Colorectal Cancer Cells Induce Lymphocyte Apoptosis by an Endothelial Monocyte-Activating Polypeptide-II-Dependent Mechanism[1]

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Endothelial monocyte-activating polypeptide-II (EMAP-II) was first isolated from cell growth medium conditioned by tumor cells, and is closely related or identical with the p43 component of the mammalian multisynthase complex. In its secreted form, EMAP-II has multiple cytokine-like activities in vitro, inducing procoagulant activity on the surface of endothelial cells, increasing expression of E- and P-selectins and TNF-R1, and directing migration of monocytes and neutrophils. EMAP-II has also been shown to induce apoptosis in endothelial cells, leading to the suggestion that it is a proinflammatory polypeptide with antiangiogenic activity. The role of secreted EMAP-II in tumors remains poorly understood, and we hypothesized that EMAP-II may play a role in immune evasion by tumor cells. We investigated its effects on lymphocytes, using recombinant protein, or colorectal cancer cell lines, as a source of native EMAP-II. Recombinant EMAP-II inhibits DNA synthesis and cell division, and induces apoptosis in mitogen-activated lymphocytes in PBMC preparations, and in Jurkat T cells. Native EMAP-II, released by or expressed on the surface of colorectal carcinoma cells, also induces activation of caspase 8 and apoptosis of PBLs and Jurkat cells, which are partially blocked by addition of Abs against EMAP-II. Thus, activated lymphocytes, along with proliferating endothelial cells, are targets for the cytotoxic activity of EMAP-II. Membrane-bound and soluble EMAP-II appear to play multiple roles in the tumor microenvironment, one of which is to assist in immune evasion. ***The Journal of Immunology*****, 2004, 172: 274–281.**

Endothelial monocyte-activating polypeptide-II (EMAP-II)[3] is a novel molecule with pleiotrophic activities toward endothelial cells, monocytes/macrophages, and neutrophils, which was first detected in supernatants of cultured murine tumor cells (1), and subsequently human melanoma cells (2; for reviews, see Refs. 3 and 4). Purified EMAP-II protein induces expression of E- and P-selectin on endothelial cells in vitro, release of von Willebrand factor, chemotaxis of neutrophils and macrophages, and activation of the neutrophil respiratory burst (5). In vivo, injection of EMAP-II stimulates an inflammatory infiltrate in the mouse footpad and some regression of transplanted tumors (5). More recently, EMAP-II has been implicated in lung morphogenesis (6), and has been shown to induce endothelial cell apoptosis and inhibit angiogenesis (7, 8). On the basis of these findings, EMAP-II has been described as a proinflammatory cytokine with antiangiogenic activity. The true function of soluble EMAP-II outside the cell remains unclear, although one role would certainly appear to be that of a modulator of capillary development.

The sequences of full-length cDNAs encoding murine and human EMAP-II (5, 9) suggest that EMAP-II is synthesized as a 34-kDa precursor molecule, which is proteolytically cleaved to produce the 20-kDa mature polypeptide. Quevillon et al. (10) showed that hamster p43 protein, a component of the multisynthase complex, is essentially identical with the human EMAP-II protein. They suggest 34-kDa EMAP-II may be a proteolytic product of p43, and that cytokine activity is a secondary role, only expressed in abnormal situations such as tumors, where protein synthesis is often dysfunctional. Furthermore, it has been suggested that in vivo EMAP-II is only released from cells as a consequence of activation of programmed cell death (11). We showed that in prostate cancer cell lines (as well as in a wide range of cultured cells) 34-kDa precursor EMAP-II is retained in the cytoplasm, and in some cases low levels of processed EMAP-II are released constitutively (12). Enhanced processing and release of mature protein are initiated in response to cellular stress, such as that caused by exposure to hypoxia, or by treatment with certain chemotherapeutic agents, and activation of apoptosis is not a prerequisite of this release (12). In addition to release, EMAP-II synthesis de novo may also be enhanced by hypoxia (12).

