Differentiation between Cell Death Modes Using Measurements of Different Soluble Forms of Extracellular Cytokeratin 18

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ABSTRACT

Cytokeratins are released from carcinoma cells by unclear mechanisms and are commonly used serum tumor markers (TPA, TPS, and CYFRA 21–1). We here report that soluble cytokeratin-18 (CK18) is released from human carcinoma cells during cell death. During necrosis, the cytosolic pool of soluble CK18 was released, whereas apoptosis was associated with significant release of caspase-cleaved CK18 fragments. These results suggested that assessments of different forms of CK18 in patient sera could be used to examine cell death modes. Therefore, CK18 was measured in local venous blood collected during operation of patients with endometrial tumors. In most patient sera, caspase-cleaved fragments constituted a minor fraction of total CK18, suggesting that tumor apoptosis is not the main mechanism for generation of circulating CK18. Monitoring of different CK18 forms in peripheral blood during chemotherapy of prostate cancer patients showed individual differences in the patterns of release. Importantly, several examples were observed where the increase of apoptosis-specific caspase-cleaved CK18 fragments constituted only a minor fraction of the total increase. These results suggest that cell death of epithelia derived tumors can be assessed in patient serum and suggest that tumor apoptosis may not necessarily be the dominating death mode in many tumors in vivo.

INTRODUCTION

Cytokeratins (CKs) 8, 18, and 19 are expressed by most types of carcinomas, including those of the breast, prostate, lung, colon, and ovary. CKs are released from tumor cells by unclear mechanisms and provide useful serum markers for evaluating the clinical progression of patients with epithelial malignancies (1, 2). Tissue polypeptide antigen was originally identified as a tumor antigen present in the insoluble fraction of human tumor cells (3), and was later shown to consist of fragments of CK8, 18, and 19 (4). Tissue-polypeptide-specific antigen is defined by a monoclonal antibody recognizing a COOH-terminal epitope on CK18 (5), and CYFRA 21–1 detects CK19 (6–8).

The bulk of cellular CK is part of the intermediate filament system and insoluble at physiological salt concentrations. Proliferating cells have a substantial pool of soluble CK8 and 18, and G2-M arrest induces an increase of this pool (9). Soluble CK fragments can also be produced by apoptosis. Type I CKs (CK18 and CK19) are cleaved by caspas during apoptosis, and the resulting fragments are relatively stable (10–13). A positive association has been described between the levels of tissue polypeptide antigen in breast cancer cytotos and apoptosis (14). Furthermore, release of tissue-polypeptide-specific antigen and CYFRA 21–1 from tumor cells into the extracellular space has been demonstrated to occur during apoptosis (15, 16).

Many effective anticancer drugs interfere with DNA synthesis and cell division, leading to induction of apoptosis in susceptible tumor cells (17, 18). Anticancer agents may induce other forms of cell death (i.e., necrosis), and may also induce growth arrest and senescence (19). Altered cell susceptibility to apoptosis after drug treatment is believed to be an important mechanism of acquired drug resistance (20). The sensitivity to apoptosis induction is particularly important for drug sensitivity of malignancies of lymphoid origin (21) but may be less important for solid tumors (19, 22, 23). We have here measured different forms of CK18 in serum from cancer patients as an approach to evaluate the death mode of epithelial tumors in vivo.

MATERIALS AND METHODS

ELISA Assays. The levels of the CK18-Asp396 neo-epitope were measured using a commercially available ELISA kit, M30-Apoptosense (PEVIVA AB, Bromma, Sweden). This ELISA uses antibody 5 as catcher and horseradish peroxidase-conjugated antibody M30 for detection (see Fig. 1 for details). The units of the M30-ELISA are defined using a synthetic peptide (Ref. 24; 1.24 pmol = 1 unit). The M65-ELISA assay (PEVIVA AB) measures total soluble CK18. Two monoclonal antibodies that recognize epitopes between residues 300 and 380 in the CK18 molecule are used in this assay (Fig. 1). The M65-ELISA shows similar, but not identical, properties as the tissue-polypeptide-specific antigen assay; analysis of 36 samples by both assays gave a Spearman Rho coefficient of 0.645 (P < 0.0001). The units of the M65-ELISA were defined on the basis of the M30-ELISA units to allow direct comparisons between the two assays. This was accomplished by adsorption of cell extracts and patient sera to immobilized M30 antibody followed by measurements of CK18 and CK18-Asp396-NE content using the two ELISA assays.

