Immunohistochemical Analysis of Endothelial-Monocyte-Activating Polypeptide-II Expression *in Vivo*

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Endothelial-monocyte activating polypeptide (EMAP)-II is a novel molecule with cytokine-like pro-inflammatory properties, inducing procoagulant activity on the surface of endothelial cells and monocyte/macrophages *in vitro*, as well as up-regulating E- and P-selectin expression. EMAP-II is chemotactic for monocytes/macrophages and neutrophils, and stimulates myeloperoxidase release from neutrophils. Injection of EMAP-II into the mouse footpad induces an acute inflammatory response, although some regression occurs in response to direct injection of EMAP-II into murine tumors. Very little is known about the expression of EMAP-II in normal tissues of mice or humans, or about its function *in vivo*. We developed polyclonal antibodies against EMAP-II using recombinant protein produced in *Escherichia coli*, and used these antibodies to carry out an immunohistochemical study of the occurrence and distribution of EMAP-II in human tissues. The distribution of EMAP-II protein is relatively restricted, occurring primarily in endocrine organs, in cells of neuroendocrine origin, but also in tissues with high turnover. EMAP-II is strongly expressed in secretory epithelial cells of the thyroid, pancreas, adrenal and salivary glands, among others, as well as in neurons and subsets of monocytes/macrophages. It is also found in the epithelium of the small and large intestines. We conclude that EMAP-II expression is usually, but not always, associated with tissues that display high turnover and high levels of protein synthesis. (Am J Pathol 2000, 157:2045–2053)

EMAP-II is a novel molecule with pleiotropic activities toward endothelial cells, monocytes/macrophages, and neutrophils.^{1,2} EMAP-II was first detected in the supernatants of cultured MethA murine fibrosarcoma cells. Its

existence was suggested by the observation that infusion of tumor necrosis factor- α into mice bearing MethA fibrosarcomas induces intravascular coagulation at the tumor site, resulting in a major decrease in tumor blood flow.³ This intravascular coagulation is initiated by enhanced expression of tissue factor (or thromboplastin) on the luminal surface of the tumor-associated endothelial cells, in turn leading to the deposition of insoluble fibrin within the tumor vasculature.³ These effects seem to be confined specifically to the tumor vasculature, leading to speculation that factors produced by tumor cells prime the tumor-associated endothelial cells to respond to tumor necrosis factor- α .

Several polypeptides with tumor necrosis factor- α -potentiating activity have been isolated from MethA tumor cell supernatants and characterized. One such factor is the murine homologue of vascular endothelial cell growth factor⁴ which, in addition to its ability to modulate endothelial cell function, has effects on neutrophils and monocytes.⁴ Two additional proteins with similar activities were isolated from MethA cells; the 44-kd endothelial-monocyte-activating polypeptide-I (EMAP-I)⁵ and 22-kd EMAP-II.⁶ Unlike vascular endothelial cell growth factor, these factors are not mitogenic for endothelial cells. Based on its mobility on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and range of activities, MethA-derived EMAP-II is likely to be the mouse homologue of a bladder carcinoma-derived cytokine,⁷ and of the FO-1⁸ and HS-1⁹ proteins, derived from the FO-1 and BLM human melanoma cell lines, respectively.

EMAP-II possesses a wide range of activities toward endothelial cells, neutrophils, and monocytes/macrophages *in vitro*. In addition to the induction of tissue factor-dependent coagulation on endothelial cells and monocytes, EMAP-II up-regulates endothelial E- and P-selectin expression and release of von Willebrand Factor.¹⁰ It is also chemotactic for neutrophils and monocytes, and induces the release of myeloperoxidase activity from neutrophils.¹⁰ *In vivo*, local injection of EMAP-II into the mouse footpad evokes an acute inflam-

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matory response characterized by edema and a neutrophil-rich infiltrate.⁶ Furthermore, direct injection of EMAP-II into subcutaneous tumors in mice leads to hemorrhage and inflammatory infiltrates, followed by a decrease in tumor volume.¹⁰ These activities are consistent with those of a pleiotropic, pro-inflammatory cytokine. More recently, however, EMAP-II has been shown to induce endothelial apoptosis *in vitro*, leading to speculation that its anti-tumor activity may be conferred, at least in part, by anti-angiogenic activity based on its ability to induce programmed cell death in tumor-associated endothelium.¹¹