On the assumption that the induction of an inflammatory response could be detrimental to the growth and progression of a solid tumor, we have sought a biological rationale for the release of EMAP-II by tumor cells, other than acting as a chemoattractant for phagocytic cells. Although there is a need for the presence of phagocytic cells to clear debris associated with high cell turnover and death due to apoptosis and necrosis, there is also a need to suppress the activity of T cells directed toward tumor Ags. We hypothesized that EMAP-II might act in an immune-suppressive role, simultaneously attracting phagocytic cells and inhibiting cell-mediated cytotoxicity, and therefore investigated the effects of rEMAP-II on lymphocytes. Using cocultures, we then attempted to model indirect and direct interactions between EMAP-II-expressing colorectal tumor cells and lymphocytes, to determine the effects of the native protein.

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[3] Abbreviations used in this paper: EMAP, endothelial monocyte-activating polypeptide; Ac-IETD, acetyl-Ile-Glu-Thr-Asp; CHO, Chinese hamster ovary; FasL, Fas ligand; PI, propidium iodide; pNA, *p*-nitroaniline; tyrRS, tyrosyl-tRNA synthetase.

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Materials and Methods

Cell lines

The human leukemic T cell line Jurkat; colorectal adenocarcinoma cell lines HT29, DLD-1, LS513, HCT-15 (all from American Type Culture Collection, Manassas, VA), and Colo-205; and the normal human fibroblast line MRC-V were cultured in RPMI 1640 medium (Life Technologies, Paisley, U.K.) supplemented with 10% FCS (PAA Laboratories, Lintz, Austria) and 100 U/ml penicillin/streptomycin solution (Sigma-Aldrich, Poole, U.K.). Cells were maintained at 37°C in 5% CO_2 in a humidified incubator, and routinely subcultured by removal from flasks with 0.05% trypsin/1 mM EDTA (Sigma-Aldrich).

Preparation of PBMC

Anticoagulated blood from normal donors was diluted 1/2 with RPMI medium containing HEPES buffer and penicillin/streptomycin. Twenty milliliters of diluted blood were layered onto 5 ml of Histopaque (Sigma-Aldrich), and centrifuged at 1500 × *g* for 15 min. Medium was removed, and the PBMC layer was resuspended in fresh medium. PBMC were then centrifuged at 1500 × *g* for a further 10 min. This wash step was repeated twice, and PBMC were pooled and resuspended in RPMI medium containing 5% FCS, HEPES, and penicillin/streptomycin.

Abs and recombinant proteins

Rabbit polyclonal Abs against human EMAP-II (R2B2) were used for immunohistochemistry, Western blotting, and flow cytometry in this study. The characteristics of R2B2, which recognizes the 34- and 20-kDa, as well as several intermediate forms of human EMAP-II, have been described elsewhere (9, 12, 13). Anti-Fas ligand (FasL) polyclonal Abs Ab-3 (Oncogene Research Products, Nottingham, U.K.) and N20 goat and rabbit polyclonals (Santa Cruz Biotechnology, Santa Cruz, CA) were used to detect expression of FasL. HRP- and FITC-labeled anti-rabbit or anti-goat IgG Abs (Sigma-Aldrich) were used for detection of primary Abs. Recombinant human TNF-α and IFN-γ were obtained from PeproTech (London, U.K.). Recombinant human EMAP-II was generated in house, as described (9), or purchased from PeproTech. Recombinant FasL was obtained from R&D Systems (Abingdon, U.K.)

Immunohistochemistry

Archived samples of colorectal material preserved in paraffin wax were analyzed for EMAP-II expression. Material was mounted on 3-aminopropyl-triethoxysilane-coated slides. Slides were dewaxed in Histolene, before being rehydrated in graded ethanol solutions (100–30%). Ag retrieval was performed by boiling the slides for 10 min in citrate buffer (10 mM citric acid, 25 mM sodium hydroxide). Slides were blocked with normal goat serum for 20 min. EMAP-II was identified by incubating the slides with purified polyclonal Abs R2B2 (1 μg/ml in PBS) for 1 h at room temperatures (9). Secondary detection was performed using the Vectastain Elite kit, according to manufacturer's instructions (Vector Laboratories, Burlingame, CA). Slides were counterstained with hematoxylin solution (Vector Laboratories), dehydrated in ethanol, and mounted with depex polystyrene solution (BDH, Poole, U.K.).