Cell Culture. MDA-MB-231 is an estrogen receptor-negative human breast epithelial cell line. Cells were grown in DMEM (Life Technologies, Inc., Paisley, Scotland) supplemented with 5% FCS, 50 μg of streptomycin, and 50 units of penicillin/ml (Life Technologies, Inc.). On the day before addition of cisplatin, cells were plated at 104 cells/well in 24-well plates (Techno Plastic Products AG, Trasadingen, Switzerland) in 1 ml of medium. The next day, cells received fresh medium containing 50 μM cisplatin (Bristol-Myers Squibb) with or without 40 μM Z-VAD(Ome)FMK (ESP, Livermore, CA). At the times indicated, culture medium was removed, and the cells were collected and centrifuged. Cells were lysed in 1 ml of 10 mM Tris (pH 7.4), 150 mM NaCl, and 0.5% NP40. CK18 forms were measured in medium and cell extracts by ELISA. For induction of necrosis, cells were incubated for 24 h in glucose-free 15-M medium supplemented with 10% FCS. The medium was then changed to glucose-free 15-M medium without serum, and oligomycin (Sigma-Aldrich, St. Louis, MO) was then added to 5 μM. Culture medium and cell extracts were prepared and analyzed as described above.

Annexin V/Propidium Iodide (PI) Flow Cytometry. Redistribution of plasma membrane phosphatidyl serine is a marker of apoptosis and was assessed using Annexin V FLUOS (Roche Molecular Biochemicals). Cells were collected, washed in PBS, pelletted, and resuspended in 10 mM HEPES, NaOH (pH 7.4), 140 mM NaCl, 5 mM CaCl2, 1% annexin V, and 0.5 μg/ml PI. The samples were incubated for 15 min in the dark and analyzed on a Calibur flow cytometer (Becton Dickinson) using Cell Quest software.

Patients and Serum Collection. Two patient materials were examined. The first material consisted of 56 patients who underwent abdominal hysterectomy with salpingo-oophorectomy because of endometrial cancer (n = 37) or benign gynecologic conditions (n = 19). All of the patients were postmenopausal, and the tumors were of the following grades: 15 grade I, 10 grade II, and 12 grade III + IV. Blood was sampled from an antecubital vein

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Cytokeratin 18

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Fig. 1. Cytokeratin (CK) 18 is cleaved by caspases at two distinct sites (Asp238 and Asp396) during apoptosis. Cleavage after Asp396 leads to exposure of the M30 neo-epitope (CK18-Asp396-NE). Soluble CK18 cleaved at this site is detected by the M30-ELISA assay that uses antibodies M30 and 5. Total soluble full-length CK18 and soluble COOH-terminal fragments are measured with the M65-ELISA assay that uses antibodies 6 and 5. The approximate positions of these epitopes are indicated.

preoperatively and from the utero-ovarian plexus at either side preoperatively. When blood was collected from both sides, the average CK18 values are presented. The second material consisted of 25 patients with hormone-refractory prostate cancer treated at the Department of Urology at the Vienna General Hospital. These patients were treated with i.v. estramustine (300 mg) on days 1, 2, and 3, and subsequently with i.v. vinorelbine (30 mg/m²) on day 5. Blood was collected on days 1, 3, 5, and 7 for most patients. Ethical approval and patient consent were obtained for collection of serum samples. Prostate-specific antigen (PSA) decreases of >50% from baseline were determined according to the response guidelines from the Prostate-Specific Antigen Working Group (25). The PSA nadir was observed after an average of 4 cycles.

