Journal of Cellular Biochemistry 85:670–677 (2002)

ARTICLES

Release of Cytokeratin-18 and -19 Fragments (TPS and CYFRA 21-1) Into the Extracellular Space During Apoptosis

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Abstract Serum fragments of cytokeratins-18 and -19 (measured as TPS and CYFRA 21-1, respectively) have traditionally been considered as markers of tumor proliferation, although the evidence is scarce for a causative relationship between proliferation and levels of TPS and CYFRA 21-1. We examined whether apoptosis might produce TPS and CYFRA 21-1 fragments. MCF-7 breast cancer cells were treated with mitomycin C or agonistic anti-CD95 antibody, and levels of TPS and CYFRA 21-1 in tissue culture supernatants were compared with the frequency of cells exhibiting the following markers of cell death: intracellular cytokeratin-18 cleavage, surface staining with annexin-V, propidium iodide uptake, DNA fragmentation. Twenty-four hours after inducing apoptosis, levels of TPS and CYFRA 21-1 were elevated $\geq$ 4-fold in culture supernatants. Elevations in TPS and CYFRA 21-1 coincided with apoptosis measured by the first three cell death markers but preceded DNA fragmentation. These mitomycin C- and CD95-mediated elevations were completely inhibited by co-incubation with the caspase inhibitors Z-VAD.fmk and Z-IETD.fmk, respectively. We conclude that TPS and CYFRA 21-1 can be abundantly released into the extracellular space during the intermediate stage of epithelial cell apoptosis. J. Cell. Biochem. 85: 670–677, 2002.

Key words: tumor marker; proliferation; caspase; breast cancer; CD95/Fas

Apoptosis is a process in which damaged or unneeded cells commit suicide. Apoptosis requires activation of a biochemical pathway of proteases known as caspases (cysteine proteases with specificity for aspartic acid residues). Apoptosis can be intitiated by release of cytochrome c from mitochondria or by signaling through cell surface death receptors such as CD95 (Fas) [reviewed in Green, 2000]. The substrates of caspases are diverse. Through cleavage, caspases can activate other caspases and inactivate both DNA repair enzymes and structural proteins such as actin and certain cytokeratins. Cleavage of these substrates prepares for, and participates in, the dismantling of the cell in an orderly fashion that may substantially reduce inflammation [Bellamy et al., 1995], although perhaps not completely [Restifo, 2000].

Serum fragments of cytokeratins-18 and -19 can be detected and referenced as TPS (Tissue Polypeptide Specific antigen) and CYFRA 21-1 (Cytokeratin-19 Fragment), respectively. Serum concentrations of TPS and CYFRA 21-1 correlate with disease progression in various malignancies and thus have been used as tumor markers [Nisman et al., 1998; Rebhandl et al., 1998; Tempfer et al., 1998; Nagler et al., 1999; Doweck et al., 2000]. TPS and CYFRA 21-1 have traditionally been considered as proliferation markers, although the evidence for this association is limited, and the biology behind the release of soluble cytokeratin fragments

Grant sponsor: Internal Grant Agency of the Ministry of Health; Grant numbers: IGA 6402-3, IGA 6404-3, IGA 6403-3, MZ00020980501.

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Received 17 September 2001; Accepted 1 February 2002
DOI 10.1002/jcb.10173

actually remains poorly understood. Interestingly, intracellular cleavage of cytokeratin-18 was recently shown to be mediated by caspases 6, 7, and 3 during apoptosis [Caulin et al., 1997]. Cleavage of cytokeratin-19 has been postulated to occur through spontaneous caspase 3 activity, resulting in release of CYFRA 21-1 into tissue culture supernatants [Dohmoto et al., 2001]. We therefore investigated whether the apoptotic program in epithelial cells can induce release of TPS and CYFRA 21-1 into the extracellular space in vitro.