Full-length cDNAs encoding murine and human EMAP-II have been isolated from MethA,¹⁰ U937,¹⁰ and normal peripheral blood cells,¹² respectively. The deduced amino acid sequences seem to be 86% identical between the two species. The amino-terminal region of mature EMAP-II has minor homologies with interleukin (IL)-8, IL-1 β , and von Willebrand Factor Antigen II. The cDNA sequence suggests that EMAP-II is synthesized as a 34-kd precursor molecule, which is cleaved at a critical aspartate residue to produce an ~18-kd mature polypeptide.¹⁰ The putative precursor, 34-kd EMAP-II, lacks a classic hydrophobic signal peptide necessary for membrane translocation, indicating that the mature molecule may be secreted by a novel pathway; however, little is known about the mechanism of this processing or its control. It has been suggested¹⁰ that EMAP-II may be processed in a similar manner to the leaderless precursor of IL-1 β , which undergoes proteolytic cleavage at the plasma membrane with subsequent release into the extracellular space.¹³

The relationship between the mature form of EMAP-II and the putative precursor as described by Kao et al¹⁰ has recently become less clear, since Quevillon et al¹⁴ have noted the high degree of amino acid identity between EMAP-II and the p43 auxiliary component of the mammalian multisynthase complex. This complex is a high-molecular weight structure composed of nine aminoacyl-tRNA synthetases and three auxiliary proteins with molecular weights of 18, 38, and 43 kd. Hamster p43 component is composed of 359 amino acids with a predicted molecular weight of 40 kd. This protein shares 86 and 85% amino acid identity with human and murine 34-kd EMAP-II, respectively, whereas the human p43 and EMAP-II homologues seem to share 100% identity.¹⁴ We recently reported the isolation of recombinant human EMAP-II¹² and have raised polyclonal antibodies against this material. In Western blots, the antibodies detect a 34-kd EMAP-II precursor molecule in lysates of U937 cells as well as an ~18- to 20-kd mature form. Both 34- and 18- to 20-kd species can be detected in culture medium conditioned by U937 cells.¹² Significantly, our antibodies fail to detect a protein band in the region of 40 to 43 kd, corresponding to a putative human p43.

By reverse transcriptase-polymerase chain reaction, EMAP-II transcripts are found in a wide range of human tissues, as well as normal and tumor cell lines (Tas et al, unpublished observations; Knies et al, personal communication). To date, however, there is little information on the distribution of EMAP-II protein in normal tissues or

tumors. Recently Schluessener et al¹⁵ reported the first immunohistochemical study of EMAP-II, in this case of the rat. Using monoclonal antibodies raised against a synthetic peptide corresponding to the N-terminal sequence of mature EMAP-II, these authors found expression of EMAP-II antigen restricted to subsets of macrophages and microglial cells. A recent immunohistochemical and *in situ* hybridization study of the mouse lung suggests that EMAP-II is highly expressed in the developing lung, but subsequently diminishes and remains low throughout adult life.¹⁶ We now report a comprehensive immunohistochemical survey of EMAP-II expression in normal human tissues using polyclonal antibodies against recombinant human EMAP-II.

Materials and Methods

Antibody Preparation

The preparation and characterization of polyclonal antibodies against recombinant human EMAP-II has been described in detail elsewhere.¹² Briefly, rabbits were immunized with recombinant human EMAP-II (rEMAP-II) expressed in *Escherichia coli* as a fusion protein with glutathione-S-transferase. Serum was tested for reactivity with rEMAP-II by enzyme-linked immunosorbent assay, and the animals were exsanguinated. Reactivity with recombinant glutathione-S-transferase 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 also tested for reactivity with recombinant glutathione-S-transferase and found to be negative.

Immunohistochemistry

All tissues were fixed in 4% paraformaldehyde before embedding in paraffin. Four-micron sections were cut onto glass slides and incubated at 60°C for 30 minutes.