Statistics. CK18 levels in serum are presented as medians (25th-75th percentile) and graphically displayed by box plots. The possible statistical significance of differences between different groups was analyzed by the non-parametric Wilcoxon test.

RESULTS

Caspase-Cleaved Fragments Constitute a Subset of Soluble CK18 Proteins in MDA-MB-231 Breast Carcinoma Cells. CK18 is an insoluble intermediate filament protein expressed in cells of simple epithelia. A pool of soluble CK18 is also present, mostly consisting of full-length protein (9). CK18 is cleaved at Asp238 and Asp396 by caspases during apoptosis, generating soluble protein fragments (10–12). We used two ELISA assays for quantitation of total soluble CK18 and of caspase-cleaved fragments (Fig. 1). The M65-ELISA assay measures full-length soluble CK18 and CK18 COOH-terminal fragments. The M30-ELISA assay is based on the epitope-specific M30 antibody, which only recognizes soluble CK18 fragments cleaved at Asp396 by caspases (26, 27). These fragments are referred to as CK18-Asp396-NE (due to the formation of a neo-epitope, NE, after cleavage at Asp396) during apoptosis. After 24 h of oligomycin treatment, ~90% of the cellular content of soluble CK18 had been released into the medium (Fig. 2, E and F). Approximately 1% of the extracellular CK18 consisted of CK18-Asp396-NE. Similar results were observed using HCT116 colon cancer cells.

Release of Caspase-Cleaved CK18 to the Extracellular Compartment during Apoptosis. Cisplatin induces apoptosis of MDA-MB-231 cells, as assessed by nuclear fragmentation and caspase activation (data not shown) and by annexin V/PI staining (Fig. 3, A–C). An increasing number of apoptotic (annexin V positive/PI negative) cells were detected after 24 and 48 h of treatment (Fig. 3C). A population of PI-positive cells was also observed, consistent with previous reports that the plasma membrane becomes leaky before complete disintegration of cells during the apoptotic process (30). Increases in soluble CK18, consistent with the occurrence of dead cells, were observed in culture medium at 24 and 48 h (Fig. 3D). The caspase inhibitor z-VAD-fmk did not decrease the release of soluble CK18. The high levels of release of total CK18 in z-VAD treated cells is believed to reflect retardation (but not complete inhibition) of cell death, leading to prolonged production of CK18. The increases in

Release of Soluble CK18 to the Extracellular Compartment during Necrosis. MDA-MB-231 cells were treated with oligomycin in glucose-free medium to deplete cellular ATP, a standard treatment for induction of necrosis (28, 29). Analysis by annexin V binding showed that ~40% of the cells were dead (PI-positive) after 8 h and 90% dead after 24 h (Fig. 2, B–D). Dead cells were initially annexin V positive, but subsequent cellular decay resulted in decreased annexin V binding. Oligomycin treatment did not induce significant apoptosis. After 24 h of oligomycin treatment, ~90% of the cellular content of soluble CK18 had been released into the medium (Fig. 2, E and F). Approximately 1% of the extracellular CK18 consisted of CK18-Asp396-NE. Similar results were observed using HCT116 colon cancer cells.

Fig. 2. Release of soluble cytokeratin (CK) 18 to the extracellular compartment from MDA-MB-231 cells induced to undergo necrosis. Cells were treated with 5 μM oligomycin in glucose-free medium. A–C, cells were stained with annexin V and propidium iodide and analyzed by flow cytometry; A, untreated cells; B, cells treated with oligomycin for 8 h; C, cells treated for 24 h. Dead cells (propidium iodide positive) are observed in the upper left and right quadrants. D, the number of viable (●), necrotic (○), and apoptotic (□) cells at different times of treatments; E, soluble CK18 was measured after 24 h in medium and cytosols using the M65-ELISA assay; F, caspase-cleaved CK18 (CK18: Asp396-NE) was measured after 24 h in medium and cytosols using the M30-ELISA assay. Results are shown as means; bars, ±SD.