MATERIALS AND METHODS

Cell Culture and Reagents

MCF-7 breast adenocarcinoma cells were plated at subconfluence in 75 cm^2 tissue culture flasks (Nalge Nunc International, Denmark) and maintained in 5% CO_2 at 37°C in Dulbecco's modified eagle's medium (DMEM) containing 10% fetal bovine serum, glutamine, and antibiotics. Agonistic anti-CD95 IgM antibody (clone CH11) and antagonistic anti-CD95 antibody (ZB4) were purchased from Immunotech (Westbrook, ME). Cycloheximide (CHX) (Sigma, St. Louis, MO) was solubilized in culture medium immediately prior to use. Mitomycin C (Sigma) was solubilized in distilled water and stored at −20°C until use. Z-VAD.fmk was obtained from RBI/Sigma (MA) and Z-IETD.fmk from R&D Systems (Weisbaden-Nordenstadt, Germany). M30 antibody was obtained from Boehringer Mannheim (Mannheim, Germany). Monoclonal antibodies CO8 [Bartek et al., 1991] and DC-10 [Lauerova et al., 1988] were developed in our laboratory and are specific for human cytokeratin-18; monoclonal antibody BA-17 is specific for cytokeratin-19 [Bartek et al., 1986]; the epitopes recognized by these antibodies have not been characterized.

Immunoblotting

Total cellular protein lysates were prepared by harvesting cells in hot Laemmli electrophoresis sample buffer. Proteins were separated by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) on 10 or 12.5% gels, and transferred onto a nitrocellulose membrane in a Bio-Rad Mini Trans-Blott Electrophoretic Transfer Cell for 2 h at 4°C, applying 150 mA in transfer buffer (242 mmol Tris, 190 mmol glycine, 20% methanol). Prestained molecular weight markers (Bio-Rad, Hercules, CA) were run in parallel. The blotted membranes were blocked with 5% milk and 0.1% Tween 20 in phosphate buffered saline (PBS) for 2 h and probed overnight with specific monoclonal antibodies (final concentration 1 μg of antibody in 1 ml of PBS containing 5% milk). After washing 3 × in PBS plus 0.1% Tween 20, peroxidase-conjugated rabbit anti-mouse immunoglobulin antiserum (Dako, Denmark) diluted 1:1,000 in PBS containing 5% milk and 0.1% Tween 20 was used as the secondary antibody. To visualize peroxidase activity, ECL reagents (Amersham Pharmacia Biotech, Buckinghamshire, UK) were used according to the manufacturer's instructions.

Quantitation of Soluble Cytokeratin Fragments

The amounts of soluble cytokeratin-18 fragments in tissue culture supernatants were determined using the TPS enzyme immunoassay (Beki Diagnostics, Bromma, Sweden), which employs the M3 antibody specific for cytokeratin-18 residues 322–340 [Rydlander et al., 1996]. The TPS assay was used as recommended by the manufacturers, followed by quantitation using a Cobas CORE analyzer (Roche, Basel, Switzerland). Soluble cytokeratin-19 fragments were measured using the highly specific [Bodenmuller et al., 1994] CYFRA 21-1 electrochemiluminescence immunoassay (Roche Diagnostics, Mannheim, Germany) on an Elecsys 2010 (Roche Diagnostics). Briefly, after establishing that centrifugation over a wide range of speeds had no effect on the levels of TPS and CYFRA 21-1 in the supernatant of normal and apoptotic cultures, 400 μl of tissue culture supernatants were centrifuged at 800*g* for 20 min to pellet floating cells, and the supernatants were stored at −20°C until analysis. After thawing, 100- and 5-fold dilutions were typically used for TPS and CYFRA 21-1 assays, respectively, as determined experimentally to be within the linear measuring ranges of the assays. Pelletted cells were pooled with the main fraction of floating and adherent cells during simultaneous staining for apoptosis, described below.

Detection of Apoptosis

All apoptotic parameters were quantitatively assessed on an EPICS® XL flow cytometer (Coulter, Hialeah, FL) using the manufacturer's analysis software. DNA content (sub-G1) analyses were performed as previously described [Sheard et al., 1999]. Cells containing

intracellular cytokeratin-18 fragments were stained using the M30 antibody that is specific for a neo-epitope of fragmented cytokeratin-18 expressed during early apoptosis [Leers et al., 1999]. Briefly, adherent and floating cells were pooled, fixed for 2 h in methanol at −20°C, washed 1 × in washing solution (PBS, 0.1% Triton X-100), washed 1 × in staining solution (PBS, 0.1% albumin, 0.1% Triton X-100), incubated for 2 h at room temperature in the dark with the M30 antibody diluted 1:250 in staining solution, washed 2 × in washing solution, and resuspended in 0.5 ml PBS. Subcellular debris were eliminated from analysis by appropriate gating on forward scatter and side scatter parameters.

