Early Detachment of Colon Carcinoma Cells During CD95(APO-1/Fas)-mediated Apoptosis
I. De-adhesion from Hyaluronate by Shedding of CD44

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Abstract. Ligation of CD95 (APO-1/Fas) cell surface receptors induces death in apoptosis-sensitive cells. Induction of apoptosis in adherent gamma interferon-stimulated HT-29 and COLO 205 colon carcinoma cells by cross-linking CD95 with anti-APO-1 monoclonal antibody resulted in detachment of the cells from hyaluronate starting about 1 h after antibody exposure. Loss of adhesion was paralleled by a substantial reduction of the multifunctional cell surface adhesion molecule CD44. As evidenced by cycloheximide treatment, this effect was not caused by impaired protein synthesis. Depletion of surface CD44 was also not due to membrane blebbing, since cytochalasin B failed to inhibit ascension from hyaluronate. Instead, ELISA and time kinetics showed increasing amounts of soluble CD44 in the supernatant of CD95-triggered cells. SDS-PAGE revealed that soluble CD44 had an apparent molecular mass of about 20 kD less than CD44 immunoprecipitated from intact cells. Thus, CD95-triggering induced shedding of CD44. Shedding is a novel mechanism operative in early steps of CD95-mediated apoptosis. Shedding surface molecules like CD44 might contribute to the active disintegration of dying epithelial cells in vivo.

Apoptosis is an irreversible intracellular program induced by a variety of internal and external stimuli and which leads to the cell’s extinction. Apoptosis does not cause any tissue reaction except phagocytosis (Cohen and Duke, 1992). An early feature of cells undergoing apoptosis in tissues is detachment from neighboring cells or the basement membrane (Searle et al., 1982). This also applies to colonic epithelium where senescent enterocytes undergo apoptosis, detach from the basement membrane, and are released into the gut lumen (Gavrieli et al., 1992; Strätter et al., 1995). There is good evidence that this phenomenon is maintained in colonic carcinoma cell lines in vitro. Induction of apoptosis in HT-29 cells by factors like transforming growth factor β, etoposide, or teniposide leads to detachment from the substratum, and cells are found in the supernatant (Oberhammer et al., 1993; Desjardins and MacManus, 1995).

CD95 (APO-1/Fas) is a 48-kD cysteine-rich type I transmembrane glycoprotein and is a member of the tumor necrosis factor receptor family (Itoh et al., 1991; Oehm et al., 1992). Upon cross-linking of CD95 by antibody (Trauth et al., 1989; Dhein et al., 1992) or by its natural ligand, CD95-positive cells undergo apoptosis, provided the cells are sensitive. It has been shown that CD95 is expressed at high levels on colonic epithelial cells along the crypt axis and at the mucosal surface (Möller et al., 1994). CD95 is often down-modulated in colon carcinoma in situ and on colon carcinoma cell lines (Möller et al., 1994). Stimulation of cells by gamma interferon (IFN-γ), however, increases CD95-expression (Möller et al., 1994) and sensitizes cells for CD95-induced cell death (Yonehara et al., 1989).

CD95 ligand is a 40-kD type II membrane protein belonging to the tumor necrosis factor family of cytokines (Suda et al., 1993; Smith et al., 1994; Takahashi et al., 1994). Like other members of this cytokine family, CD95 ligand exists in a membrane-bound and soluble form (Dhein et al., 1995). CD95 ligand in its biologically active form is a trimer (Nagata and Golstein, 1995).

CD44 is a type I transmembrane glycoprotein involved in homo- and heterotypic cell adhesion and cell-matrix interaction. The CD44 core protein of 37 kD is N and O-glycosylated to an 85-95-kD form, the so-called standard (CD44s) or hematopoietic form or, by linkage of chondroitin sulfate (Dhein et al., 1992). Upon cross-linking of CD44 by antibody (Trauth et al., 1989; Dhein et al., 1992) or by its natural ligand, CD44 is shed as a 20-kD fragment in the supernatant of CD95-stimulated cells. CD44 is a member of the RHAMM family of cell surface glycoproteins that are involved in the development of tumors and malignant cells (Dhein et al., 1995).

Abbreviations used in this paper: CD44s, CD44 standard form; DCI, 3,4-dichloroisocoumarin; HMW, high molecular weight; ICE, interleukin-1β converting enzyme; IFN-γ, gamma interferon; PI, propidium-iodine.
The most selective CD44 ligand, however, is hyaluronate antibodies (Goldstein et al., 1991; Stamencovic et al., 1991). Further structural complexity of the CD44 molecule comes from alternative cosaminoglycan substitutions as the hematopoietic form of one of the early phenomenon of CD95-induced apoptosis. We show here that CD44 and is primarily expressed on epithelial cells (Brown et al., 1989). An intermediate-size form of 110–160 kD shows similar glycosaminoglycan substitutions as the hematopoietic form of CD44 and is primarily expressed on epithelial cells (Brown et al., 1991; Stamencovic et al., 1991). Further structural complexity of the CD44 molecule comes from alternative splicing of at least 10 exons of the CD44 gene, giving rise to the so-called CD44 variants with altered adhesion properties (Güntert, 1993). CD44 molecules act as receptors for fibronectin (Jalkanen and Jalkanen, 1992), type I and IV collagen (Carter and Wayner, 1988), and sulphated proteoglycan (Toyama-Sorimachi and Miyasaka, 1994). The most selective CD44 ligand, however, is hyaluronate (Stamencovic et al., 1989).