Detection of EMAP-II by Western blotting

Western blotting was conducted essentially as previously described (9). Cells were trypsinized and washed with cold PBS, and counted 5×10^6 cells were lysed in 400 μl SDS buffer (0.5 M Tris-HCl, 10% glycerol, 10% w/v SDS, 2% 2-ME, 0.1% bromphenol blue). Supernatant proteins were precipitated with ice-cold acetone and resuspended in 400 μl SDS buffer. Samples were boiled for 10 min, electrophoresed by SDS-PAGE on 12% gels, and transferred onto nitrocellulose membranes (Amersham, Bucks, U.K.). Membranes were blocked overnight with 5% nonfat dried milk in 0.5% PBS/Tween 20, and exposed to anti-EMAP-II Abs R2B2, diluted in 0.5% PBS/Tween 20, for 2 h at room temperature. Proteins were visualized with HRP-conjugated goat anti-rabbit IgG (Sigma-Aldrich) using the ECL system (Amersham)

DNA synthesis

Freshly prepared PBMC were diluted to a final concentration of 2×10^6/ml in growth medium, and 1 ml of cells was added to each well of a 24-well plate (Nunc, Rochester, NY). To some wells, the mitogen PHA was added to a final concentration of 2.5 μg/ml. Plates were then incubated in an atmosphere of 5% CO_2/95% air at 37°C for up to 5 days. To assay DNA synthesis, 300 μl of medium containing resuspended cells was removed from each well and transferred to a 96-well plate (Nunc). To each well, 10 μl (1.85 kBq) [^{3}H]thymidine (Amersham) was added and the plate was returned to the incubator for 24 h. The contents of each well were harvested onto a UniFilter plate (Packard Instrument, Meriden, CT) and counted on a liquid scintillation counter (Packard Instrument). All assays were conducted in triplicate.

CFSE dye dilution assay for cell division

The method used was essentially that described by Lyons (14). PBMC were resuspended in serum-free RPMI medium before staining. To 9 ml fresh medium, 10 μl CFSE (5 mM stock solution in DMSO; Molecular Probes, Eugene, OR) was added, then immediately mixed thoroughly with 1 ml PBMC. The cells were incubated for 10 min at 37°C, centrifuged at 600 × *g*, and resuspended in RPMI medium containing 5% FCS. The wash step was repeated. PBMC were counted by hemocytometer and diluted to a final concentration of 2×10^6/ml. One milliliter of cell suspension was added to each well of a 24-well plate. The plate was incubated at 37°C, and the cells were harvested on days 3, 4, 5, and 6 of incubation. For analysis by flow cytometry, PBMC were resuspended and 1×10^5 cells were transferred from each well into an Eppendorf centrifuge tube. Cells were centrifuged for 30 s at 15,000 × *g*. The supernatants were removed and the cells were resuspended in 300 μl of PBS. The samples were analyzed for fluorescence in the fluorescein (FL1) channel using a FACScan flow cytometer (BD Biosciences, San Diego, CA), after gating on forward and right-angle scatter to electronically isolate the lymphocyte population, and presented as fluorescence histograms. The numbers of cell division were estimated from the number of peaks appearing at equally spaced intervals to the left of the control peak. The proportion of cells in each peak was also determined and divided by 2^n, where n is the number of divisions to which that peak corresponds. The sum of these terms represents the total starting population from which the dividing cells arose, and this number was used to calculate the expansion of the cell population.

