Prostate Adenocarcinoma Cells Release the Novel Proinflammatory Polypeptide EMAP-II in Response to Stress

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ABSTRACT

The proinflammatory protein endothelial monocyte-activating polypeptide II (EMAP-II) was first detected in supernatants of murine tumor cells by virtue of its ability to stimulate endothelial-dependent coagulation in vitro. The purified protein has pleiotropic effects on endothelial cells, monocytes, and neutrophils; however, its function in vivo is unknown, and the mechanism whereby it is released from cells is poorly understood. We investigated the expression of EMAP-II in human prostate adenocarcinoma specimens by immunohistochemistry and in LNCaP and DU-145 human prostate adenocarcinoma cells by reverse transcription-PCR, flow cytometry, and Western blotting. We then examined the effects of chemical and physiological stress on release and processing of EMAP-II by LNCaP and DU-145 cells. These cells constitutively express a Mr = 34,000 form of EMAP-II that is retained intracellularly. Exposure to agents that induce apoptosis or, in some cases, necrosis induces the release of the Mr = 34,000 form and further processing to the Mr = 27,000 and Mr = 22,000 forms. Hypoxia, but not heat shock, is a potent inducer of release and processing of biologically active EMAP-II by LNCaP and DU-145 cells. We suggest that release of EMAP-II by prostate adenocarcinoma cells as a consequence of treatment with anticancer agents or as a result of constitutive hypoxia may potentiate the effects of those agents through the localized activation of host effector mechanisms.

INTRODUCTION

The existence of EMAP-II (1) reviewed in Refs. 1 and 2) was first suggested by the observation that infusion of TNF-α into murine fibrosarcomas results in intravascular coagulation at the tumor site as well as a major decrease in tumor blood flow (3). This led to speculation that soluble factors produced by tumor cells prime tumor-associated endothelial cells to respond to TNF-α. EMAP-II protein was subsequently identified and purified from supernatants of cultured murine fibrosarcoma cells, based on its ability to potentiate the endothelial procoagulant-inducing activity of TNF-α (4).

Purified EMAP-II protein possesses a wide range of activities toward endothelial cells, neutrophils, and monocyte/macrophages in vitro. In addition to the induction of TF-dependent coagulation on endothelial cells and monocytes, EMAP-II up-regulates endothelial E- and P-selectin expression and release of von Willebrand factor (4). It is also chemotactic for neutrophils and monocytes and induces release of myeloperoxidase activity from neutrophils (4). In vivo, local injection of EMAP-II into the mouse footpad evokes an acute inflammatory response characterized by edema and a neutrophil-rich infiltrate (5). Furthermore, direct injection into s.c. tumors in mice leads to hemorrhage and inflammatory infiltrates, followed by a decrease in tumor volume (4), consistent with the activities of a pleiotropic, proinflammatory cytokine. EMAP-II is likely to be identical to the bladder carcinoma-derived cytokine BCDC and to the PO-1 and HS-1 proteins derived from the FO-1 and BLM human melanoma cell lines, respectively (6–8).

Murine EMAP-II and human EMAP-II have been cloned and expressed in bacteria (4, 9). The cDNA sequence of EMAP-II is consistent with a Mr = 34,000 precursor molecule, which is cleaved at a critical aspartate residue to produce the mature polypeptide (4). This precursor lacks a classic hydrophobic signal peptide necessary for membrane translocation, and the mature molecule may be secreted via a novel pathway, in a manner similar to that of the leaderless precursor of interleukin-1β, which undergoes proteolytic cleavage at the plasma membrane with subsequent release into the extracellular space (10).

Given the potency of the mature EMAP-II molecule, it is important to understand how its biological availability and activity are regulated. A recent study of EMAP-II expression in the mouse embryo noted the coincidence of EMAP-II transcripts and the presence of macrophages in areas with high levels of apoptosis (11). These findings led the authors to speculate that EMAP-II is released by apoptotic cells in tissues undergoing remodeling, thereby providing a chemoattractant signal for phagocytic cells required to remove cellular debris (11). This hypothesis is consistent with a role for caspase-like enzymes in the cleavage of the precursor molecule, which was predicted earlier by Kao et al. (4), based on the amino acid sequence of the putative cleavage site.