Expression of phosphatidyl serine on the outer leaflet of the surface membrane, which occurs on both apoptotic and non-apoptotic dead cells, was quantitated using the Annexin-V assay (Boehringer Mannheim). Cells were trypsinized, washed 2 × in cold incubation buffer (10 mmol HEPES/NaOH, pH 7.4, 140 mmol NaCl, 5 mmol $CaCl_2$), and resuspended in 100 µl of staining solution containing 1 µg/ml propidium iodide and annexin-V-fluorescein diluted 1:50 in incubation buffer. Cells were incubated for 10 min and analyzed by flow cytometry using a 530 nm bandpass filter to detect annexin-V-fluorescein and a 620 nm bandpass filter to detect propidium iodide.

RESULTS

Intracellular Fragmentation of Cytokeratin-18 During Apoptosis

To examine cleavage of cytokeratin-18 during the apoptotic program, we induced apoptosis in MCF-7 breast carcinoma cells with mitomycin C or anti-CD95 antibody and stained intracellularly with the M30 antibody. Induction of cell death by mitomycin C elicited easily detectable levels of cleaved, intracellular cytokeratin-18 in a time-dependent manner (Fig. 1A). Similarly, signaling through the CD95 death receptor also induced a substantial level of intracellular cytokeratin-18 fragments (Fig. 1B), which was preceded by activation of caspase-8 detected 14 h after treatment (data not shown). CD95-mediated generation of intracellular cytokeratin-18 fragments was almost completely blocked by pretreatment with an antagonistic antibody (Fig. 1B). To confirm these findings, immunoblotting was performed using two distinct anti-cytokeratin-18 antibodies. A strong band corresponding to unfragmented cytokeratin-18 was observed at 45 kDa in all treatment groups, and a second band exhibiting increased migration (indicative of formation of a smaller fragment) was observed at ~29 kDa, 24 and 48 h after treatment with either mitomycin C or anti-CD95 agonistic antibody (Fig. 2A). Furthermore, immunoblotting with the M30 antibody

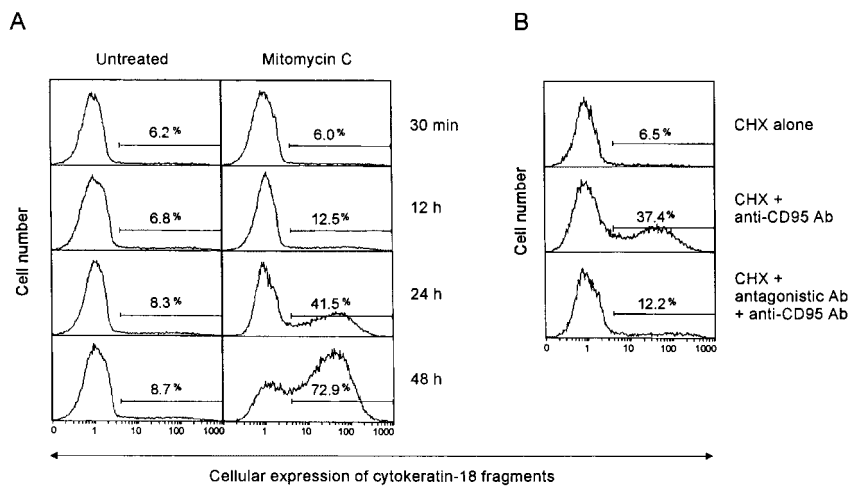

Fig. 1. Detection of intracellular cytokeratin-18 cleavage in MCF-7 cells after apoptosis-inducing treatments. **A**: Cellular expression of cytokeratin-18 fragments after treatment with mitomycin C. Cells were treated at various times with mitomycin C (8 µg/ml) and harvested together for staining. Plasma membranes were permeabilized, and cells were stained with the M30 antibody for flow cytometric analysis. **B**: Expression of cytokeratin-18 fragments after treatment with anti-CD95 agonistic antibody. Cell cultures were pretreated with the CD95-sensitizing agent CHX (3 µg/ml), and one culture received an antagonistic anti-CD95 antibody (0.5 µg/ml), as shown. Six hours later, specified cultures were treated with agonistic antibody (0.1 µg/ml), incubated for an additional 46 h, and stained as above. Results are representative of three independent experiments.