Flow cytometric analysis of cell surface expression of EMAP-II

The expression of EMAP-II on the external membranes of tumor cells was determined by flow cytometry. Cells were incubated in RPMI medium with or without 20 ng/ml TNF-α/IFN-γ for 48 h. After removal of medium, cells were harvested without trypsin to reduce membrane damage, and fixed in 0.25% glutaraldehyde/PBS for 10 min to restrict access of Abs to cell surface Ag only. Cells were washed in PBS and incubated with either anti-EMAP polyclonal Abs (R2B2) or purified IgG from preimmune rabbit serum (R&D Systems) at 1 μg/ml for 2 h. Surface Ag expression was detected by subsequent incubation with FITC-conjugated secondary Abs (Sigma-Aldrich) for 30 min. Additional cell samples were incubated with the secondary FITC-conjugated Ab, but without primary Ab, as controls. The cells were immediately analyzed by flow cytometry. A total of 20,000 events was stored for each sample.

ELISA for soluble EMAP-II and FasL

Detection of soluble EMAP-II and FasL was performed after incubating confluent cells with or without TNF-α/IFN-γ (20 ng/ml each) diluted in serum-free RPMI medium for 48 h. Supernatant was withdrawn from the flasks and centrifuged at 600 × *g* for 5 min to pellet cellular debris. Recombinant EMAP-II, FasL, TNF-α, and IFN-γ were dissolved in RPMI medium to a final concentration of 50 ng/ml for use as external standards or negative controls. Samples were analyzed using human EMAP-II (BioSource International, Camarillo, CA) or soluble FasL ELISA kits (R&D Systems), in accordance with the manufacturers' instructions.

Assessment of apoptosis by FITC-labeled annexin-V and propidium iodide (PI)

Annexin-V binds to phosphatidylserine exposed on the external surface of the plasma membrane during the early phase of the apoptotic program, while DNA within the nucleus only becomes accessible to PI during late apoptosis. Therefore, these two reagents can be used together to distinguish early and late apoptosis. PBMC and Jurkat cells, following treatment with rEMAP-II or tumor cell-conditioned medium, or after coculture with tumor cell monolayers, were assessed for apoptosis. Untreated control cells and cells treated with 20 ng/ml TNF-α/IFN-γ were also prepared. Samples were immediately analyzed using the Apotest-FITC kit (DAKO, Glostrup, Denmark), in accordance with the manufacturer's instructions, by flow cytometry. Analysis of PBMC was restricted to the lymphocyte population by gating on forward and right-angle scatter. Twenty thousand events were acquired for each analysis.

Caspase 8 assay

A colorimetric assay based on the specific cleavage of the peptide substrate acetyl-Ile-Glu-Thr-Asp-*p*-nitroaniline (Ac-IETD-pNA) by caspase 8, releasing pNA, was used as directed by the manufacturers (Sigma-Aldrich). Caspase 8 activity was measured in extracts of control Jurkat cells, cells cocultured for 24 h with HT29 tumor cells, and cells cocultured with HT29 cells pretreated with TNF-α in the presence or absence of R2B2 Abs. A specific caspase 8 inhibitor Ac-IETD-Chinese hamster ovary (Ac-IETD-CHO) was included in matched assay samples.

Conditioned medium and coculture experiments

DLD-1 cells were grown for 48 h in serum-free RPMI medium, in medium supplemented with TNF-α/IFN-γ (20 ng/ml each), or in medium under hypoxic conditions ($\sim$1–2% O_2). Used culture medium was centrifuged at $2000 \times g$ for 5 min to remove cellular debris and stored at -80°C. Jurkat cells were pelleted, resuspended in conditioned medium, and grown in normoxia for 48 h, before analyzing for apoptosis, as described above. In some experiments, Jurkat cells (4×10^6) were cocultured with adherent monolayers of HT29 or DLD-1 colorectal cells in complete RPMI medium for 16 h. Before addition of Jurkat cells, tumor cells were either untreated, treated with TNF-α/IFN-γ (20 ng/ml), or treated with TNF-α/IFN-γ and 10 μg/ml R2B2 to block endogenous EMAP-II. We have previously demonstrated functional blocking with this concentration of R2B2 Abs (9). After 16 h, the nonadherent Jurkat cells were removed and centrifuged at $1000 \times g$ for 5 min, then resuspended in PBS. Samples were analyzed using the annexin-V/PI technique described above.