Because processing and release of EMAP-II may be associated with programmed cell death (4, 11), we decided to examine the expression of EMAP-II in two established prostate adenocarcinoma cell lines, LNCaP and DU-145. In general, prostate tumor cells proliferate slowly (12), making them poor targets for conventional antimitotic chemotherapy (13). In the absence of cell proliferation, programmed cell death can be activated in androgen-dependent prostatic cancer cells by androgen withdrawal (14). On the other hand, androgen-independent tumor cells are resistant to androgen ablation because they have a defect in the initiating step of the apoptosis pathway (15). However, these cells retain downstream components of the pathway (14) and can be induced to undergo apoptosis by agents that elevate intracellular calcium levels, such as ionomycin (16) and thapsigargin (17), suggesting some potentially novel therapeutic approaches. We therefore examined the effects of chemical stress on LNCaP and DU-145 cells using known inducers of apoptosis to determine whether these might cause the release of biologically active EMAP-II. We compared these effects with those of necrosis-inducing agents. We also examined the effects of physiological stresses, in particular, hypoxia, which is often a constitutive component of the tumor microenvironment.

Our data indicate that LNCaP and DU-145 cells express EMAP-II in vitro, but the protein is normally retained intracellularly as a Mr = 34,000 precursor. Treatment of these cells with hypoxia or chemical agents known to induce apoptotic or necrotic cell death leads to the release and partial processing of EMAP-II. Importantly, EMAP-II release can occur in the absence of apoptosis. We suggest that the release of EMAP-II and its subsequent interaction with other host cells may contribute to the overall response of tumors to cytotoxic therapy.
Materials and Methods

Cell Culture and Treatment. The LNCaP-FGC (LNCaP) and the DU-145 human prostate adenocarcinoma cell lines were obtained from the American Type Culture Collection (Manassas, VA). The cell lines were routinely grown in 75-cm² tissue culture flasks (Becton Dickinson, Oxford, United Kingdom) in 15 ml of RPMI 1640 supplemented with 5% or 10% heat-inactivated bovine calf serum (PAA Laboratories, GmbH, Austria) and cultured in a humidified incubator at 37°C with 5% CO₂.

All experiments were performed with cells at 80–90% confluence, unless otherwise indicated. For chemical treatment of cells, medium was replaced with fresh medium supplemented with freshly dissolved drug at a concentration either derived from the literature or based on initial dose-ranging experiments (data not shown). Incubations with drugs were carried out for up to 48 h. Oxygen tension was monitored by the built-in indicator as well as by the use of a Fireright Oxygen monitor (LEEC Ltd., Nottingham, United Kingdom). The atmosphere also contained 5% CO₂. Control flasks were incubated in an ACCISS oxygen-controlled incubator (Boro Labs, Berkshire, United Kingdom). The cell lines were routinely grown in 75-cm² tissue culture flasks (Becton Dickinson, Oxford, United Kingdom) and cultured in a humidified incubator at 37°C with 5% CO₂.

Viability was assessed by Hoechst/PI staining, and cells were categorized as described in “Materials and Methods.” Data are given as the mean of duplicate estimations. Viability assay was not performed on cells treated with hormone withdrawal (ND, not determined).

Recombinant M₉, 22,000 and M₉, 34,000 EMAP-II. These forms of EMAP-II were prepared as described previously (9).

Antibodies. Preparation and characterization of polyclonal antibodies against recombinant human EMAP-II (R2B2) have been described in detail elsewhere (9). Briefly, rabbits were immunized with recombinant human EMAP-II expressed in Escherichia coli as a fusion protein with GST. Serum

