that are resistant to complement and is a significant limitation for the use of monoclonal antibodies (52). Inactivation of complement regulators by mAbs restores the tumor vulnerability to complement (52). We found here for the first time that the multifunctional cell surface protein CD46 belongs to the group of proteins whose levels can be regulated by ADAM10. It was possible to stimulate the shedding of sCD46 by UVB, staurosporine, and calcimycin treatment that activates ADAM10-mediated sCD46 shedding. Identification of the CD46 shedding will open new possibilities for studies that aim at increasing cancer cell vulnerability by reducing their complement resistance.
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REFERENCES

1. Seya, T., Turner, J. R., and Atkinson, J. P. (1986) J. Exp. Med. 163, 837–855
2. Lindahl, G., Sjöbring, U., and Johnson, E. (2000) Curr. Opin. Immunol. 12, 44–51
3. Karp, C. L., Wysocka, M., Wahl, L. M., Ahearn, J. M., Cuomo, P. J., Sherry, W. C., and Heinecke, J. W. (2001) J. Biol. Chem. 276, 2620–2629
4. Sbarba, P. D., and Rovida, E. (2002) Mol. Biol. Cell. 13, 44–51

17. Fu, X., Kassim, S. Y., Parks, W. C., and Heinecke, J. W. (2000) J. Clin. Immunol. 20, 345–358
18. Kaneko, E., Ueda, M., Budiyanto, A., Bito, T., Oka, M., Fukunaga, M., Ichihashi, M., Ueda, M., Budiyanto, A., Bito, T., Oka, M., Fukunaga, M., Kuriyama, S., Hasegawa, T., Nagasawa, S., and Okumura, K. (1999) J. Immunol. 162, 727–736