Before antibody staining, sections were dewaxed with Histolene clearing agent (Cell Path PLC, Hemel Hempstead, UK), and rehydrated by passing through a graded series of alcohols (100 to 30%), then to phosphate-buffered saline (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 minutes in 0.1 mol/L citrate buffer (2.1 g/L citric acid, 1.0 g/L sodium hydroxide). For all immunohistochemical reactions a Vectastain Elite ABC Kit (Vector Laboratories, Peterborough, UK) was used, and all incubations were performed at room temperature in a humidified chamber. Nonspecific binding of antibodies was blocked by incubating sections in 20% normal goat serum for 20 minutes. After shaking off excess blocking solution, rabbit antibodies against rEMAP-II were added at a 1:500 dilution. Pre-immune rabbit serum at the same dilution was used as a control. Sections were incubated for a further 60 minutes and then washed 3 times with PBS. Sections were incubated

for a further 30 minutes with a biotinylated goat anti-rabbit secondary antibody, followed by washing 3 times with PBS. Slides were then incubated with the ABC (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 with Fujichrome 100 ASA film.

Western Blotting

Western blotting was used to confirm the specificity of antibodies for EMAP-II, and to determine whether they cross-react with other proteins in human tissues. Tissue and cell extracts were electrophoresed on 12% SDS-polyacrylamide gels under reducing conditions. Antibodies bound to membrane-immobilized proteins were visualized by enhanced chemiluminescence using the Amersham enhanced chemiluminescence Western blotting protocol (Amersham Life Science, Little Chalfont, UK). Rabbit anti-EMAP-II antibodies were diluted 1:2,000 for this purpose. The secondary antibody was horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin at 1:2,000 dilution (DAKO, UK).

To produce tissue lysates, small pieces of frozen tissue (~2 mm³) were placed directly in 2× SDS-PAGE loading buffer, and boiled for 2 minutes. Insoluble material was removed by centrifugation, and the supernatant stored at -20°C until required. For studies with U937 cells (a kind gift of Dr Matthias Clauss, Max-Planck-Institut, Bad Nauheim, Germany), cells were grown as monolayers on tissue-culture grade plastic dishes in RPMI 1640 medium (Gibco BRL, Paisley, UK) supplemented with 10% fetal calf serum and 100 U/ml penicillin/10 µg/ml streptomycin. To produce lysates of the U937 monocytic cell line, cells were grown to ~80% confluence. They were then transferred into serum-free medium for 24 hours before harvesting by scraping. Cells (4 × 10⁶) were lysed in 120 µl of SDS-PAGE loading buffer. To examine protein in supernatants from the U937 cells, 15 ml of serum-free, spent culture medium was acetone-precipitated at 4°C, centrifuged, and the resulting pellet resuspended in 120 µl of loading buffer.

In Situ Hybridization

In situ hybridization was used to detect mRNA transcripts for EMAP-II in normal human tissues. A 540-bp cDNA product corresponding to the transcript encoding EMAP-II was generated by polymerase chain reaction. The polymerase chain reaction mixture contained 0.5 nmol/L dATP, 0.5 nmol/L dCTP, 0.5 nmol/L dGTP, 0.5 nmol/L dTTP, 100 pmol/L each forward and reverse primer, 10 mmol/L Tris-HCL, pH 8.3, 1.5 mmol/L MgCl₂, 50 mmol/L KCl, and 1 U *Taq* polymerase (Roche). Amplification proceeded for 40 cycles at 94°C for 1 minute, 60°C for 1 minute, 72°C for 2 minutes, and an additional extension period of 5 minutes at 72°C. Anti-sense se-

quence: 5'-TCATTTGATTCCACTGTTGCTCATGG-3'; sense sequence: 5'-AGGATGGACTCTAAGCCAATAGAT-3'. The EMAP-II cDNA product was then cloned into the pCR2.1-TOPO vector (Invitrogen) and run-off transcripts in the sense and anti-sense direction labeled *in vitro* (Promega) with biotin (Sigma). Paraffin sections of normal human thyroid and large intestine were digested with proteinase K before overnight incubation with the EMAP-II probe in mRNA hybridization buffer (DAKO, UK) at 40°C. The tyramide-catalyzed signal amplification technique¹⁷ was used in the form of a GenPoint- kit (DAKO) according to the supplier's instructions to detect the probe *in situ*. This was followed by counterstaining with hematoxylin. Several negative controls were incorporated, including use of a sense probe, omission of probe, or digestion of the mRNA target with 100 µg/ml RNase A for 60 minutes before hybridization.