Table 1 Effect of chemical and physiological stresses on viability of LNCaP and DU-145 cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Treatment h</th>
<th>Percentage of cells (%)</th>
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<tr>
<td></td>
<td>Healthy</td>
<td>Early</td>
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<tr>
<td>Control</td>
<td>24</td>
<td>88</td>
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<tr>
<td>Irradiation (8 Gy)</td>
<td>24</td>
<td>87</td>
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<tr>
<td>Etoposide (100 µM)</td>
<td>24</td>
<td>88</td>
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<tr>
<td>Campthothecin</td>
<td>24</td>
<td>93</td>
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<tr>
<td>Ionomycin (1 µM)</td>
<td>24</td>
<td>91</td>
</tr>
<tr>
<td>Thapsigargin</td>
<td>24</td>
<td>91</td>
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<tr>
<td>H₂O₂ (20%)</td>
<td>24</td>
<td>91</td>
</tr>
<tr>
<td>Saponin (0.2%)</td>
<td>24</td>
<td>91</td>
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<tr>
<td>Antimycin A (10 µM)</td>
<td>24</td>
<td>91</td>
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<tr>
<td>Hyposia (2.5% oxygen)</td>
<td>48</td>
<td>91</td>
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Fig. 1. Expression of EMAP-II mRNA and protein by LNCaP and DU-145 cells. A, expression of EMAP-II transcripts was assessed by RT-PCR as described in “Materials and Methods.” RT-PCR with EMAP-II-specific primers, Lanes 3, 5, and 7. Control primers for human porphobilinogen deaminase, Lanes 2, 4, and 6. Reaction mixtures in Lanes 6 and 7 contained no cDNA; B, fluorescence histograms of LNCaP and DU-145 cells stained with polyclonal antibodies against human EMAP-II. Cells were fixed and permeabilized as described in “Materials and Methods.”
was tested for reactivity with recombinant EMAP-II and recombinant GST by ELISA (9), and animals were exsanguinated. Reactivity with recombinant GST and E. coli antigens was removed by cross-absorption on a column of immobilized extract of E. coli BL21 transformed with the expression plasmid pGEX-2T coupled to CNBr-activated Sepharose 4B (Pharmacia Biotech). Polyclonal antibodies were tested for reactivity with recombinant GST and recombinant EMAP-II by ELISA and showed no reactivity for GST.

Fluorescence-activated Cell-sorting Analysis. Immunofluorescent detection of EMAP-II was performed on LNCaP and DU-145 cells with R2B2 antibodies. Staining was carried out on fixed, permeabilized cell suspensions with R2B2 antibodies diluted 1:1000 in TBS. The secondary antibody was goat antirabbit FITC conjugate (Sigma). Samples were analyzed on a Becton Dickinson FACScan using the LYSIS program.

Western Blotting. PAGE was used to separate proteins from LNCaP and DU-145 cell extracts and supernatants, and EMAP-II was detected by Western blotting with the R2B2 polyclonal antibodies as described previously (9). Antibody binding was revealed by enhanced chemiluminescence. An unexpected band corresponding to a molecular weight of ~66,000 was frequently seen on Western blots. We have established that this is an artifact of the blotting technique. B, Western blot of lysates and concentrated media from LNCaP (Lanes 1–8) and DU-145 (Lanes 9–14) cells treated or not treated with thapsigargin. Lane 1, untreated cell lysate at 24 h; Lane 2, CM from untreated cells at 24 h; Lane 3 and 9, cell lysate treated with 1 μM ionomycin for 24 h; Lanes 4 and 10, medium with 1 μM ionomycin for 24 h; Lanes 5 and 11, cell lysate treated with 10 μM ionomycin for 24 h; Lanes 6 and 12, medium treated with 10 μM ionomycin for 24 h. M, molecular weight marker; R, recombinant mature EMAP-II (units are in thousands). The band at Mr ~66,000 is an artifact of the blotting technique.

Immunohistochemistry. All tissues were fixed in 4% paraformaldehyde before embedding in paraffin. Four-μm sections were cut onto glass slides and incubated at 60°C for 30 min. Before antibody staining, sections were dewaxed with Histolene clearing agent (Cell Path Plc, Hemel Hempstead, United Kingdom) and rehydrated by passing through a graded series of alcohols (100–30%), followed by PBS (pH 7.4). Endogenous peroxidase activity was quenched by incubation of all slides in 0.3% (v/v) hydrogen peroxide in methanol. Sections were microwaved in an 800 W oven for 10 min in 0.1 M citrate buffer (2.1 g/liter citric acid and 1.0 g/liter sodium hydroxide). For all immunohistochemical reactions, a Vectastain Elite ABC Kit (Vector Laboratories, Peterborough, United Kingdom) was used, and all incubations were carried out at room temperature in a humidified chamber. Non-specific binding of antibodies was blocked by incubating sections in 20% normal goat serum for 20 min. After shaking off excess blocking solution, R2B2 rabbit polyclonal antibodies against EMAP-II were added at a 1:500 dilution. Preimmune rabbit serum at the same dilution was used as a control. Sections were incubated for an additional 60 min and then washed three times with PBS. Sections were incubated for an additional 30 min with a biotinylated goat antibacter secondary antibody and then washed three times with PBS. Slides were then incubated with the avidin-biotin complex reagent, followed by diaminobenzidine substrate. After washing with distilled water, slides were counterstained with Mayer's hematoxylin. Finally, the slides were dehydrated with graded alcohols and mounted with DPX. Sections were viewed with a Nikon Optiphot microscope and photographed on Kodak Elite 100 ASA film.