Unpublished observations.

Fig. 3. Cytokeratin (CK) 18 is cleaved by caspases at two distinct sites (Asp238 and Asp396). The results showed that caspase-cleaved CK18-Asp396-NE fragments constituted only ~1% of total CK18 in MDA-MB-231 cell extracts (Fig. 2, E and F).
the group of patients with malignant disease, significantly higher levels were observed in local compared with peripheral serum [178 versus 145 units/liter; \( P = 0.01 \)]. Similarly, total CK18 levels were higher in local serum from malignant tumors [1303 (1006–2383) units/liter] than benign conditions [333 (290–537) units/liter; \( P < 0.0001 \)], and significantly higher CK18 levels were observed in local serum from malignant tumors compared with peripheral from the same patients [1303 (1006–2383) units/liter versus 165 (93–278); \( P < 0.0001 \)] (Fig. 4B). The demonstration of elevated levels of both CK18-Asp396-NE and CK18 in local tumor venous blood suggests that these proteins are tumor-derived.

Large differences in CK18-Asp396-NE:total CK18 ratios were observed in local venous sera from different patients (Fig. 4C). Ratios as low as 0.01 were observed in some sera and the median ratio (25th to 75th percentile) was 0.16 (0.09–0.37). Interestingly, lower CK18-Asp396-NE:CK18 ratios were observed in high-grade tumors (III and IV; Fig. 4D). We conclude that the median CK18-Asp396-NE:CK18 ratio in endometrial tumor venous blood was lower (0.16) than the corresponding ratio in the medium from apoptotic MDA-MB-231 cells (0.85).

Different Patterns of Increases of Caspase-Cleaved and Total CK18 during Treatment. Induction of apoptosis is a major cytotoxic mechanism of many anticancer drugs in vitro but not necessarily in vivo (31). We examined the levels of soluble CK18 proteins in sera from 25 patients with hormone-refractory prostate cancer during extracellular CK18 were paralleled by decreases in intracellular levels compared with untreated control cells (Fig. 3E).

Caspase-cleaved CK18-Asp396-NE fragments were measured in the same samples, and increases were observed both in culture medium and cell extracts after 24 h of treatment (Fig. 3, F and G). These increases were inhibited by z-VAD-fmk. After 48 h, the medium content of CK18-Asp396-NE was ∼3200 units/liter and the total CK18 content 3700 units/liter. Therefore, ∼85% of total CK18 released from cisplatin-treated cells was comprised by caspase-cleaved material.

Caspase-Cleaved Fragments Constitute a Small and Variable Portion of Total CK18 in Cancer Patient Sera. The in vitro experiments suggested that the relative levels of total and caspase-cleaved CK18 proteins in the extracellular compartment reflect differences in cell death modes. Therefore, we examined the patterns of CK18 release from human carcinoma cells in vivo. Serum samples were collected from local pelvic blood during operation of 37 patients with endometrial cancer and from operation of 19 patients with benign endometrial conditions. Peripheral serum was collected from the same patients. Higher levels of CK18-Asp396-NE were observed in local serum obtained from patients with malignant tumors [median (25th to 75th percentile): 178 (137–262) units/liter] than in patients with benign conditions [125 (109–202) units/liter; \( P = 0.009 \)] (Fig. 4A). In