In Vitro Transcription/Translation of 34-kd EMAP-II

A full-length cDNA sequence encoding human 34-kd EMAP-II was generated by polymerase chain reaction as previously described,¹² and cloned into pCR3 (Invitrogen BV, Leek, The Netherlands). The circular plasmid was then used to drive the synthesis of [³⁵S]methionine-labeled 34-kd EMAP-II protein by coupled transcription/translation (TnT; Promega, UK).

In Vitro Cleavage of [³⁵S]-EMAP-II by U937 Membrane Extracts

Microsomal membrane extracts were prepared from U937 cells as described by Nicholson et al.¹⁸ Briefly, U937 cells were washed in ice-cold PBS, after which they were suspended (10⁸ cells/ml) in lysis buffer (10 mmol/L HEPES/KOH, 2 mmol/L ethylenediaminetetraacetic acid, 0.1% (w/v) CHAPS, 5 mmol/L dithiothreitol, pH 7.4) for 1 minute at 4°C. The resulting lysate was then centrifuged at 1,000 × *g* for 10 minutes at 4°C. The supernatant was removed and centrifuged at 10,000 × *g* for a further 10 minutes at 4°C. Finally the supernatant from the previous step was centrifuged at 100,000 × *g* (30 minutes at 4°C). The pellet from this step, containing the microsomal membrane fraction, was resuspended to an equivalence of 10⁸ cells/ml, aliquoted and stored at -80°C until required.

To demonstrate cleavage of 34-kd EMAP-II by membrane-associated enzymes, [³⁵S]EMAP-II was incubated with aliquots of membrane fraction in reaction buffer (50 mmol/L HEPES/KOH, 0.1%CHAPS, 2 mmol/L ethylenediaminetetraacetic acid, 10% (w/v) sucrose, 5 mmol/L dithiothreitol, pH 7.0) at 37°C for different times. The reaction mixtures were then denatured at 100°C for 3 minutes. An aliquot of each reaction product was analyzed by SDS-PAGE on 12.5% polyacrylamide gels. Gels were fixed, dried under vacuum, and protein bands visualized by exposure of the gel to Hyperfilm βmax overnight at room temperature.

Figure 1. a: Western blot analysis of EMAP-II and its related polypeptides with polyclonal antibodies raised against recombinant human EMAP-II. Antibodies binding to membrane-immobilized proteins were visualized by enhanced chemiluminescence. Anti-EMAP-II antibodies were diluted 1:2,000; the secondary antibody was horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin. **Lane 1,** Cell lysate of U937 monocytic cells. **Lane 2,** Twenty-four-hour-conditioned growth medium from the same U937 cells. **Lane 3,** Recombinant human EMAP-II expressed in *E. coli* as a glutathione-S-transferase fusion product, cleaved with thrombin and purified to homogeneity by affinity chromatography. Note that rEMAP-II migrates more slowly than native mature EMAP-II because of the presence of extra amino acid residues at the N-terminus. **Lane 4,** Extract of fresh normal human tonsil. m = molecular weight markers; numbers indicate molecular weights of standards in kd. **b:** Time course of cleavage of radiolabeled 34-kd EMAP-II by membrane extracts of U937 cells, demonstrating the production of intermediate cleavage products.