Detection of Apoptotic and Necrotic Cells with H342/PI. This method (18) allows distinction of the nuclei of living and dying cells by fluorescence microscopy after staining with the fluorochromes H342 and PI. H342 stains all cell nuclei, whereas PI stains only the nuclei of cells with disrupted plasma membranes. Therefore, viable and necrotic cells will have blue round nuclei and pink round nuclei, respectively. Apoptotic cells will have condensed/fragmented blue or pink nuclei, depending on whether they are in the “early” or “late” stages of apoptosis. Cells were harvested by trypsinization, centrifuged in 10 ml of cell culture medium at 1500 rpm for 5 min, and then resuspended at a density of about 1 × 10^6 cells/ml in medium. Cells were stained with H342 (10 μM) and PI (10 μM) on ice for 5 min in the dark. A 50-μl aliquot was then dropped onto a glass microscope slide, a coverslip was applied, and the cells were examined under a Nikon fluorescence microscope with UV (UV-1A) and green (G-2A) filters to detect H342 and PI staining, respectively. A minimum number of 100
cells/sample were counted, and the percentage of viable, necrotic, and apoptotic cells was calculated.

Coagulation Assay. Conditioned media from control and treated LNCaP cells were tested for procoagulant activity using a two-stage coagulation assay (19). HUVECs were grown to confluence on 0.2% gelatin-coated, 24-well tissue culture plates in HUVEC medium (M199 medium; Life Technologies, Inc., Paisley, United Kingdom) supplemented with 14 mM HEPES, 0.15% sodium hydrogen carbonate, 2 mM l-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, 90 μg/ml heparin, 20 μg/ml endothelial cell growth supplement (Boehringer Mannheim), and 20% FCS. Tumor CM was diluted 1:1 in HUVEC medium, and 50 units/ml polymixin B was added to all test solutions to quench the effects of the contaminating lipopolysaccharide. As a positive control, TF was added to some test solutions. HUVEC medium was carefully removed from plates by aspiration and replaced with test medium (500 μl/well). The samples were incubated for 4.5 h at 37°C and then assayed for procoagulant activity.

Test medium was removed by careful inversion of the plate onto tissue paper, and each well was washed twice with 1 ml of PBS. Owen’s (barbital) buffer (100 μl) and 100 μl of citrated human plasma from a normal donor were then added to each well. The plate was placed in a 37°C water bath for 5 min to equilibrate. Coagulation was then initiated by the addition of 100 μl of 30 mM calcium chloride. Time to formation of visible fibrin strands/gel was measured with a stopwatch. Clotting times were converted to TF equivalents (pg/10⁶ cells) by reference to a bilogarithmic calibration curve constructed using purified reconstituted human TF (19).

RESULTS

EMAP-II Is Expressed by LNCaP and DU-145. We used RT-PCR to determine whether LNCaP and DU-145 cells expressed mRNA transcripts encoding EMAP-II. cDNA prepared from both cell lines demonstrated strong, single bands corresponding to the expected cDNA fragments of 305–310 bp (Fig. 1A, Lanes 3 and 5).

We then examined the expression of EMAP-II protein in the two cell types using flow cytometry and Western blotting. Proteins reactive with R2B2 anti-EMAP-II polyclonal antibodies were detected intracellularly in both LNCaP and DU-145 cells by flow cytometry (Fig. 1, B and C). To determine the size distribution of the immunoreactive polypeptides, we carried out Western blotting on extracts of LNCaP and DU-145 cells from both cell lines with R2B2. We have previously shown (9) that these antibodies detect a major band of Mr = 34,000 corresponding to the precursor of EMAP-II in lysates of U937 transformed human monocytic cells and a weaker band of Mr = 22,000 in concentrated growth medium from the same cells. Lysates of LNCaP and DU-145 cells showed a major band on Western blots of Mr = 34,000, indicating the presence of a precursor of EMAP-II within both cell types (Fig. 2A, Lanes 1 and 7). Concentrated media in which the same cells had been grown also contained detectable levels of Mr = 34,000 EMAP-II antigen, more in the case of LNCaP cells (Fig. 2A, Lanes 2 and 8). We concluded from these experiments that both prostate
cancer cell lines constitutively express EMAP-II protein as the $M_r$ 34,000 form, releasing minimal levels under normal conditions of culture. We therefore examined the effects of a variety of cellular stresses known to cause apoptotic or necrotic death or both to determine whether these might stimulate the release of EMAP-II.