To investigate detachment of cells upon induction of apoptosis, we used HT-29 and COLO 205 colon carcinoma cells line as models for epithelial cells. We show here that one of the early phenomenon of CD95-induced apoptosis is the loss of surface CD44 by proteolytic cleavage. This loss of CD44 abrogates the hyaluronate-binding capacity and therefore contributes to the early detachment of adherent cells seen during CD95-induced cell death.

### Material and Methods

#### Reagents

3,4-dichloroisocoumarin (CDI), 1,10-phenanthroline, Na-p-tosyl-l-lysine chloro-methyl ketone (TLCK), cytochalasin B, leupeptin, cycloheximide, etoposide, okadaic acid, and propidium-iodide (PI) were purchased from Sigma Chemical Co. (St. Louis, MO). Boc-Asp(OBzl)-CMK was obtained from Sigma Chemical Co. (St. Louis, MO). 3,4-dichloroisocoumarin (CDI), 1,10-phenanthroline, Na-p-tosyl-l-lysine chloro-methyl ketone (TLCK), cytochalasin B, leupeptin, cycloheximide, etoposide, okadaic acid, and propidium-iodide (PI) were purchased from Sigma Chemical Co. (St. Louis, MO). Boc-Asp(OBzl)-CMK was obtained from Sigma Chemical Co. (St. Louis, MO). 3,4-dichloroisocoumarin (CDI), 1,10-phenanthroline, Na-p-tosyl-l-lysine chloro-methyl ketone (TLCK), cytochalasin B, leupeptin, cycloheximide, etoposide, okadaic acid, and propidium-iodide (PI) were purchased from Sigma Chemical Co. (St. Louis, MO). Boc-Asp(OBzl)-CMK was obtained from Sigma Chemical Co. (St. Louis, MO).

#### Antibodies

CD44a(A-1) [IgG1 isotype], CD44s(F10-44-2) [IgG2a isotype], CD29 (PAC10) [IgG2b isotype], and FITC-conjugated goat anti-mouse IgG affinity purified IgG was purchased from DSMO (Hamburg, Germany). CD44s(MEM85) [IgG1 isotype] was purchased from Serva (Heidelberg, Germany). CD21 lF18) [IgG1 isotype] and CD30(Ki-1) [IgG1 isotype] were obtained from Dako (Copenhagen, Denmark). CD95(anti-APO-1) [IgG1 isotype] and PA-1 [IgG1 isotype] were generated by the laboratories of two of us (P.H. Krammer and G. Moldenhauer, respectively).

#### Cell Culture Conditions

Human colon carcinoma cell lines HT-29 and COLO 205 were purchased from American Type Culture Collection (Rockville, MD). Cells were kept in RPMI 1640 (GIBCO, BRL, Gaithersburg, MD) containing 10% fetal calf serum (FCS, PAA, Linz, Austria), 5 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C. 5% CO2-atmosphere and were cultured on either plastic or hyaluronate. Culture plates were coated with hyaluronate (5 mg/ml) by incubating plates overnight at 4°C. Finally, pre-coated plates were blocked with BSA and cells were added. To sensitize cells to CD95-triggered apoptosis, HT-29 and COLO 205 were treated with 100 U/ml IFN-γ for 72 h (Möller et al., 1994). Apoptosis was induced by cross-linking surface CD95 with 500 ng/ml anti-APO-1 (Dhein et al., 1992). To remove adherent cells from culture plates, cells were incubated with trypsin/EDTA (Biochrom-KG, Berlin, Germany) for 5 min at 37°C. Subsequently, cells were washed twice with either RPMI or Hank’s salt solution.

#### DNA Fragmentation Assay

For DNA fragmentation analysis, a pellet of 10⁸ cells was lysed in 20 μl lysis buffer (0.5% N-lauroylsarcosine, Sigma Chemical Co.; 10 mM EDTA; 50 mM Tris, pH 8.0, 0.5 mg/ml protease K) for 1 h at 50°C. After addition of 1 μl of RNase A (1 mg/ml) and further incubation of 1 h at 50°C, lysates were electrophoresed in a 2% agarose gel containing ethidium bromide and photographed under UV illumination. Characterization of high molecular weight (HMW)-DNA damage was achieved by subjecting samples of sarcosyl-lysates (1% N-lauroylsarcosine, Tris-buffer, 2mg/ml protease K, pH 8.0) to pulse field gel electrophoresis on 1% agarose gels. The separated fragments were stained with ethidium bromide and photographed under a transilluminator. DNA fragments were compared to a lambda DNA ladder.

### Immunocytochemistry

Acetoine-fixed cytoplasm preparations of cells were incubated for 1 h with primary monoclonal antibodies at appropriate dilutions. A biotinylated anti-mouse antibody, streptavidin/biotin-peroxidase (Amersham Corp., Buckinghamshire, UK), and aminoethyl-carbazole (Sigma Chemical Co.) served as a detection system for the primary antibody resulting in an intense red precipitate. After counterstaining with Harris' hematoxylin, slides were mounted with glycerol gelatine.