Results

Characterization of Antibodies

On Western blots, the polyclonal antibodies detect a single protein band of ~20 to 22 kd in preparations of recombinant human EMAP-II (Figure 1, lane 3). In lysates of U937 cells (lane 1), a major band of ~34 kd, corresponding to the putative precursor previously identified

by us in these cells,¹² was detected, as well as minor bands of ~32 kd and 18 to 20 kd. In some cases, samples of lysates were pre-incubated with anti-EMAP-II antibodies and immune complexes adsorbed with Protein A beads. The supernatants were then subjected to SDS-PAGE. After this procedure, all three bands disappeared from Western blots (data not shown), suggesting that the bands represent intermediates and the fully processed

form of EMAP-II. Concentrated conditioned medium from U937 cells contained an extra band with a molecular weight in the region of 27 to 28 kd (lane 2). Extracts of normal human tonsil (lane 4) demonstrated major bands at 34 kd, corresponding to precursor EMAP-II, and further bands at ~32 kd and ~18 kd. The smaller band corresponds to fully processed EMAP-II, which has a predicted molecular mass of 18 kd. Similarly, native murine EMAP-II contains one less amino acid and has a mobility on SDS-PAGE corresponding to that of a ~18-kd polypeptide.¹¹

Cleavage Products of 34-kd EMAP-II

In an attempt to characterize unidentified, intermediate-sized bands appearing on Western blots, we examined the cleavage products of radiolabeled 34-kd EMAP-II incubated with a membrane fraction from U937 cells and separated by SDS-PAGE. Figure 1b shows a time course for this reaction. By 4 hours there is complete conversion of the 34-kd EMAP-II substrate to a 20- to 22-kd form, which corresponds to recombinant EMAP-II (Figure 1a, lane 3). At shorter times, intermediate-size bands appear, falling into discrete size ranges. In particular, major bands can be seen at 31 to 32 kd, 27 to 28 kd, and 25 kd. The first two of these groupings demonstrate correspondence with bands on the Western blot (Figure 1a), a 31- to 32-kd band being present in U937 cell lysate and tonsil extract (lanes 1 and 4), and a 27- to 28-kd band in U937-conditioned medium and tonsil (lanes 2 and 4).

The results of the cleavage study of radiolabeled 34-kd EMAP-II suggest that the intermediate-sized protein bands appearing on our Western blots between the 34-kd precursor and 18- to 20-kd fully-processed EMAP-II are partially processed forms of the precursor molecule.

Immunohistochemical Analysis of EMAP-II Distribution

Cardiovascular System

Antibodies against EMAP-II stained the cytoplasm of smooth muscle cells very weakly. There was occasional weak cytoplasmic staining of endothelial cells in lung, heart, liver, cervix, ovary, and small and large intestine, although in general blood vessels of all sizes were negative. In the heart there was weak cytoplasmic staining of muscle, with darker staining of some capillaries.

Gastrointestinal Tract

EMAP-II immunostaining was evident in several cell types associated with the gastrointestinal tract. In the salivary gland there was very weak staining of the luminal surface of ducts, whereas the acini were negative. The esophagus showed weak reactivity in the serous and mucinous glands, whereas the squamous epithelium was negative. Strong EMAP-II staining was present in the gastric pits of the stomach, particularly in the peptic cells located at the base of the glands (Figure 2a). The hydro-

chloric acid-secreting parietal cells were negative. There was strong staining of hepatocytes in the liver (Figure 2b). Studies in the rat have suggested that EMAP-II expression is restricted to the Kupffer cells.¹⁵ In the pancreas, there was moderate staining of the Islets of Langerhans (Figure 2c), as well as some weak staining of acini. Within the large (Figure 2d) and small (Figure 2e) intestine there was staining of the surface epithelium, which tended to become weaker toward the base of the crypts.

Respiratory Tract

The lung parenchyma was generally negative. Occasional staining of alveolar macrophages was observed, but pneumocytes were negative. In the bronchus the epithelium did not stain, but there was weak staining of serous glands. Mucinous glands were negative.

Musculoskeletal System

Skeletal muscle, cartilage, and bone were generally negative for EMAP-II staining.

Renal System

In the kidney there was moderate staining of epithelium of the proximal tubules (Figure 2f). Glomerular endothelium was negative.

Reproductive Organs

In the male, weak EMAP-II staining was seen in the secretory glands of the prostate (Figure 2g), in the seminiferous tubules of the testis, and in the epididymis. The Leydig cells of the testis were generally negative although occasional cells showed some granular staining.