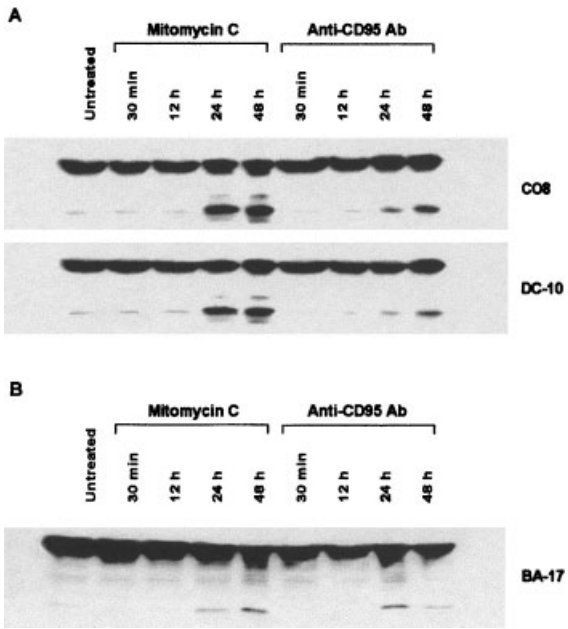

Fig. 2. Detection of intracellular cytokeratin-18 and -19 cleavage by immunoblotting. Cultures of MCF-7 cells were treated together and harvested at the time-points shown. Floating cells were pelletted by centrifugation, adherent cells were rinsed 3x with PBS and lysed, and floating cells were pooled with corresponding lysates before immunoblotting. **A**: Cytokeratin-18 cleavage. Upper and lower gels were probed with distinct anti-cytokeratin-18 antibodies, clones C08 and DC-10. Mitomycin C was used at a concentration of 8 μg/ml. Anti-CD95 antibody-treated cultures were pretreated with CHX (3 μg/ml) for 6 h prior to addition of 0.1 μg/ml of the anti-CD95 agonistic antibody. **B**: Cytokeratin-19 cleavage was detected using anti-cytokeratin-19 antibody BA-17.

identified similar bands of cytokeratin-18 fragments within cells (data not shown). Paralleling cytokeratin-18 fragmentation, cleavage of cytokeratin-19 (~40 kDa) was also observed, with fragments detected at ~27 kDa in treated cells (Fig. 2B). Collectively, these results indicate that cytokeratin-18 and -19 are cleaved intracellularly during apoptosis in MCF-7 cells.

Cellular Release of TPS and CYFRA 21-1 During Apoptosis

To examine whether cytokeratin fragments might be released from cells during apoptosis, aliquots were collected from tissue culture supernantants of mitomycin C-treated cells and assayed for their content of soluble cytokeratin-18 and cytokeratin-19 fragments by TPS and CYFRA 21-1 assays. Although no increase in soluble cytokeratin fragments was detectable within the first 12 h of treatment with 8 μg/ml mitomycin C, a greater than 4-fold increase was observed 24 and 48 h after treatment (Fig. 3A). A lower dose of 2 μg/ml, chosen to approximate the serum concentration of mitomycin C after bolus administration of 20–25 mg/m^2 [McEvoy, 1999], consistently induced a small increase in soluble cytokeratin levels. A low spontaneous level of apoptosis in untreated cultures correlated with a detectable level of soluble cytokeratin fragments in corresponding culture supernatants. No fragments were detectable in cell-free culture medium containing 10% fetal bovine serum (data not shown).

The increases in soluble fragments of cytokeratins induced by mitomycin C correlated temporally with the initiation of apoptosis. Large increases in the frequencies of cells exhibiting intracellular fragments of cytokeratin-18, as well as phosphatidyl serine exposure on the outer leaf of the plasma membrane and uptake of the vital dye propidium iodide, were detected 24 and 48 h after mitomycin C treatment (Fig. 3A). In contrast, an increase in the frequency of cells exhibiting DNA fragmentation (a late marker of apoptosis in most epithelial cells) was not detectable until 48 h after treatment with mitomycin C (Fig. 3A) or agonistic anti-CD95 antibody (Fig. 3B and data not shown).