![Fig. 3. Release of cytokeratin (CK) 18-Asp396-NE from cisplatin-treated MDA-MB-231 cells. Cells were treated with 50 μM cisplatin. A–C, cells were stained with annexin V and propidium iodide (PI) and analyzed by flow cytometry; A, untreated cells; B, cells treated with cisplatin for 48 h [apoptotic cells (annexin V positive and PI negative) are observed in the lower right quadrant; PI-positive dead cells are observed in the upper quadrants]. C, the number of viable (), necrotic (○), and apoptotic (□) cells at different times of treatment; D–G, medium and cytosol fractions were assayed for CK18 at the times indicated. D and E, levels of soluble CK18 from cisplatin-treated cells in media (D) and cell extracts (E) measured by the M30-ELISA; F and G, CK18-Asp396-NE levels from cisplatin-treated cells in media (F) and cell extracts (G) measured by the M30-ELISA. Results are shown as means in the presence or absence of the caspase inhibitor z-VAD-fmk (40 μM); bars, ±SD.](image-url)
Fig. 5. Measurements of cytokeratin (CK) 18 and CK18-Asp396-NE in sera of patients with hormone-refractory prostate cancer during second or third line chemotherapy. A, increases in CK18 forms during second line therapy with estamustine/vinorelbine (cycle 1 and 2). The patient was a prostate-specific antigen (PSA) responder (patient 1; Table 1). B, increases of both CK18 and CK18-Asp396-NE during second line therapy with estamustine/vinorelbine and third line therapy with estamustine and docetaxel (patient 2; cycle 1 shown from both treatments). C and D, increases of CK18 forms during third line therapy (first cycle) with estamustine/vinorelbine of two different patients. Patient 3 showed no decreases in PSA; patient 4 showed a PSA decrease of 15% between cycle 1 and 2 and PSA levels were then stable during subsequent cycles. E, CK18 forms in patient 5 (a PSA responder) during second line estamustine/vinorelbine treatment. Note the increase in total CK18 but not in CK18-Asp396-NE during the 2nd and 4th cycle of treatment. F, increases of CK18-Asp396-NE during cycle 1 and 3 during second line estamustine/vinorelbine treatment (a PSA responder). Note increases in both forms, but a relatively small contribution by CK18-Asp396-NE during cycle 1 and 3 during second line estamustine/vinorelbine treatment of 2 patients with hormone refractory prostate cancer. These patients did not respond to therapy.

One complicating factor is that PSA gene expression or release can be maintained 10 patients were classified as responders. Increases of CK18 of ≥2-fold were observed in one or more treatment cycles in 6 of 10 responders (Table 1). Two patients showed >90% decreases in PSA, and both of these patients were classified as CK18 responders. One of these patients (patient 5) did not show increases in CK18-Asp396-NE in any of 4 cycles investigated, suggesting that the antitumor effect was primarily associated with tumor necrosis. None of the 3 nonresponding patients showed increases in CK18-Asp396-NE and/or CK18 of ≥2-fold during treatment.

DISCUSSION

CKs are expressed at high levels in epithelial cells, and expression is usually maintained or even increased after oncogenic transforma-

Table 1  Changes in the serum levels of CK18-Asp396-NE and CK18 during second line therapy of prostate cancer patients

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<td>0.6/2.4</td>
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<td>+</td>
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<td>6</td>
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<td>+</td>
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* Changes in CK18-Asp396-NE/CK18 in each cycle (calculated as peak value or nadir value divided by pretreatment value). Increases of ≥2 are indicated with bold figures. Note that peak values and nadir values for CK18-Asp396-NE and CK18 were not necessarily observed at the same time points during treatment cycles (for example, see patient 2, Fig. 5B).