Statistical analysis

All analysis of FACScan data was performed using WinMDI software (TSRI Cytometry, La Jolla, CA). Analysis of ELISA data was performed with Microsoft Excel (Redmond, WA).

Results

Colorectal cancer cells express EMAP-II in vivo and in vitro

Initially, we examined expression of EMAP-II in archival specimens of colorectal carcinomas by immunohistochemistry. Fig. 1*A* shows the typical pattern of a section of normal bowel, with staining essentially restricted to the lumenal epithelium. We previously demonstrated a similar pattern of expression in the normal bowel by in situ hybridization and immunohistochemistry (13). Fig. 1*B* shows a moderately differentiated adenocarcinoma of the colon stained with the same Ab. There is strong cytoplasmic staining within the malignant epithelial cells, while the stromal component of the tumor remains essentially negative.

We then examined expression of EMAP-II in a range of cell lines derived from colorectal tumors, to determine whether expression persisted in vitro, and whether these cells retained EMAP-II within the cell, or released it into culture medium. For this purpose, we used the R2B2 polyclonal Ab, which we have previously shown detects the 34-kDa as well as other processed forms of EMAP-II by Western blotting (9, 12, 13). Fig. 2 shows a blot of extracts from DLD-1 and HT29 colorectal cancer cells. Both cell lines expressed EMAP-II within the cell, predominantly in the 34-kDa form (*lanes 2* and *4*). This is consistent with our observation that all human cells, both normal and neoplastic, express the 34-kDa form of EMAP-II intracellularly, as detected by Western blotting (data not shown). Growth medium conditioned by DLD-1 cells (*lane 3*) contained detectable amounts of a $\sim$18- to 20-kDa form of EMAP-II, normally associated with the extracellular space (12). Note that this form appears to have a lower mass than the rEMAP-II standard (*lane 1*). Conditioned medium from HT29 cells (*lane 5*), as well as that from the LS513, HCT-15, and Colo-205 colorectal cell lines (data not shown) did not contain detectable levels of soluble EMAP-II, although, as previously mentioned, the intracellular 34-kDa form could be detected in all these lines by Western blotting.

We next quantified the levels of EMAP-II Ag secreted by the colorectal tumor cells into culture medium (Fig. 3*A*). Consistent with the Western blotting data, DLD-1 cells secreted EMAP-II Ag, which reached a maximum concentration of $\sim$2 ng/ml ($\sim$0.1 nM) after 24-h incubation. As expected, HT29 cells (Fig. 3*A*) and the three remaining lines (data not shown) secreted barely detectable levels of soluble Ag. Treatment of DLD-1 cells with a combination of TNF-α and IFN-γ resulted in a $\sim$6-fold increase in release of EMAP-II Ag. Combination TNF-α/IFN-γ had no effect on HT29 or the other three cell lines. Similarly, exposure to hypoxia ($<$2% O_2) for 24 h resulted in a $\sim$4-fold increase in release of EMAP-II by DLD-1, but had no effect on the other lines. By ELISA, none of the colorectal cancer cell lines released FasL into the culture medium (data not shown).

Cell surface expression of EMAP-II on colorectal cancer cell lines and MRC-V normal human fibroblasts was examined by flow cytometry using R2B2 polyclonal Abs (Fig. 3*B*). Both DLD-1 and HT29 demonstrated cell surface expression of EMAP-II, showing increases in mean fluorescence over negative controls in the range of 3- to 4-fold (as did the other three tumor cell lines; data not shown). MRC-V fibroblasts showed no cell surface expression. The specificity of this staining was confirmed by the use of a preimmune Ab control, which showed no membrane staining in any of the cell lines. Treatment with the combination of TNF-α/IFN-γ caused a further $\sim$4-fold increase in mean fluorescence associated with R2B2 Ab binding to HT29 cells, but no change in the DLD-1 cells, which, as shown earlier, release EMAP-II into the culture medium in response to the cytokine treatment.