Effect of Stress on Tumor Cell Viability. We first carried out experiments to determine the effect of known stressors on cell viability. After exposure for up to 48 h to chemical agents or physiological stresses, cells were analyzed by the H342/PI technique (described in “Materials and Methods”) to assess the proportion of apoptotic and necrotic cells in the cultures. The results of this analysis are summarized in Table 1. In brief, the most potent inducers of apoptosis were ionomycin, camptothecin, and thapsigargin. Etoposide induced approximately equal levels of necrosis and apoptosis by 48 h. Overall, DU-145 cells appeared to be somewhat more resistant to the effects of apoptosis-inducing agents than LNCaP cells. Hydrogen peroxide, saponin, and antimycin rapidly induced necrosis in both cell lines. Both cell lines appear resistant to 8 Gy of X-rays given as a single dose. Hypoxia (2.5% $O_2$ for 48 h), on the other hand, is clearly a potent physiological stress, because approximately 49% of LNCaP cells and 11% of DU-145 cells were necrotic by 48 h. Hypoxia appears to primarily induce necrosis and not apoptosis in these cell lines. In response to hormone withdrawal, androgen-sensitive LNCaP cells ceased proliferating and showed morphological alterations, but there was no evidence of apoptosis or necrosis after 7 days of observation (data not shown). Hormone withdrawal had no effect on DU-145 cells. Heat shock produced extremely variable and inconsistent results (data not shown).

Effect of Stress on Processing and Release of EMAP-II. We examined the effect of different stresses on the release and processing of EMAP-II by Western blotting. Fig. 2A shows a Western blot of lysates and concentrated media from LNCaP and DU-145 cells after treatment for 24 h with ionomycin, which we found to be a potent inducer of apoptosis. The blots show a relative increase in $M_t$ 34,000 EMAP-II in the supernatants of LNCaP and DU-145 cells after treatment (Fig. 2A, compare Lane 2 with Lanes 4 and 6 and compare Lane 8 with Lanes 10 and 12). Furthermore, in the treated samples, a strong immunoreactive band of $M_t$ ~26,000–28,000 appears in supernatants. Using radiolabeled $M_t$ 34,000 precursor, we have shown that a $M_t$ 26,000–28,000 band is a common intermediate in the processing of EMAP-II. In the DU-145 cells, ionomycin treatment induces essentially the complete disappearance of $M_t$ 34,000 EMAP-II from the cells and the appearance of the $M_t$ 26,000–28,000 form in the supernatants (Fig. 2A, Lanes 10 and 12). On the other hand, thapsigargin seems to have less effect on EMAP-II processing or release from DU-145 cells, with some release into the medium and conversion after 48 h (compare Fig. 2B, Lanes 10 and 14). Interestingly, DU-145 cells do not undergo significant apoptosis or necrosis in response to thapsigargin (see Table 1). A $M_t$ 22,000 band corresponding to the fully processed protein and migrating with the same mobility as recombinant EMAP-II (Fig. 2A, Lane R) is just detectable in supernatant samples from LNCaP cells treated with 10 µM ionomycin (Fig. 2A, Lane 6). Etoposide treatment gave rise to a similar profile; camptothecin also produced a $M_t$ 26,000–28,000 band, but not a $M_t$ 20,000–22,000 band (see Table 2).

Western blots of lysates and concentrated supernatants from cells treated with inducers of necrosis, including hydrogen peroxide, saponin, and antimycin-A, were also performed. Antimycin-A is a mitochondrial respiratory chain inhibitor. In conjunction with glucose-free growth medium, the effects of antimycin-A are similar to hypoxia, inducing cellular necrosis (18, 20). The results of treatment of LNCaP cells with antimycin-A, in the presence or absence of glucose, are shown in Fig. 3. The data indicate changes similar to those seen with ionomycin, thapsigargin, and etoposide, i.e., increased $M_t$ 34,000 EMAP-II in the cell supernatants combined with the appearance of a $M_t$ ~26,000–28,000 band. Excluding glucose from the medium, which increases the levels of necrosis, enhances this effect, and the appearance of the fully processed $M_t$ ~20,000–22,000 form of EMAP-II can be noted (Fig. 3B, Lanes 23 and 24). Partial processing to the $M_t$ 26,000–28,000 forms was also observed in DU-145 cells (data not shown).