### Flow Cytometry

We used several methods to analyze antigen expression. DNA content, or both in parallel. The first procedure was to resuspend 10⁶ cells in 50 μl Hank’s salt solution containing 0.1% sodium azide, 1.5% Hepes, and 2% FCS. 50 μl of diluted primary antibody was added and incubated on ice for 1 h. After washing, FITC-conjugated goat anti-mouse IgG at appropriate dilution was added. The cell suspension was incubated on ice for 1 h. Cells were washed in Hank’s salt solution and immediately analyzed by flow cytometry on a FACScan® (Becton Dickinson Immunocytometry Sys., Mountain View, CA) using LYSIS II software. To quantify cells with advanced DNA degradation, we used a procedure similar to that described by Nicoletti et al. (1991). A pellet containing 1 × 10⁶ cells was gently resuspended in 500 μl hypotonic fluorochrome solution containing 0.1% Triton X-100 (Sigma Chemical Co.), 0.1% sodium-citrate, and 50 μg/ml PI. The cell suspensions were placed at 4°C in the dark overnight before flow cytometry analysis of cellular DNA content. To compare the immune phenotype of apoptotic and nonapoptotic cells, cells were immunostained with antibodies as described above. Subsequently, cells were washed with Hank’s salt solution and then fixed in 2 ml ice-cold ethanol and incubated on ice for 1 h. After washing, cells were resuspended in 250 μl staining solution containing PI (80 μg/ml) and RNase (0.4 μg/ml) in PBS. Cells were kept in the dark at room temperature for 15 min and then incubated at 4°C overnight before analysis.

### Enzyme-linked Immunosorbant Assay (ELISA)

Soluble CD44 in the culture supernatant was quantified using a soluble CD44 ELISA kit (Bender MedSystem, Vienna, Austria) containing CD44s (SFF-2) [IgG1 isotype] and polyclonal anti-mouse IgG as detection system. Samples were measured at 450 and 620 nm reference wavelength, respectively.

### Pulse-chase Labeling of Cells, Incubation with Monoclonal Antibodies In Situ, Immunoprecipitation, and Enzymatic Treatment of Precipitates

HT-29 cells preincubated with 100 U/ml IFN-γ for 3 d were biosynthetically labeled with a mixture of [35S]methionine and -cysteine. To this end, two tissue culture flasks (75-cm² growth area) containing a semiconfluent cell layer were washed twice with RPMI 1640 medium containing 2mM methionine and cysteine (ICN Flow Laboratories, Kielheim, Germany). Adherent cells were incubated with 10 ml RPMI 1640 medium without methionine and cysteine and supplemented with 5% dialyzed FCS and 2 mM glutamine for 1 h at 37°C, to which subsequently 2 μCi [35S]methionine and [35S]cysteine solution (Promix, specific activity >1000 Ci/mmol, Amershams-Buchler, Braunschweig, Germany) was added. After a labeling period of 1 h at 37°C, the supernatant was washed off and cells were chased in complete RPMI 1640 medium containing 100 U/ml IFN-γ for 16 h at 37°C. After washing with complete medium, cells in one flask were
treated in situ with 3 ml anti-APO-1 monoclonal antibody (500 ng/ml), whereas cells in the control flask were subjected to the same amount of CD30(Ki-) monoclonal antibody, which served as an isotype-matched control. Supernatants were harvested and centrifuged for 30 min at 12,000 × g before further processing. Cells of both flasks were washed twice with PBS and detached by means of 0.25% EDTA. Supernatants were precleared by three sequential cycles of nonspecific precipitations using protein A-Sepharose CL-4B (50% saturation: Pharmacia Fine Chemicals, Uppsala, Sweden). Cells were solubilized in lysis buffer containing 1% Nonidet P-40 and proteinase inhibitors (10 mM PMSF and 20 μg/ml aprotinin, both from Sigma Chemical Co.). Insoluble material was removed by centrifugation at 12,000 × g for 30 min. The cell lysate containing supernatant was prefiltered by three sequential cycles of nonspecific precipitations using affinity-purified polyclonal mouse IgG at 15 μg/50 μl lysate together with 100 μl/50 μl protein A-Sepharose CL-4B. For specific immunoprecipitation, samples either from the supernatant or from the cell lysate were mixed with 10 μg of purified monoclonal antibody and 20 μl protein A-Sepharose CL-4B. After overnight incubation at 4°C, with gentle rotation, the adsorbent was washed extensively and then boiled in reducing SDS sample buffer. Samples were subjected to PAGE employing a discontinuous buffer system. Gels were enhanced with treatment with DMSO/PPD and fluorographed on Kodak X-OMAT AR film (Rochester, NY). To remove N-linked sugar moieties from the precipitated molecules, the washed adsorbents were resuspended in 100 mM Tris/HCl, pH 8.0, containing 0.5% SDS, 1% 2-mercaptoethanol, and 10 mM EDTA, and then were boiled and centrifuged. The supernatants were incubated with 0.5 U/sample endoglycosidase F/N-glycosidase F (Boehringer-Mannheim Gmbtt) for 3 h at 37°C. Subsequently, treated immuno-precipitates were reconstituted with threefold-concentrated reducing sample buffer and run on SDS-PAGE.