† CK18 responder defined as patient with a ≥2-fold increase of either CK18-Asp396-NE or CK18 in at least one cycle.
‡ Lowest PSA value observed during treatment as compared to pretreatment value.
§ This patient showed an objective clinical progression during treatment despite the PSA decrease and was classified as a nonresponder.
tation (34). CKs can be detected in serum from cancer patients and are widely used serum tumor markers (1, 2). The mechanisms of release of soluble CKs into the extracellular compartment have been unclear. It was shown recently that apoptosis leads to the release of soluble CK fragments from tumor cells, suggesting that the widely used CK tumor markers tissue polypeptide antigen, tissue-polypeptide-specific antigen, and CYFRA 21–1 may reflect tumor apoptosis (14–16). The results of the present study confirmed previous findings of release of soluble CK18 to the extracellular compartment during apoptosis (Fig. 3), but also showed that large amounts of soluble CK18 were released from cells induced to undergo necrosis (Fig. 2). Release of CK18 is therefore a marker of epithelial cell death and not a specific marker of apoptosis.

The in vitro data suggesting that the relative levels of total soluble CK18 and caspase-cleaved fragments in the extracellular compartment mirror the mode of cell death of epithelially derived tumor cells prompted us to measure CK18 forms in serum from cancer patients. We have found previously increases in CK18-Asp396-NE fragments in serum from patients with breast, liver, and lung cancer (24), interpreted to reflect spontaneous apoptosis of tumor cells. In the material of endometrial cancer patients studied here, higher levels of CK18-Asp396-NE were observed in local compared with peripheral blood from patients with malignant disease, consistent with release from tumors. Large variations were observed in the relative levels of caspase-cleaved and total CK18 proteins. These variations in local blood are likely to reflect differences in the type of CK18 proteins released from tumors and less likely to reflect differences in stability. Interestingly, in most endometrial cancer patient sera, the caspase-cleaved CK18-Asp396-NE fragments constitute only a fraction of total CK18. The implication of this finding is that apoptosis is not the major mechanism of spontaneous cell death in those tumors. In the limited material of venous blood available, lower ratios of CK18-Asp396-NE to total CK18 were associated with high-grade tumors, consistent with reports of more necrosis in higher-grade tumors (35).

Anticancer therapy is generally believed to induce tumor apoptosis (36, 37). Apoptosis is an attractive clinical end point for assessment of treatment efficiency (38). It has been recently demonstrated that 99mTc-Annexin V can be used for imaging tumor cell death in patients (39–41). This method is likely to measure both apoptotic and necrotic cell death. Increased uptake of 99mTc-Annexin V in human tumors after one course of treatment was observed to predict tumor response (39). We here observed different relative contributions of increases of the CK18-Asp396-NE marker relative to the increases in total CK18 (Fig. 5; Table 1). In patient 5, who showed a strong PSA decline during therapy, increases in total CK18 but not in apoptosis-specific CK18-Asp396-NE were observed. Other patients showed relatively small increases in CK18-Asp396-NE (Fig. 5, D and F). These observations suggest that apoptosis is not the dominating death mode of the corresponding tumors during therapy, and suggest that it may be important to not only monitor apoptosis but also total cell death during treatment.

It is well established that under conditions of deficient cellular ATP generation, cells fail to execute the apoptotic program and instead undergo necrosis (28, 42). Apoptosis and necrosis may therefore represent "two extremes of a continuum of possible types of cell demise" (28). The individual variations in the relative proportion of caspase-cleaved CK18 fragments released from tumors observed in this study may reflect tumor-specific differences of this continuum. Future investigations will address whether tumor hypoxia will lead to low ratios of caspase cleaved to total CK18. Tumor hypoxia is associated with treatment resistance (43, 44), and CK18 measurements before therapy could potentially be used for prediction of response.

Survival is generally considered as the gold standard for approval of new drugs. However, the use of survival as a clinical end point requires long observation periods and could be confounded by effective second-line therapy (45). Assessment of increases of serum CK18 forms is a candidate surrogate marker to demonstrate treatment efficiency during clinical trials. The sensitivity and specificity of CK18 serum measurements for detection of cell death of epithelially derived tumors will have to be established to evaluate the utility of this approach. The present study did not include sufficient number of patients to warrant any statement with regard to the clinical utility of CK18 serum measurements, but larger studies are ongoing.

REFERENCES

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