Fig. 4 shows the results of exposure of LNCaP and DU-145 cells to hypoxia, which induced high levels of necrosis and very little apoptosis. EMAP-II Western blot profiles were similar to those seen with antimycin, which mimics some effects of hypoxia, and other inducers of apoptosis. Hypoxia induced the appearance of a strong $M_t$ 22,000 band that was absent from control cultures. Although treatment with hypoxia appeared to induce increased immunoreactive material, there was no significant change in total EMAP-II antigen in hypoxic cultures by competitive ELISA (data not shown).

Hypoxia induces the Release of Biologically Active EMAP-II. To establish whether the released EMAP-II was biologically active, we assayed the ability of supernatants from cells subjected to stress to potentiate TNF-induced coagulation activity on the surface of human endothelial cells, a defining activity of EMAP-II. It was not possible to assay supernatants from cells exposed to chemical agents because control experiments indicated that in almost every case, medium
containing the agent in question induced endothelial-dependent coagulation, regardless of whether or not it had been exposed to tumor cells. This phenomenon was attributed to direct toxic effects of the agent on the endothelial cells in the coagulation assay. However, we were able to assay medium from hypoxic cells, where there could be no subsequent effect of hypoxia per se on the endothelial cells. The results of experiments with CM from LNCaP cells are shown in Fig. 5. The first four bars of the graph demonstrate the well-documented response of endothelial cells to EMAP-II, whereby recombinant EMAP-II and TNF act synergistically to increase procoagulant activity. The next two bars show that LNCaP CM has very low levels of procoagulant-inducing activity; the addition of TNF produces a supraadditive effect (Control CM + TNF). Medium from cells subjected to hypoxia for 48 h does not have significantly elevated coagulation-inducing activity. However, when TNF is added to this medium, a strong coagulation response is seen, which is approximately equal to the level obtained with recombinant EMAP-II and TNF combined. Similar data were obtained with DU-145 CM (data not shown).

Because it is not clear that the M, 22,000 form of EMAP-II is the only biologically active form of the molecule, we also carried out TNF-induced coagulation assays in the presence of recombinant M, 22,000 or M, 34,000 EMAP-II. The data in Fig. 6 indicate that the M, 34,000 form of EMAP-II is only slightly but not significantly less potent than the M, 22,000 form in terms of activity in the endothelial coagulation assay.

**DISCUSSION**

EMAP-II is expressed at the mRNA and protein level in LNCaP and DU-145 prostate adenocarcinoma cells. Transcripts encoding EMAP-II have been found in a wide range of human tissues as well as normal and tumor cell lines (9, 11). To date, however, there is little information on the distribution of EMAP-II protein in normal tissues or tumors. Recently, Schluesser et al. (21) reported the first immunohistochemical study of EMAP-II in adult rat tissues, in which they found expression of EMAP-II antigen restricted to subsets of macrophages and microglial cells. An immunohistochemical and in situ hybridization study of the mouse lung suggests that EMAP-II is highly expressed in the embryo, but expression subsequently diminishes and remains low throughout adult life (22). We recently carried out a comprehensive immunohistochemical study of normal human tissues and concluded that EMAP-II protein is expressed primarily in endocrine organs, particularly in cells of neuroendocrine origin. In the normal prostate, we found weak staining of the epithelial component only (Fig. 7A). Adenocarcinomas of the prostate show stronger staining of the malignant epithelial component, with occasional weak staining of the stromal component (Fig. 7B). This pattern, which occurred in all prostatic adenocarcinomas we studied, is consistent with the intracellular expression of EMAP-II by the prostate adenocarcinoma cell lines LNCaP and DU-145 in vitro. Although the present study is limited to two cell lines, we believe they accurately reflect EMAP-II expression in the human disease.