In the female, the squamous epithelium of the cervix was negative while there was slight positivity in the glandular epithelium. Within the endometrium there was also weak staining of the proliferative glandular epithelium. There was strong focal staining of the luminal surface and cytoplasm of epithelium lining the fallopian tube (Figure 2h), whereas the ovary itself was negative in all compartments. Normal breast tissue demonstrated weak staining of the luminal surface of lobules, and running into the ducts.

Central Nervous System

In the cerebral cortex, neurons were strongly positive (Figure 2i). There was also some staining of microglial cells.

Endocrine Organs

In the thyroid gland there was strong staining of the T₃ and T₄ hormone-secreting follicular epithelium (Figure 2j). There was occasional staining of parafollicular or

Figure 2. a–h: Immunohistochemical staining of normal human tissues with polyclonal antibodies (1:500 dilution) raised against recombinant human EMAP-II. Six- μ m sections were cut from formalin-fixed, paraffin-embedded blocks, and stained by the avidin-biotin complex (ABC) peroxidase technique. Binding of ABC complex was revealed with diaminobenzidine substrate, which produces a brown color. Sections were counterstained with hematoxylin. **a:** Gastric pits of stomach. **Arrow** indicates strong staining of peptic cells. Original magnification, $\times 400$. **b:** Liver. Generalized cytoplasmic staining of hepatocytes. Original magnification, $\times 200$. **c:** Pancreas. There is weak staining of islets of Langerhans (**arrow**). Original magnification, $\times 200$. **d:** Large intestine. Diffuse cytoplasmic staining of epithelium. Original magnification, $\times 200$. **e:** Small intestine. Diffuse staining of epithelium. Original magnification, $\times 200$. **f:** Kidney. Cytoplasmic staining of epithelium of proximal tubules, indicated by **arrow**. Original magnification, $\times 400$. **g:** Prostate. Weak staining in secretory epithelium. Original magnification, $\times 200$. **h:** Fallopian tube. **Arrow** indicates strong focal staining of luminal surface. Original magnification, $\times 400$. **i:** Cerebral cortex; showing strong staining of neurons (**arrow**) and some microglial cells. Original magnification, $\times 400$. **j:** Thyroid; strong staining of T3- and T4-producing follicular epithelium (**double arrow**), and weaker staining of calcitonin-producing parafollicular (clear) cells (**single arrow**). Original magnification, $\times 400$. **k:** Adrenal; strong membrane-associated staining of secretory cells of zona glomerulosa. Original magnification, $\times 400$. **l:** Thymus; weak staining of Hassall's corpuscles (**arrow**). Original magnification, $\times 200$.

clear cells. The parathyroid gland in contrast was generally negative for EMAP-II staining.

There was positive staining in both the cortex and medulla of the adrenal gland. There was particularly strong staining of the secretory cells of the zona glomerulosa, which demonstrated intense membrane-associated reaction product (Figure 2k). There was also staining of the steroid-secreting cells of the zona fasciculata. The catecholamine-secreting cells of the adrenal medulla were weakly positive.

The endocrine component of the pancreas, the islets of Langerhans, was also positive for EMAP-II staining (Figure 2c). This staining clearly delineated the islets, in

contrast to the exocrine component of the pancreas, which was negative apart from a few acinar cells.

Immune System

The thymus was negative, apart from weak staining of the Hassall's corpuscles (Figure 2l), which are believed to represent nests of degenerated epithelial cells within the thymic medulla. Lymph nodes were negative, although there was strong staining of subcapsular macrophages, which localize to the periphery of nodes. Staining of the spleen was restricted to the red pulp, which contains anastomosing cords of highly phagocytic cells,

Figure 3. *In situ* detection of mRNA transcripts for EMAP-II in paraffin-embedded sections of normal human thyroid and large intestine, using the tyramide-catalyzed signal amplification technique. **a:** Thyroid section hybridized with biotinylated anti-sense probe. Note intense cytoplasmic staining of follicular cells (arrows) and parafollicular cells. **b:** Thyroid section hybridized with anti-sense probe after digestion with RNase A, eliminating cytoplasmic staining. **c:** Section of large intestine hybridized with anti-sense probe. Note weak staining of mucosa epithelium (arrow). **d:** Large intestine hybridized with anti-sense probe after RNase A digestion.