25. Solovyov, V. T., and Keski-Oja, J. (2006) J. Cell. Physiol. 207, 445–453
26. Gaipl, U. S., Kuenkele, S., Voll, R. E., Beyer, T. D., Kolowos, W., Heyder, P., Kalden, J. R., and Herrmann, M. (2001) Cell Death Differ. 8, 327–334
27. Murai, T., Miyazaki, Y., Nishinakamura, H., Sugahara, K. N., Miyasaka, M., Sako, Y., Yanakida, T., and Miyasaka, M. (2000) J. Biol. Chem. 279, 4541–4550
28. Tsuji, S., Kaji, K., and Nagasawa, S. (1994) J. Biochem. 116, 794–800
29. Hara, T., Matsumoto, M., Tsuji, S., Nagasawa, S., Hiraoka, A., Masaoka, T., Kodama, K., Horai, T., Sakuma, T., and Seya, T. (1996) Immunobiology 196, 491–503
30. Jones, I., and Morgan, B. P. (1995) Immunology 86, 651–660
31. Gescher, A. (2000) Crit. Rev. Oncol. Hematol. 34, 127–135
32. Vermes, I., Haanen, C., and Reutelingsperger, C. (2000) J. Immunol. Methods 243, 167–190
33. Amour, A., Knight, C. G., Webster, A., Slocombe, P. M., Stephens, P. E., Knauper, V., Dockerty, A. J., and Murphy, G. (2000) FEMS Lett. 473, 275–279
34. Guzman, E., Langowski, J. L., and Owen-Schaub, L. (2003) Apoptosis 8, 315–323
35. Ito, M., Kachi, Y., and Madeo, D. (2002) FASEB J. 16, 362–372
36. Steinhusen, U., Weiske, J., Bauck, T., Bammert, K., and Huber, O. (2001) J. Biol. Chem. 276, 4972–4980
37. Sordet, O., Khan, Q. A., Plo, I., Pourquier, P., Ursaki, Y., Yoshida, A., Antony, S., Kohlhagen, G., Solary, E., Sarapaa, M., Laval, J., and Pommier, Y. (2004) J. Biol. Chem. 279, 50499–50504
38. Simizu, T., Numata, T., and Okada, Y. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 6770–6773
39. Weiss, S. I. (1989) New Engl. J. Med. 320, 365–376
40. Frisch, S. M., and Francis, H. (1994) J. Cell Biol. 124, 619–626
41. Navratil, J. S., Watkins, S. C., Wisnieski, J. J., and Ahearn, J. M. (2001) J. Immunol. 166, 3231–3239
42. Mevorach, D., Mascarenhas, J. O., Gershov, D., and Elkon, K. B. (1998) J. Clin. Invest. 101, 913–923
43. Riley-Vargas, V. C., Lanzendorf, S., and Atkinson, J. P. (2005) Apoptosis 10, 281–289
44. Fisher, P. G., and Fontana, A. (2003) Semin. Immunopathol. 25, 345–358
45. Yoshimura, T., Tomita, T., Dixon, M. F., Axon, A. T. R., Robinson, P. A., and Crabtree, J. E. (2002) J. Exp. Med. 196, 445–453
46. Fishelson, Z., Doniin, N., Zell, S., Schultz, S., and Kirschfink, M. (2003) Cell Death Differ. 8, 327–334
47. Murai, T., Miyazaki, Y., Nishinakamura, H., Sugahara, K. N., Miyasaka, M., Sako, Y., Yanakida, T., and Miyasaka, M. (2000) J. Biol. Chem. 279, 4541–4550
48. Tsuji, S., Kaji, K., and Nagasawa, S. (1994) J. Biochem. 116, 794–800
49. Hara, T., Matsumoto, M., Tsuji, S., Nagasawa, S., Hiraoka, A., Masaoka, T., Kodama, K., Horai, T., Sakuma, T., and Seya, T. (1996) Immunobiology 196, 491–503
50. Jones, I., and Morgan, B. P. (1995) Immunology 86, 651–660
51. Gescher, A. (2000) Crit. Rev. Oncol. Hematol. 34, 127–135
52. Vermes, I., Haanen, C., and Reutelingsperger, C. (2000) J. Immunol. Methods 243, 167–190
53. Amour, A., Knight, C. G., Webster, A., Slocombe, P. M., Stephens, P. E., Knauper, V., Dockerty, A. J., and Murphy, G. (2000) FEMS Lett. 473, 275–279
54. Guzman, E., Langowski, J. L., and Owen-Schaub, L. (2003) Apoptosis 8, 315–323
55. Ito, M., Kachi, Y., and Madeo, D. (2002) FASEB J. 16, 362–372
56. Steinhusen, U., Weiske, J., Bauck, T., Bammert, K., and Huber, O. (2001) J. Biol. Chem. 276, 4972–4980
57. Sordet, O., Khan, Q. A., Plo, I., Pourquier, P., Ursaki, Y., Yoshida, A., Antony, S., Kohlhagen, G., Solary, E., Sarapaa, M., Laval, J., and Pommier, Y. (2004) J. Biol. Chem. 279, 50499–50504
58. Simizu, T., Numata, T., and Okada, Y. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 6770–6773
59. Weiss, S. I. (1989) New Engl. J. Med. 320, 365–376
60. Frisch, S. M., and Francis, H. (1994) J. Cell Biol. 124, 619–626
61. Navratil, J. S., Watkins, S. C., Wisnieski, J. J., and Ahearn, J. M. (2001) J. Immunol. 166, 3231–3239
62. Mevorach, D., Mascarenhas, J. O., Gershov, D., and Elkon, K. B. (1998) J. Clin. Invest. 101, 913–923
63. Riley-Vargas, V. C., Lanzendorf, S., and Atkinson, J. P. (2005) J. Clin. Investig 115, 1241–1249
64. Duffy, M. J., Lynn, D. J., Lloyd, A. T., and O’Shea, C. M. (2002) Transplantation 89, 622–631
65. Lizewska, K., Kemper, C., Price, J. D., and Atkinson, J. P. (2005) Springer Semin. Immunopathol. 27, 345–358
66. Yoshimura, T., Tomita, T., Dixon, M. F., Axon, A. T. R., Robinson, P. A., and Crabtree, J. E. (2002) J. Infect. Dis. 185, 332–340
67. Lemjabbar, H., and Basbaum, C. (2002) Nature Med 8, 41–45
68. Fishelson, Z., Doniin, N., Zell, S., Schultz, S., and Kirschfink, M. (2003) Mol. Immunol. 40, 109–123
69. Durrant, L. G., and Spendlove, I. (2001) Curr. Opin. Investig. Drugs 2, 959–966