A 5–7-fold increase in levels of soluble cytokeratin fragments was observed during CD95-mediated apoptosis (Fig. 3B). Pretreatment with an antagonistic anti-CD95 antibody substantially blocked both apoptosis and the release of soluble cytokeratin fragments induced by agonistic antibody. Taken together, these results show that the same monoclonal antibody-based assays used for detection of cytokeratin-18 and -19 fragments in patient sera also detect cytokeratin-18 and -19 fragments in cell culture supernatants after apoptosis induction. Thus, drug-induced and death receptor-mediated apoptosis can involve the release of TPS and CYFRA 21-1 from epithelial cells.

An Essential Role of Caspases in Producing Extracellular TPS and CYFRA 21-1

To examine the role of caspases in the production of soluble cytokeratin fragments during apoptosis, cells were simultaneously treated with mitomycin C and the broad-spectrum caspase-inhibitor Z-VAD.fmk. Z-VAD (25 μmol) abrogated both the intracellular cleavage of cytokeratin-18 and the release of TPS and CYFRA 21-1 occurring 24 h after mitomycin C

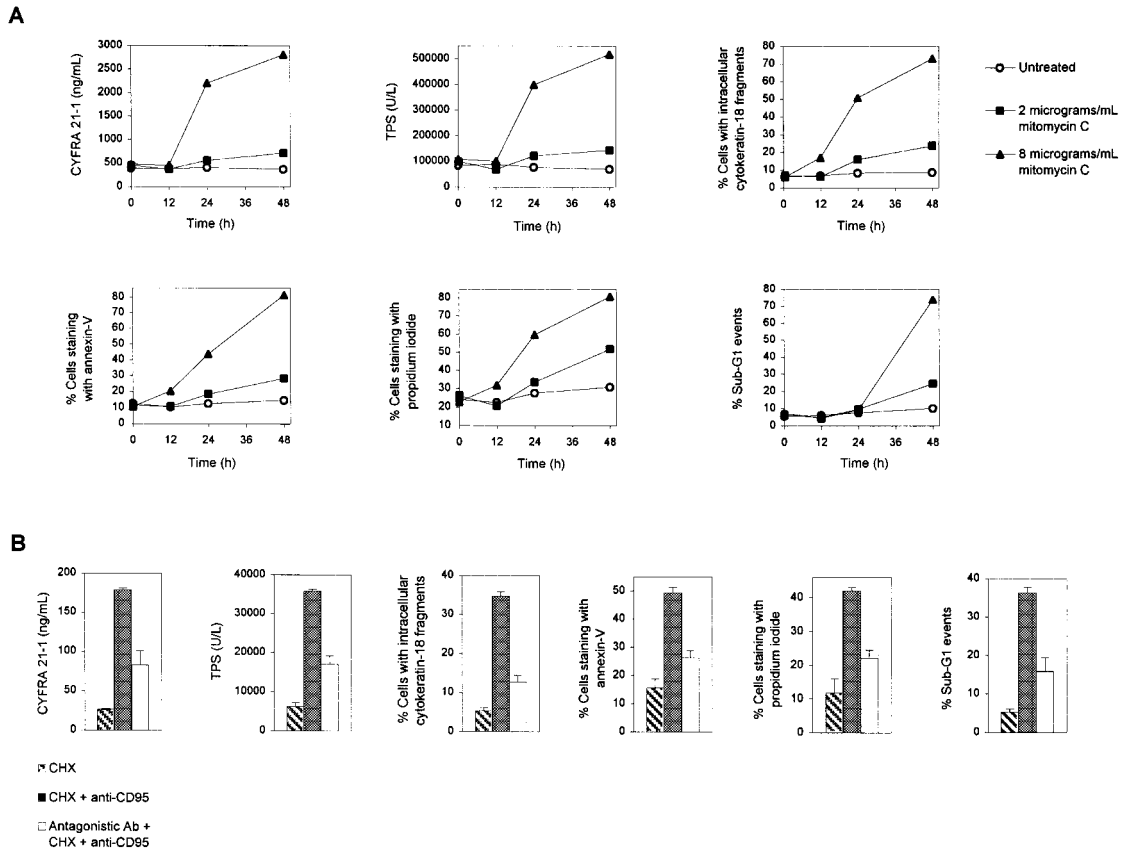

Fig. 3. Generation of TPS and CYFRA 21-1 during apoptosis. MCF-7 cells were induced to undergo apoptosis and cultures were assayed for TPS, CYFRA 21-1 and for both intracellular and surface markers of cell death. Results are representative of three independent experiments. **A**: Time-course for detection of TPS and CYFRA 21-1 during mitomycin C-induced apoptosis. Cell cultures were plated together, treated at different time-points with mitomycin C (8 μg/ml, triangles; 2 μg/ml, squares; untreated, circles), and aliquots of tissue culture supernatants were collected at the same time for analyses. Simultaneous assays were performed to determine the percentages of cells exhibiting: (i) intracellular fragments of cytokeratin-18, (ii) surface staining with annexin-V, (iii) uptake of the vital dye propidium iodide, and (iv) a sub-G1 amount of DNA. **B**: Generation of TPS and CYFRA 21-1 during CD95-mediated apoptosis. MCF-7 cells were pretreated with CHX (3 μg/ml) and antagonistic anti-CD95 antibody for 6 h, and subsequently incubated for 48 h with agonistic anti-CD95 antibody. Means of triplicates are shown ± standard deviations.

treatment (Fig. 4A). Z-VAD did not completely eliminate mitomycin C-induced cell death as measured by annexin V and propidium iodide staining, indicating that a fraction of cells had undergone caspase-independent death in the presence of the caspase inhibitor, without releasing TPS or CYFRA 21-1.

To confirm the essential role of caspases in generating soluble cytokeratin-18 and -19 fragments in culture supernatants, quantitation of TPS, CYFRA 21-1, and apoptosis was performed 24 h after treatment of cells with anti-CD95 agonistic antibody in the presence or absence of 10 μmol Z-IETD.fmk (a specific inhibitor of caspase-8, the apical caspase in the CD95 signaling pathway). Z-IETD.fmk strongly inhibited both the generation of soluble cytokeratins and cell death after signaling through CD95 (Fig. 4B). Altogether, these results obtained with two distinct caspase inhibitors indicate that caspase activity is critically required for drug-induced and death receptor-induced increases in the levels of extracellular TPS and CYFRA 21-1.

DISCUSSION

The data presented here demonstrate that apoptosis-inducing treatment of MCF-7 epithelial cells causes multi-fold elevations in extracellular TPS and CYFRA 21-1 concommitantly with significant increases in intracellular cytokeratin fragments, exposure of phosphatidyl serine on the outer leaflet of the plasma