the primary function of which is to effect the destruction of aged or damaged red cells. Similar findings have been reported for the rat, where EMAP-II immunostaining was restricted to subpopulations of monocytes in spleen and lymph nodes.¹⁵

In Situ Hybridization

In situ hybridization was used to demonstrate the correspondence of expression of transcripts for EMAP-II with accumulation of protein. Figure 3a shows a section of normal thyroid hybridized with the anti-sense biotinylated probe for EMAP-II. Staining is restricted to the hormone-producing parafollicular and follicular epithelial cells of the tissue, and is absent from the stromal component. In the large intestine somewhat weaker staining is seen, primarily in the mucosal epithelial cells (Figure 3c). Control sections (Figure 3, b and d), hybridized with the same probe after digestion with RNase A, show no reaction product confirming the specificity of this reaction. The patterns obtained with the anti-sense probe should be com-

pared with the patterns of EMAP-II protein expression revealed by immunohistochemistry (Figure 2, d and j).

Discussion

Early interest in EMAP-II focused on its role in tumors, and in particular the nature of its effects on tumor-associated endothelial cells. However, *in vitro* studies demonstrated that this molecule has activity toward other cell types, in particular monocytes/macrophages and polymorphonuclear leukocytes, raising the possibility that EMAP-II is a pleiotropic cytokine with pro-inflammatory properties. Nevertheless, the limited information available to date on the biological activities of EMAP-II provides little in the way of clues concerning its possible distribution *in vivo*.

Our immunohistochemical survey shows that a form (or forms) of EMAP-II can be detected in a limited number of normal human tissues. Furthermore, based on *in situ* hybridization, protein expression corresponds with expression of mRNA transcripts in at least two normal tissues,

thyroid and colon. An earlier study in the rat,¹⁵ using monoclonal antibodies raised against synthetic peptides, found highly restricted normal tissue expression of EMAP-II. In that study, immunostaining was primarily restricted to cells of the monocyte/macrophage lineage, and was observed in lymphoid tissues such as spleen, lymph nodes, and follicles of the gut. We, on the other hand, have observed more widespread staining, the highest levels of expression being seen in cells/tissues with high turnover and/or levels of protein synthesis, such as neuroendocrine tissues. The significance of high-level production of EMAP-II by these tissues is not clear, because they are not normally invested with inflammatory cells. However, those tissues that do undergo rapid turnover might be expected to produce significant levels of cellular debris, in which case the potent chemotactic properties of EMAP-II may serve to recruit phagocytic cells into the tissue to effect clearance.

An alternative explanation for high levels of expression in such tissues derives from the hypothesis that the 34-kd precursor of EMAP-II may itself be a proteolytically processed form of the p43 auxiliary protein of the multisynthase complex, and not the product of a dedicated and unique EMAP-II gene.¹⁴ If this hypothesis is correct, it is not surprising that high levels of EMAP-II antigen are observed, because those tissues with high EMAP-II expression are likely to have high protein biosynthetic activity. Against this hypothesis, on the other hand, is the fact that our polyclonal antibodies do not detect a 43-kd band on Western blots. It is very unlikely that p43 could be converted so rapidly to p34 as to be essentially undetectable. Furthermore, if both proteins are present, it is also very unlikely that polyclonal antibodies raised against a common region will react with one and not the other under denaturing conditions. Therefore our data suggest that EMAP-II may be synthesized independently of p43.

The *in situ* hybridization data confirm the co-localization of EMAP-II protein and corresponding mRNA transcripts. However, they do not resolve the issue of whether transcripts for EMAP-II, p43, or both are being detected, because the EMAP-II probe corresponds to a cDNA sequence that is theoretically common to EMAP-II and the putative human p43. Because the upstream sequences, not shared with the shorter 34-kd EMAP-II, are unavailable it is not possible to probe specifically for human p43. Likewise, the lack of upstream sequence data precludes the generation of recombinant human p43, which could have been compared with 34-kd EMAP-II for protease sensitivity.