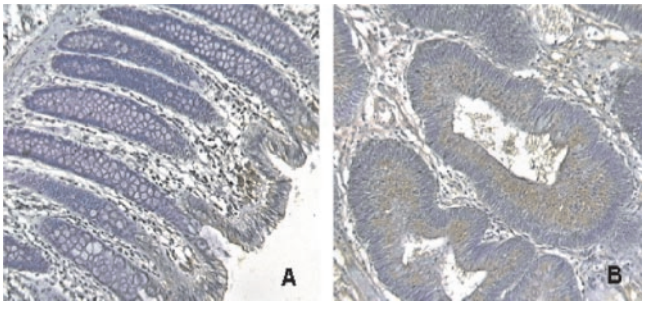

FIGURE 1. Colorectal cancers express EMAP-II in vivo. Immunohistochemical staining of archival specimens of *A*, normal colon, and *B*, a moderately differentiated adenocarcinoma of the colon with polyclonal anti-EMAP-II. Note slight brown staining of lumenal epithelial cells as well as some mononuclear cells in normal tissue, compared with more intense generalized staining of malignant epithelial cells in the tumor ($\times$20 objective).

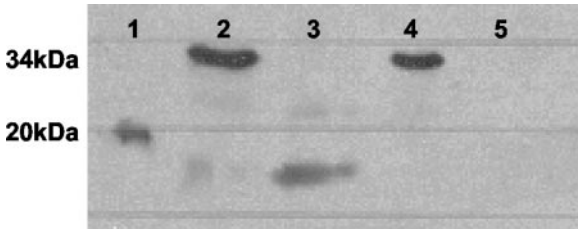

FIGURE 2. Colorectal adenocarcinoma cells express EMAP-II in vitro. Western blot of extracts of colorectal cancer cells and medium conditioned by cells, using R2B2 polyclonal Ab against EMAP-II. *Lane 1*, Recombinant EMAP-II; *lane 2*, DLD-1 cell extract; *lane 3*, medium conditioned by DLD-1 cells; *lane 4*, HT29 cell extract; *lane 5*, medium conditioned by HT29 cells.

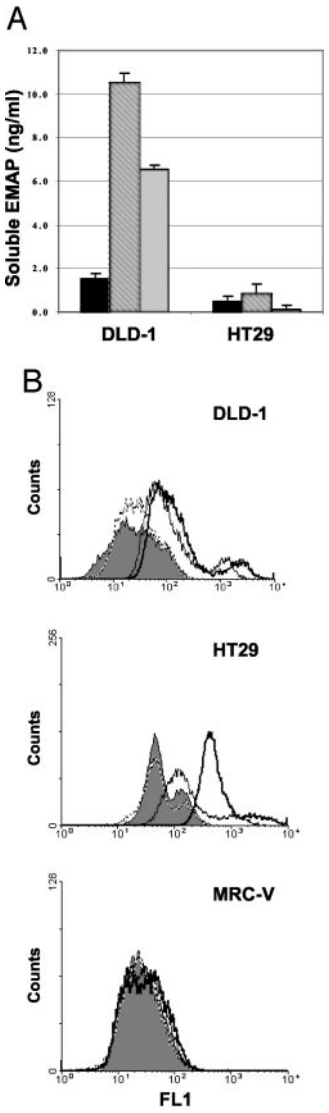

FIGURE 3. Colorectal cancer cell lines, but not normal human fibroblasts, express cell surface EMAP-II in vitro. *A*, ELISA of conditioned medium from colorectal cancer cells for soluble EMAP-II. ■, Untreated cells; ▨, cells treated with TNF/IFN; □, cells exposed to hypoxia for 18 h. Data represent mean of three determinations ± SD. *B*, Flow cytometric determinations of cell surface EMAP-II expression. Untreated DLD-1 and HT29 tumor cells, and MRC-V fibroblasts without primary Ab (filled areas); untreated cells incubated with preimmune Abs (dotted lines); untreated cells incubated with R2B2 Abs against EMAP-II (thin solid lines); cells pretreated with TNF/IFN and incubated with R2B2 Abs (thick solid lines).