### Results

#### Loss of Adhesion Is an Early Event in CD95-mediated Apoptosis

HT-29 and COLO 205 cells were grown as monolayers on plastic and on hyaluronate precoated surfaces. 24 h after treatment with UV irradiation (10 min), etoposide (100 μM), or okadaic acid (10 nM), cells showed morphological signs of apoptosis such as nuclear and cytoplasmatic condensation and, in the case of treatment with okadaic acid or UV irradiation, membrane blebbing. About 24 h, most cells featuring these morphological characteristics remained sessile. Although the surface membrane was still intact (as evidenced by PI exclusion), DNA fragmentation was in an advanced stage (see Fig. 4). Ascension of the cells into the supernatant started between 24 and 36 h and was significant after about 40 h. This observation was in line with previous experiments on HT-29 cells in which the topoisomerase II inhibitor teniposide caused loss of adhesion, which was definitely preceded by internucleosomal DNA cleavage, as a very late event in drug-induced apoptosis (Desjardins et al., 1995).

In contrast, after induction of apoptosis by anti-APO-1, the first alteration that was microscopically visible was the detachment of an increasing number of cells beginning at about 1 h after antibody administration. After 3 h of antibody treatment, adherent cells and cells in suspension were harvested and analyzed separately for morphology and DNA fragmentation. In cytopsin preparations, a major subset of detached cells showed morphological criteria of apoptosis whereas sessile cells were essentially intact (data not shown). Hypodiploid DNA content was only observed in floating cells and not in adherent cells (Fig. 1).

The experiment was repeated with cells grown on uncoated plastic and yielded comparable results.  

![Figure 1. DNA fragmentation measured by flow cytometry (Nicoletti et al., 1991).](image-url)

**Figure 1.** DNA fragmentation measured by flow cytometry. (Nicoletti et al., 1991). (Left) HT-29 cells after 72 h IFN-γ treatment with scarcely detectable DNA degradation. (Right) After 3 h of anti-APO-1 treatment. Cells in suspension were separated from those remaining adherent. Only detached HT-29 cells show characteristic apoptotic DNA fragmentation with lower fluorescence activity.

**CD95-mediated Ascension from Hyaluronate Is Due to Early Depletion of Surface CD44**

To investigate whether CD95-mediated loss of adhesion was due to changes in the surface receptor profile of the cells, expression of surface molecules was examined by immunocytochemistry and flow cytometry. In fact, HT-29 and COLO 205 cells exhibited a significant decrease in surface levels of a comprehensive panel of adhesion molecules. However, the early and extensive depletion of surface CD44 was most impressive (Fig. 2). For further specification, floating and adherent cells were measured separately. While sessile cells still expressed CD44, cells in the supernatant were almost completely devoid of surface CD44. Many of these CD44-negative cells did not yet exhibit cytoplasmatic or nuclear condensation (data not shown). Pretreatment with cytochalasin B, a drug which disrupts microfilaments and interferes with the formation of apoptotic bodies (Catter et al., 1992), failed to decrease the anti-APO-1 induced depletion of CD44 (data not shown) in contrast to integrin-type adhesion receptors (von Reyher, U., J. StrOller, T. Barth, A.R. Günthert, K. Koretz, G. Moldenhauer, P.H. Krammer, and P. Möller, manuscript submitted for publication). Consistently, cells grown on hyaluronic acid were still ascending into the supernatant by similar treatment with anti-APO-1 and cytochalasin B (data not shown) while detachment was extensively delayed when grown on plastic or collagen (v. Reyher et al., submitted).

Kinetics revealed that CD95-mediated decrease of CD44 clearly preceded advanced DNA fragmentation (Fig. 3); 1 h treatment of HT-29 cells with anti-APO-1 led to a significant depletion of surface CD44, while no loss of DNA was detected by flow cytometry. After 2 h, about 90% of cells were CD44-negative and an hypodiploid DNA content was detected in about 25%. Only 3 h after antibody administration, nearly all nuclei exhibited DNA fragmentation.

In comparison, after inducing apoptosis in HT-29 cells with either UV irradiation, okadaic acid, or etoposide, CD44 depletion was only significant in UV-exposed cells. In this case, the decrease started after about 18 h and was preceded by DNA breakdown. Okadaic acid and etoposide failed to induce significant reduction of CD44 surface expression during the first 36 h, although most cells showed advanced DNA fragmentation and nuclear condensation.
densation (Fig. 4). These data collectively suggest that ascension of epithelial cells from hyaluronate caused by extensive depletion of surface CD44 may be specific for CD95-mediated apoptosis.