Kao et al. (4) proposed that the M, 34,000 protein, which we observed intracellularly, is a precursor of M, 20,000–22,000 EMAP-II and that a caspase-like enzyme may be responsible for cleavage. The involvement of a caspase would be consistent with a requirement for the initiation of apoptosis, and Knies et al. (11) have recently provided experimental evidence in support of this hypothesis. However, our Western blot results clearly show the generation of major intermediates in the range of M, 26,000–28,000 and, in some cases, the M, 22,000.
22,000 form at relatively low levels after treatment with agents that induce either apoptosis or necrosis. Other experiments by us with recombinant Mr 34,000 EMAP-II have demonstrated that incubation with isolated cell membrane extracts gives rise sequentially to Mr 26,000–28,000 fragments, followed by the fully processed Mr 20,000–22,000 form. This processing is abrogated by inhibitors of serine proteases, and Mr 34,000 EMAP-II is readily cleaved by serine proteases with the sequential production of Mr 26,000–28,000 fragments, followed by the Mr 20,000–22,000 form.5 Moreover, whereas it had always been assumed that the Mr 20,000–22,000 form of EMAP-II was the biologically active moiety, our data indicate that recombinant Mr 34,000 and Mr 22,000 forms of EMAP-II are equally active in TNF-potentiating activity in vitro. Therefore, we conclude that release of Mr 34,000 EMAP-II alone, without further processing, may be sufficient for expression of certain biological effects. Further processing, via Mr 26,000–28,000 intermediates, may occur in both apoptotic and necrotic cells. We anticipate that the Mr 26,000–28,000 form of EMAP-II will also show biological activity.

The nature of the putative precursor of EMAP-II has recently become less clear because Quevillon et al. (23) noted the high degree of amino acid identity between EMAP-II and the p43 auxiliary component of the mammalian multisynthase complex. The hamster p43 protein is composed of 359 amino acids with a

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predicted molecular weight of \( M_t \) 40,000. This protein shares 86% and 85% amino acid identity with human and murine \( M_t \) 34,000 EMAP-II, respectively, whereas the human p43 and EMAP-II homologues appear to share 100% identity (23). Quevillon et al. (23) have suggested that \( M_t \) 20,000 EMAP-II and, by implication, its \( M_t \) 34,000 precursor are truncated forms of the p43 auxiliary protein generated by partial proteolysis of the multisynthase complex, which may occur as a consequence of the disruption of protein synthesis in tumor cells. Our data demonstrate that LNCaP expressed a unique protein and does not represent the partially processed form of a larger protein.

Under conditions of stress, however, EMAP-II is further processed and released by both cell lines into the extracellular environment. EMAP-II could play a role in two possible scenarios in vivo: regions of low oxygen tension are frequently present in rapidly growing experimental and human tumors, usually as a consequence of vascular insufficiency (24). Hypoxia contributes directly to resistance to treatment with ionizing radiation but may also indirectly induce resistance to antimitotic chemotherapeutic agents through the initiation of a cell cycle block. On the basis of our data, rapidly growing, hypoxic tumors should release higher levels of the mature protein and should be more sensitive to TNF. Prostate cancer cells proliferate slowly in vivo (12); therefore, prostate tumors might be considered less likely to contain significant regions of hypoxia. However, recent evidence, including that derived from oxygen electrode measurements in situ (25), supports the existence of hypoxic regions within prostate tumors. It might therefore be predicted that human prostate tumors do release some processed EMAP-II in these regions. Subjecting such tumors to added stress such as chemotherapeutic agents or other inducers that induce cell death should enhance this release.

Treatment of tumors with chemotherapeutic agents is frequently associated with vascular pathologies, particularly disorders of coagulation (26). Intravascular coagulation and hemorrhage are seen histologically in experimental tumors after treatment with conventional chemotherapy agents (27). Damage to the vasculature may be due to direct effects on endothelial cells (28) or may occur indirectly through the action of molecules such as EMAP-II (29) released by damaged cells. Our data show that several chemical agents, including camptothecin and etoposide (both used clinically), stimulate EMAP-II release, which could then contribute to the damage described above.

In summary, treatment of prostate tumor cells with stress-inducing agents promotes the release of biologically active EMAP-II. Furthermore, release and processing are not restricted to apoptotic cells but may also occur in cells that will ultimately die through necrosis. We suggest that released EMAP-II may act directly on tumor-associated endothelial cells, promoting a procoagulant phenotype, or may be involved in recruiting immune effector cells into the tumor milieu. These findings suggest a potential role for EMAP-II in tumors and, furthermore, mechanisms whereby its potent biological activity is expressed.

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