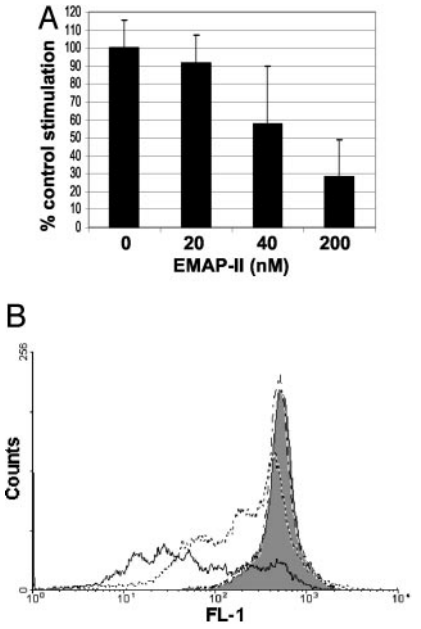

FIGURE 4. Recombinant EMAP-II inhibits DNA synthesis and cell division in PBMC. *A*, Recombinant EMAP-II inhibits [^{3}H]thymidine incorporation in PHA-stimulated PBMC in a dose-dependent manner. *B*, Recombinant EMAP-II inhibits cell division in PHA-stimulated PBMC. Lymphocyte division was assessed by CFSE dye dilution. Equally spaced fluorescent peaks to the *left* of the control peak indicate number of cell divisions. Filled area, control cells; filled line, PHA-stimulated cells; dotted line, cells treated with PHA and EMAP-II; dashed line, cells treated with EMAP-II alone.

Recombinant EMAP-II inhibits DNA synthesis and cell division in PBMC

We next examined the effects of EMAP-II on lymphocytes. Before investigating the effects of tumor cell-derived EMAP-II, we conducted experiments with rEMAP-II. Fig. 4*A* shows the effects of rEMAP-II on [^{3}H]thymidine incorporation by PHA-stimulated PBMC. There was a dose-dependent inhibition of incorporation at day 5 following treatment with rEMAP-II at day 0, with a half-maximal effect in the region of ~40 nM. This effect was also seen if EMAP-II was added to cultures at day 4, and DNA synthesis measured 24 h later (data not shown).

We examined lymphocyte division within PBMC preparations using the CFSE dye dilution technique (Fig. 4*B*). Whereas PHA stimulated approximately seven divisions within the lymphocyte population over a period of 5 days, in the presence of 100 nM rEMAP-II there was a clear reduction in the number of cell divisions. After PHA treatment alone, there was an almost 5-fold expansion of the cell population, with over half of the original cells dividing, and about one-third of the original cells dividing at least twice. In response to the combination of PHA and rEMAP-II, there was only a 1.5-fold expansion of the cell population, with about one-quarter of the original cells dividing, and only a small minority of the original cells dividing at least twice.

Recombinant EMAP-II induces apoptosis in PBMC and Jurkat T cells, but not in fibroblasts

To determine whether the apparent reduction in cell division reflected cell loss through apoptosis, we examined the viability of lymphocytes in PBMC preparations following treatment with PHA and rEMAP-II (100 nM). Fig. 5*A* shows a representative bivariate dot plot generated by flow cytometry using the annexin-V/PI technique. Early apoptotic cells appear in the *lower right*, and late apoptotic cells in the *upper right quadrants*. Recombinant EMAP-II had no effect on viability of lymphocytes in unstimulated PBMC preparations after 24-h exposure, because the sum of the percentages of cells in the *upper* and *lower quadrants* of the two panels is essentially unchanged (Fig. 5*A*, *a* compared with *b*). However, addition of rEMAP-II to PHA-stimulated PBMC preparations caused massive induction of lymphocyte apoptosis (Fig. 5*A*, *c* compared with *d*). PHA stimulation alone causes a rise in the proportion of apoptotic cell (Fig. 5*A*, *c* compared with *a*). We also examined the effect of rEMAP-II on Jurkat T cells, a leukemic cell line representing a model for T lymphocytes, and found that EMAP-II induced apoptosis in these cells without prior stimulation by mitogen. Fig. 5*B* shows a time course for the induction of