It is generally accepted that HMW-DNA cleavage, giving rise to DNA fragments of >50 kb, precedes the ladder formation, indicating internucleosomal DNA breaks (Oberhammer et al., 1993; Weis et al., 1995). To correlate loss of surface CD44 with ongoing DNA fragmentation, we simultaneously analyzed samples of CD95-triggered HT-29 cells by pulse field and conventional DNA gel electrophoresis. As shown in Fig. 5, HMW-DNA fragments appeared in parallel with loss of CD44 after 1 h, whereas a characteristic DNA ladder indicating internucleosomal cleavage was seen only after 3 h when HMW-DNA fragments disappeared due to further digestion. This indicates that detachment from hyaluronate and decrease of CD44 surface expression is an early event during CD95-triggered apoptosis, occurring at the time the early HMW-DNA fragments appear.

Depletion of Surface CD44 Is Not Due to Impaired Protein Synthesis

To determine whether the decrease/loss of CD44 was due to lack of synthesis and expression of new surface molecules, we inhibited protein synthesis of HT-29 cells using cycloheximide (5 μg/ml). By measuring the decline of surface CD44 levels after cycloheximide treatment, the half-life of CD44 on HT-29 cells was determined to be about 12 h.
Fluorescence

Figure 4. CD44 cell surface expression (black histogram) in comparison to DNA fragmentation (white histogram) in HT-29 cells after UV irradiation and after treatment with okadaic acid or etoposide. (a) HT-29 cells before treatment showing no DNA fragmentation (first) and clear-cut CD44 surface expression (second). CD21 was used as negative control (third). (b) HT-29 cells after 10 min UV irradiation showing an almost complete DNA fragmentation after 24 h, while surface level of CD44 has just slightly declined. (c) HT-29 cells during exposure to etoposide showing progressive DNA fragmentation after 36 h, while the level of CD44 surface expression has decreased to a minor extent. (d) HT-29 cells during exposure to okadaic acid. DNA fragmentation starts between 18 to 24 h and is completed after 36 h. Only a slight decrease in surface CD44 is detectable after 36 h.

(data not shown). This is in keeping with published data of the kinetics of CD44 surface expression as determined by pulse chase experiments in T-lymphoma cells indicating a half-life of CD44 molecules is in the order of magnitude of 8 h (Lokeshwar et al., 1991. Thus, the very different kinetics by which CD44 is lost from the cell surface after anti-APO-1 treatment are indicative for an active process.

CD95-trigering Induces Soluble CD44

CD44 is one of several surface molecules that can be shed by enzymatic cleavage. This was shown by cross-linking of CD44 (Bazil and Horejsi, 1992) and in phorbolester-stimulated leukocytes (Bazil and Strominger, 1994). To clarify whether shedding is operative in anti-APO-1-triggered cells, supernatants were filtered using a 0.2-μm millipore filter before analysis. In supernatants of untreated cultures of HT-29 and COLO 25 cells, levels of soluble CD44 were regularly low. After administration of anti-APO-1, levels of CD44 increased rapidly during the first 2 h (Fig. 6). These kinetics are in good accordance with the decrease of surface CD44 (Fig. 4).

Cleaved CD44 Is Released into the Supernatant of CD95-stimulated HT-29 Cells

HT-29 cells induced by IFN-γ and pulse chase labeled with [35S]methionine and -cysteine were incubated in situ for 3 h at 37°C with either CD95(anti-APO-1) or CD30(Ki-1) iso-type-matched control antibody. Supernatants of treated cells were immunoprecipitated with CD44(F10-44-2) (Fig. 7). The precipitate from anti-APO-1-treated cells showed a broad band ranging from 130-170 kD under reducing conditions (lane B), whereas Ki-1 did not yield any specific precipitate (lane D). Deglycosylation of N-linked sugar moieties by treatment of the CD44 precipitate with endoglycosidase F/N-glycosidase F resulted in a shift of the band to an apparent molecular weight of 110–150 kD (lane C). By contrast, immunoprecipitations of Ki-1 antibody treated cell supernatants using the same F10-44-2 antibody exhibited only faint background bands (lane E). For comparison, the cellular CD44 molecule was precipitated from cell lysates of anti-APO-1-treated HT-29 cells. Cleavage of N-linked sugars by enzymatic digestion revealed a reduction in apparent molecular mass to 130–170 kD (lane G). The fact that the range of the precipitated bands was not reduced by endoglycosidase F/N-glycosidase F is noteworthy. These data suggest an additional extensive O-glycosy-
Figure 6. ELISA of soluble CD44 appearing in the supernatants of IFN-γ stimulated HT-29 and COLO 205 during APO-1-mediated apoptosis. (Standard deviations were calculated on the basis of eight experiments for HT-29 and four experiments for COLO 205.)

lation of the CD44 molecule. Taken together, results from the immunoprecipitations provide evidence that a shed form of CD44 from epithelial cells exists which is specifically released upon treatment with anti-APO-1. This form of CD44 is approximately 20 kD smaller in size than the authentic cell surface molecule.