We are currently attempting to address the issue of the relationship between p43 and 34-kd EMAP-II. A possible explanation for the identity of these two proteins, other than that one is a proteolytically derived fragment of the other, is that they are the products of overlapping mRNA transcripts, with distinct transcription initiation sites. Our preliminary data suggest that U937 cells contain a single mRNA of appropriate size to translate 34-kd EMAP-II, but too short to translate p43 (Brown et al, unpublished).

Whether EMAP-II derives from a separate gene, mRNA transcript, or is a cryptic peptide derived from the p43

auxiliary protein, a fundamental question remains as to the function of such a potent pro-inflammatory molecule in these environments. Expression of paracrine pro-inflammatory activity will clearly depend on release of EMAP-II into the extracellular space, although not necessarily on conversion from the 34-kd form. We have recently shown that recombinant 34-kd and 20-kd forms of recombinant EMAP-II are equipotent on a molar basis at potentiating tumor necrosis factor-induced endothelial tissue factor.¹⁹

Currently little is known about the factors controlling the processing or release of mature EMAP-II. By immunoblotting we have observed EMAP-II expression in the form of a 34-kd intracellular precursor molecule in several cultured cell lines (unpublished data),¹² with, in some cases, no evidence of processing or release under normal conditions. However, we have shown that after exposure to stresses capable of producing necrotic and/or apoptotic cell death, EMAP-II is processed and released by LNCaP prostate cancer cells.¹⁹ We hypothesized that in these circumstances EMAP-II acts as an injury signal that may play a variety of roles involving clearance of cellular debris and tissue repair.²

A limitation of this study is the inability of polyclonal antibodies to distinguish between the putative precursor EMAP-II and the ~18- to 20-kd mature form. Based on Western analysis of cells and tissues (see Figure 1), it would seem that forms other than the 34-kd precursor are present within tissues. The Western blotting results are supported by the results of experiments on the cleavage of radiolabeled 34-kd EMAP-II by U937 membrane extracts. These data suggest that there is no single cleavage point at a critical aspartate residue (Asp¹⁴⁷) as described by Kao et al, but rather that the molecule undergoes sequential cleavage, perhaps through the activity of a number of enzymes. Intermediate species will, of course, be detected indiscriminately by immunohistochemistry with polyclonal antibodies; how the various forms are distributed within cells and tissues *in vivo* is unclear.

If the hypothesis of Kao and colleagues¹⁰ concerning the simultaneous processing and release of EMAP-II is correct, then we might expect that extracellular staining would be associated with mature EMAP-II. However, we found very little extracellular staining in any tissue. There are several possible, nonexclusive explanations for this observation: The most plausible of these is that the release of EMAP-II is a rare event under normal circumstances. As indicated, *in vitro* and *in vivo* evidence suggest that EMAP-II has powerful pro-inflammatory properties,¹⁰ therefore its release must be tightly regulated. Quevillon and colleagues¹⁴ have suggested that abnormal protein synthesis in neoplastic cells leads to the accumulation of EMAP-II, as a proteolytic product of p43. How this EMAP-II escapes from the tumor cell is unclear, although, as stated above, we have found that physiological stresses such as hypoxia may promote release.

A second explanation for the lack of extracellular staining in normal tissues is that the processed form may be rapidly cleared from the tissue. Again, given the sug-

gested potency of EMAP-II, it seems likely that mechanisms will be in place to restrict its effects to the site of injury and ensure the activity is switched off rapidly when no longer required. Nothing is known about the metabolic fate of the mature EMAP-II molecule. Although it would appear to be relatively stable *in vitro*, we have found that it contains a thrombin-sensitive site near its C-terminus (unpublished data), suggesting that under certain circumstances EMAP-II that arrives in the circulation may undergo further proteolytic processing, perhaps leading to inactivation.

Both explanations outlined above, independently or together, may account for the low extracellular staining observed. Further elaboration of the mechanism and conditions of release *in vivo* will be aided by the development of monoclonal antibodies that discriminate between the precursor and mature forms of EMAP-II.

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