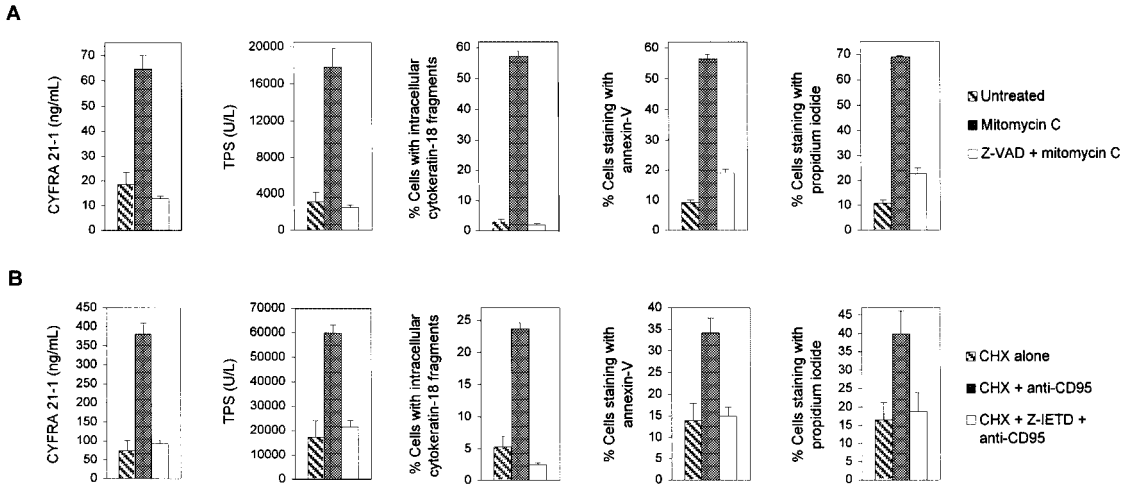

Fig. 4. Caspase inhibitors suppress the generation of intracellular cytokeratin-18 fragments, TPS, and CYFRA 21-1 after treatment of cells with apoptosis-inducing stimuli. **A**: Effect of the pan-caspase inhibitor Z-VAD.fmk on the levels of TPS, CYFRA 21-1, and cell death after mitomycin C treatment. MCF-7 cells were incubated with mitomycin C (8 μg/ml) and Z-VAD.fmk (25 μmol) for 24 h. Means of triplicates are shown ± respective standard deviations. Results are representative of three independent experiments. **B**: Effect of the caspase-8 inhibitor Z-IETD.fmk on the levels of TPS, CYFRA 21-1, and cell death induced by anti-CD95 antibody treatment. MCF-7 cells were treated with CHX overnight (12 h), certain flasks were treated with Z-IETD.fmk (10 μmol) for 1 h, and then specified cultures were incubated with agonistic anti-CD95 antibody for 24 h prior to analyses.

membrane, and loss of propidium iodide exlusion, but preceding the late apoptotic event of DNA fragmentation. Abrogation of these elevations by caspase inhibitors indicates that release of TPS and CYFRA 21-1 into the extracellular space occurs specifically during apoptosis, since caspases are not active in necrosis. Furthermore, mitomycin C-treated cells dying by caspase-independent death in the presence of the Z-VAD caspase inhibitor did not release measurable TPS and CYFRA 21-1, at least within the time period examined (Fig. 4A). Based on these findings, we conclude that cellular release of TPS and CYFRA 21-1 occurs during the intermediate stage of apoptosis, as a consequence of caspase activation.

Intracellular cleavage of cytokeratin-18 has previously been reported to occur during etoposide-induced apoptosis of SNG-M human endometrial adenocarcinoma cells and HR-9 mouse parietal endodermal cells [Caulin et al., 1997] as well as in TRAIL-induced apoptosis of MCF-7 cells [MacFarlane et al., 2000]. The data presented here extend these findings by suggesting that increased levels of cytokeratin-18 and -19 fragments can serve not only as intracellular markers, but also as an extracellular markers of apoptosis.

In contrast with necrosis, apoptosis is believed to be a form of cell death that results in an intact plasma membrane and/or membrane-bound apoptotic bodies, without inducing inflammation [Bellamy et al., 1995]. However, the presence of TPS and CYFRA 21-1 in the extracellular space indicates that apoptotic cells can release intracellular components. Consistent with this finding, it has been recently argued that apoptosis can be accompanied by a low or even substantial level of inflammation [Restifo, 2000]. Interestingly, a third form of cell death, termed autophagic cell death, has recently been proposed in which degradation of organelles occurs without substantial cleavage of cytokeratins [Bursch et al., 2000]. Thus, treatment of MCF-7 cells with inducers of autophagic cell death such as tamoxifen did not result in significant cleavage of cytokeratins [Bursch et al., 2000].

In numerous clinical laboratories, levels of TPS and CYFRA 21-1 in the sera of cancer patients have been determined as an indirect measure of tumor progression [Nisman et al., 1998; Rebhandl et al., 1998; Tempfer et al., 1998; Nagler et al., 1999; Doweck et al., 2000], although the utility of this approach has been challenged for some tumor types [Plebani et al., 1993; Wollenberg et al., 1996; Theyer et al., 1999; Valik and Nekulova, 2000]. Since the release of TPS and CYFRA 21-1 can correlate with apoptosis in vitro, further studies

are warranted to determine whether apoptotic epithelial cells can be a significant source of extracellular, soluble cytokeratins in vivo. Notably, levels of TPS have been reported to be elevated in sera of patients with hepatitis B or alcoholism [Kao et al., 1994; Gonzalez-Quintela et al., 2000], and elevated levels of CYFRA 21-1 were reported in sera of patients with interstitial pneumonia [Dobashi et al., 1999; Fujita et al., 1999]. Whether the source of elevated TPS and CYFRA 21-1 is apoptotic cells or regenerating cells is unclear. Given the current data, it has become an open question whether the constitutive low level of apoptosis occurring in most tumors is sufficient to generate a cumulative and detectable increase in the serum concentration of TPS and CYFRA 21-1.

ACKNOWLEDGMENTS

The authors thank Mirka Nekulova for critically reading the manuscript. M.A.S. was supported by a grant from the Internal Grant Agency of the Ministry of Health (I.G.A.) 6402-3; B.V. was supported by IGA 6404-3; M.S. was supported by IGA 6403-3; D.V. was supported by grant MZ00020980501.

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