Protease Inhibitors Fail to Block Shedding of Surface CD44 Without Critically Interfering with the Apoptotic Signal Cascade

To determine the type of enzymes responsible for anti-APO-1-induced cleavage of surface CD44, several protease inhibitors were used at standard effective concentrations (Beynon and Salvesen, 1989). In IFN-γ pretreated HT-29 and COLO 205 cells, CD95-triggered detachment and shedding of CD44 were completely blocked by the serine protease inhibitor DCI (250 μM). However, at the same time, DCI inhibited DNA fragmentation as well as the occurrence of the typical morphology of apoptosis. This is in line with previous reports demonstrating that CD95-induced apoptosis is completely blocked by DCI (Los et al., 1995; Weis et al., 1995). TLCK (200 μg/ml), a serine and leupeptin (100 μg/ml), and a serine/cystein-protease inhibitor affected neither CD95-induced apoptosis nor apoptosis-associated loss of CD44. The metalloprotease inhibitors 1,10-phenanthroline (5 mM) or EDTA (5 mM), slightly delayed DNA fragmentation but did not influence CD95-induced shedding of CD44 molecules. Likewise, Boc-Asp(OBzl)-CMK (50 μM), which is a specific interleukin-1β converting enzyme (ICE) inhibitor (Boudreau et al., 1995), completely blocked CD95-triggered apoptosis. Taken together, the protease responsible for shedding of CD44 could not be positively characterized since the agents known to block shedding CD44 very efficiently interfered with CD95 signaling in completely preventing apoptosis and, with it, shedding of CD44.

Discussion

We have shown that detachment of HT-29 and COLO 205 cells undergoing CD95-triggered apoptosis is a very early event in terms of ongoing DNA fragmentation, starting at the time HMW-DNA fragments first appear. The early loss of adhesion is paralleled by depletion of surface CD44 due to proteolytic cleavage with truncated soluble CD44 released in the supernatant.

Constitutively adherent cells like epithelia and endothelia are often polarized and display different molecular repertoires at their basal, lateral, and luminal surfaces. At the
basolateral surfaces, cell–cell and cell–matrix interactions take place that are mediated by different types of adhesion molecules. The interaction with the microenvironment has a fundamental influence on cytoskeletal organization (Luna and Hitt, 1992) and gene regulation of the cells. Disruption of epithelial cell–matrix (Meredith et al., 1993; Frisch and Francis, 1994) and cell–cell interactions (Bates et al., 1994) was demonstrated to eventually cause apoptosis. This route to death, recently termed “anoikis” (Frisch and Francis, 1994), may be crucial in preventing seeding of detached epithelium and endothelia at other sites. The present paper provides data suggesting that a functional link between loss of adhesion and apoptosis may be operative in both directions. We show that triggering CD95 on epithelial cells leads to loss of adhesion at early steps of programmed cell death. Such a mechanism may be advantageous for homeostasis of an organism since getting rid of apoptotic epithelia by extrusion, e.g., into a lumen, may diminish local phagocytic activity and thus stabilize the function of epithelia as a barrier.

It is well known that adherent cells in vitro can be readily removed from the substratum when subjected to limited proteolysis. It has been speculated that this might in fact be the mechanism by which cells detach from adjacent structures during apoptosis (Martin and Green, 1995). We show here that the multifunctional adhesion molecule CD44 is proteolytically shed from the cell surface early during CD95-induced cell death. These data, therefore, support the above assumption.

The question arises, which protease is responsible for the CD95-mediated shedding of CD44? CD44 was reported to be proteolytically cleaved from the cell surface of phorbol-ester-stimulated leukocytes (Bazil and Strominger, 1994) and upon cross-linking by specific antibodies (Bazil and Horejsi, 1992). Phorbol-ester-induced CD44 shedding was inhibited by 1,10-phenanthroline, EDTA, and DCl, hence by inhibition of metalloproteases and serine proteases, respectively. To investigate whether such proteases also account for CD95-induced cleavage of CD44, we applied a similar panel of protease inhibitors as they were used in these studies. In our system, however, the cation chelators 1,10-phenanthroline and EDTA, as previously reported (Schulze-Osthoff, 1994), reduced the response to anti-APO-1 treatment but had no influence on CD44 shedding from apoptotic cells. Thus, it is highly unlikely that phorbol ester–induced metalloproteases are involved in this mechanism. On the other hand, DCl prevented detachment of cells and loss of surface CD44. This, however, turned out to be due to blocking of the apoptotic program per se. It has been shown that DCl prevent CD95-induced cell death in SKW6.4 (Los et al., 1995) and Jurkat cells (Weis et al., 1995), possibly by inhibition of ICE (Los et al., 1995), which is a cystein-protease. ICE is a member of the family of ced-3-like proteases promoting apoptosis (for review see Martin and Green, 1995). Specifically blocking ICE by Boc-Asp(OBzl)-CMK had the published effect (Boudreau et al., 1995) in preventing apoptosis. In critical conclusion, the membrane-bound protease responsible for CD95-induced shedding of CD44 is as yet undefined.

Studies of Williams and Henkart (1994) have demonstrated that injection of several proteases, including a serine protease, proteinase K, into the cytoplasm of cells results in apoptosis within a few hours. Interestingly, treating HT-29 cells with extra-cellular protease K gave rise to a very selective loss of surface CD44 in the same order of magnitude and with kinetics as seen during CD95-induced apoptosis (own unpublished observations).

Shedding of surface CD44 is likely to be an important factor in the death program since it contributes to loss of cell–cell and cell–matrix anchorage and, in addition, might cut off survival-sustaining signals (Shimizu et al., 1989; Galandrini et al., 1993; Ayrodil et al., 1995).

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References

Ayrodil, E., I. Cannarile, G. Migliorati, A. Bartoli, I. Nicoletti, and C. Riccardi. 1995. CD44 (Pgp-1) inhibits CD5 and dexamethasone-induced apoptosis. Blood. 86:272-276.

Bates, R.C., A. Buret, D.F. van Helden, M.A. Horton, and G.P. Burns. 1994. Apoptosis induced by inhibition of intercellular contact. J. Cell Biol. 125: 403-415.

Bazil, V. and V. Horejsi, 1992. Shedding of the CD44 adhesion molecule from leukocytes induced by anti-CD44 monoclonal antibody simulating the effect of a natural receptor ligand. J. Immunol. 149:747-753.

Beynon, R.J., and G. Salvesen. 1991. Commercially available protease inhibitors. In Proteolytic Enzymes: a Practical Approach. R.J. Beynon and J.S. Bond, editors. Oxford University Press. Oxford. 241 pp.

Brown, T.A. T. Bouchard, T. St. John., E. Wayner, and W.G. Carter. 1991. Human keratinocytes express a new CD44 core protein (CD44E) as a heparin-sulfate intramembrane proteoglycan with additional cova. J. Cell Biol. 113:207-221.

Boudreau, N., C.J. Sympton, Z. Werb, and M.J. Bissell. 1995. Suppression of ICE and apoptosis in mammary epithelial cells by extracellular matrix. Science (Wash. DC). 267:891-893.

Carter, W.G., and E.A. Wayner. 1988. Characterization of the class III collagen receptor, a phosphorylated, transmembrane glycoprotein expressed in nucleated human cells. J. Biol. Chem. 263:4193-4201.

Cohen, S.J., and R.C. Dukx. 1992. Apoptosis and programmed cell death in immunity. Annu, Rev. Immunol. 10:267-295.

Cotter, T.G., S.V. Leenon, J.M. Glynn, and D.R. Green. 1992. Microfilament-disrupting agents prevent the formation of apoptotic bodies in tumor cells undergoing apoptosis [published erratum appears in Cancer Res. 1992; 52(12):3512]. Cancer Res. 52:997-1005.

Desjardins, L.M., and J.P. MacManus. 1995. An adherent cell model to study different stages of apoptosis. Exp. Cell Res. 216:386-387.

Dhein, J., P.T. Daniel, B.C. Trauth, A. Oehm, P. Muller, and P.H. Krammer. 1992. Induction of apoptosis by monoclonal antibody anti-APO-1 class switch variants is dependent on cross-linking of APO-1 cell surface antigens. J. Immunol. 149:3166-3173.

Dhein, J., H. Walczak, C. Kuehl, K.-M. Debatin, and P.H. Krammer. 1995. Autocrine T-cell suicide mediated by APO-1(Fas/CD95). Nature (Lond.). 373:438-440.

Frisch, M.S., and H. Francis. 1994. Dissipation of epithelial cell matrix interactions induces apoptosis. J. Cell Biol. 124(10):619-628.

Galandrini, R., N. Albi, G. Tripodi, D. Zarcone, A. Terenzi, A. Moretta, C.E. Grossi, and A. Velardi. 1993. Antibodies to CD44 trigger effector functions in human leukocytes induced by anti-CD44 monoclonal antibody simulating the effect of a natural receptor ligand. J. Immunol. 149:747-753.

Goldstein, L.A., D.F.H. Zhou, L.J. Picker, C.N. Minty, R.F. Bargatz, J.F. Ding, and E.C. Butcher. 1989. A human lymphocyte homing receptor, the Hermes antigen, is related to cartilage proteoglycan core and link protein. Cell. 56: 1003-1027.

Gunther, U. 1993. CD44: a multitude of isoforms with diverse functions. Curr. Top. Microbiol. Immunol. 184:47-63.

Horejsi, V., H. Ziegler, S. Salvesen, and K.S. Tischler. 1992. CD44 (Pgp-1) inhibits CD5 and dexamethasone-induced apoptosis. Blood. 86:272-276.

Itok, N., S. Yonehara, A. Ishii, M. Yonehara, S.-I. Mizushima, S. Sameshima, A. Hase, Y. Seto, and S. Nagata. 1992. The polypeptide encoded by the cDNA for human cell surface antigen fas can mediate apoptosis. Cell. 66:223-33.

Jalkanen, S., M. Jalkanen, R. Bargatz, M. Tammi, and E.C. Butcher. 1989. Biochemical properties of glycoproteins involved in lymphocyte recognition of high endothelial venules in man. J. Immunol. 141:1613-1623.
Lokeshwar, V.B., and L.Y. Bourguignon. 1991. Post-translational protein modification and expression of ankyrin binding site in GP85(Pgp-1/CD44) and its biosynthetic precursors during T-lymphoma membrane biosynthesis. J. Biol. Chem. 266:17983-17989.

Los, M., M. Van de Craen, L.C. Penning, H. Schenk, M. Westendorp, P.A. Bauerle, W. Dröge, P.H. Krammer, W. Fiers, and K. Schulze-Osthoff. 1995. Requirement of an ICE-CED-3 protease for Fas/APO-1-mediated apoptosis. Nature (Lond.) 375:81-83.

Luna, J.E., and A.L. Hitt. 1992. Cytoskeleton-plasma membrane interactions. Science (Wash, DC). 258:955-966.

Martin, S.J., and D.R. Green. 1995. Protease activation during apoptosis: death by a thousand cuts? Cell. 82:349-352.

Meredith, J.E., Jr., B. Fazeli, and M.A. Schwartz. 1993. The extracellular matrix as a cell survival factor. Mol. Biol. Cell. 4:953-961.

Möller, P., K. Koretz, F. Leithäuser, S. Brüderlein, C. Henne, A. Quintmeier, and P.H. Krammer. 1994. Expression of APO-1 (CD95), a member of the NGF/TNF receptor superfamily, in normal and neoplastic colon epithelium. Int. J. Cancer. 57:371-377.

Nagata, S., and P. Golstein. 1995. The Fas death factor. Science (Wash. DC). 267:1449-1456.

Nicoletti, I., G. Migliorati, M.C. Pagliacci, F. Grignani, and C. Riccardi. 1991. A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. J. Immunol. Methods. 139:271-279.

Oberhammer, F., J.W. Wilson, C. Dive, L.D. Morris, J.A. Hickman, A.E. Wakeling, P.R. Walker, and M. Sikorska. 1993. Apoptotic death in epithelial cells: cleavage of DNA to 300 and/or 50 kb fragments prior to or in the absence of internucleosomal fragmentation. EMBO (Eur. Mol. Biol. Organ.) J. 12:3679-3684.

Oehm, A., I. Behrmann, W. Falk, M. Pawlita, G. Maier, C. Klas, M. Li-Weber, S. Richard, J. Dhein, B.C. Trauth, W. Ponsting, and P.H. Krammer. 1992. Purification and molecular cloning of the Apo-1 cell surface antigen, a member of the tumor necrosis factor/nerve growth factor receptor superfamily. J. Biol. Chem. 267:10709-10715.

Schulze-Osthoff, K., P. H. Krammer, and W. Dröge. 1994. Divergent signaling via APO-1/Fas and the TNF receptor, two homologous molecules involved in physiological cell death. EMBO (Eur. Mol. Biol. Organ.) J. 13:4587-4596.

Searsle, J., J.F. Kerr, and C.J. Bishop. 1982. Necrosis and apoptosis: distinct modes of cell death with fundamentally different significance. Pathol. Annu. 17:229-259.

Shimizu, Y., G.A. Van Seventer, R. Siragian, L. Wahl, and S. Shaw. 1989. Dual role of the CD44 molecule in T cell adhesion and activation. J. Immunol. 143:2457-2463.

Smith, C.A., T. Farrah, and R.G. Goodwin. 1994. The TNF receptor superfamily of cellular and viral proteins: activation, costimulation, and death. Cell. 76:959-962.

Stamencovic, I., M. Amiot, J.M. Pesando, and B. Seed. 1989. A lymphocyte molecule implicated in lymph node homing is a member of the cartilage link protein family. Cell. 56:1057-1062.

Stamencovic, I., A. Aruffo, and M. Amiot. 1991. The hematopoietic and epithelial forms of CD44 are distinct polypeptides with different adhesion potentials for hyaluronate-bearing cells. EMBO (Eur. Mol. Biol. Organ.) J. 10:343-348.

Sträter, J., K. Koretz, A.R. Günther, and P. Möller. 1995. In situ detection of enterocytic apoptosis in normal colon mucosa and in familial adenomatous polyposis. Gut. 37:819-825.

Suda, T., T. Takahashi, P. Golstein, and S. Nagata. 1993. Molecular cloning and expression of the Fas ligand, a novel member of the tumor necrosis factor family. Cell. 75:1169-1178.

Takahashi, T., M. Tanaka, J. Inazawa, T. Abe, T. Suda, and S. Nagata. 1994. Human Fas ligand: gene structure, chromosomal location and species specificity. Int. Immunol. 10:1567-1574.

Toyama-Sorimachi, N., and M. Miyasaka. 1994. A novel ligand for CD44 is sulfated proteoglycan. Int. Immunol. 6:167-175.

Trauth, B.C., C. Klä, A.M.J. Peters, S. Matzku, P. Möller, W. Falk, K.-M. Debatin, and P.H. Krammer. 1989. Monoclonal-antibody-mediated tumor regression by induction of apoptosis. Science (Wash DC). 245:302-305.

Tsuji, A., A. Torres-Rosado, T. Arai, M.M. le Beau, R.S. Lemons, S.H. Chou, and K. Kurachi. 1991. Hepsin, a cell membrane-associated protease. Characterization, tissue distribution, and gene localization. J. Biol. Chem. 266:16948-16953.

Williams, M.S., and P.A. Henkart. 1994. Apoptotic cell death induced by intracellular proteolysis. J. Immunol. 153:4247-4255.

Yonehara, S. A. Ishii, and M. Yonehara. 1989. A cell-killing monoclonal antibody (anti-Fas) to a cell surface antigen co-downregulated with the receptor of tumor necrosis factor. J. Exp. Med. 169:1747-1756.