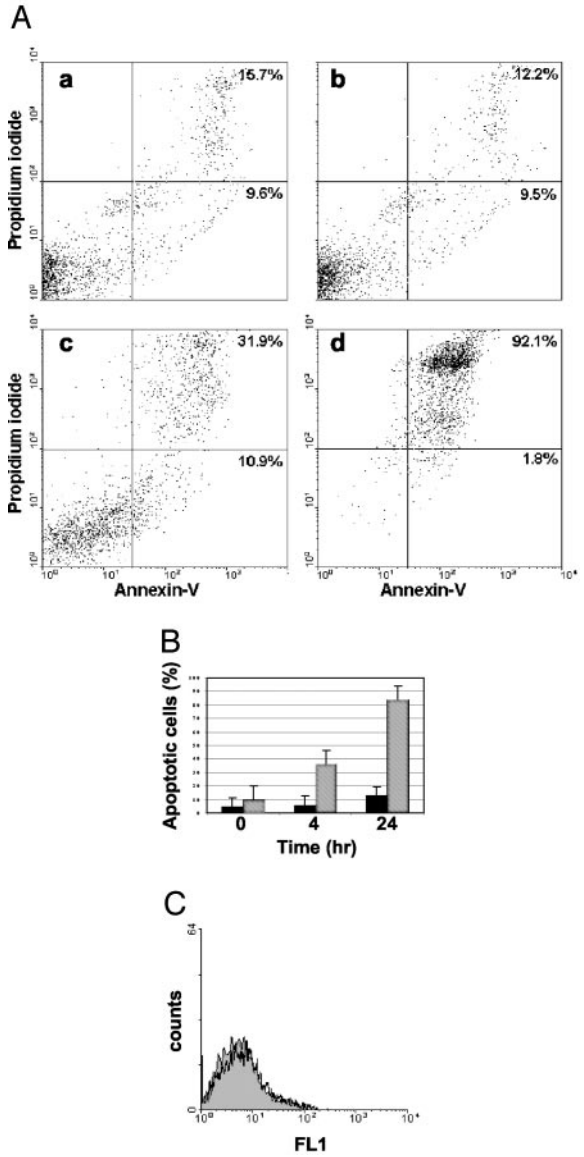

FIGURE 5. Recombinant EMAP-II induces apoptosis in PBMC and Jurkat T cells, but not in normal fibroblasts. *A*, Recombinant EMAP-II induces apoptosis in PBLs. *a*, Control cells; *b*, control cells treated with rEMAP-II (100 nM) for 24 h; *c*, PHA-stimulated cells; *d*, PHA-stimulated cells treated with EMAP-II (100 nM) for 24 h. *B*, Bar graph showing time course for effect of rEMAP-II (100 nM) on apoptosis in Jurkat cells. ■, Untreated cells; ▨, cells treated with EMAP-II. Data represent mean of three determinations ± 1 SD. *C*, Histogram showing annexin-V labeling of untreated MRC-V fibroblasts (filled area), and fibroblasts treated with 200 nM EMAP-II for 24 h (filled line).

apoptosis in Jurkat cells. Eighty-five percent of the Jurkat cells were in early or late apoptotic phase by 24 h of treatment with 100 nM EMAP-II, compared with 12% in the control population.

We also examined apoptosis in MRC-V fibroblasts at relatively high (200 nM) concentrations of EMAP-II. After 24-h exposure of monolayers of log-phase fibroblasts, there was no evidence of apoptosis or necrosis (Fig. 5*C*).

Having demonstrated that rEMAP-II induces lymphocyte apoptosis, and that colorectal cancer cells release EMAP-II into medium or express it on the external cell surface, we hypothesized that native EMAP-II produced by tumor cells might provide a protective mechanism against immune attack. We therefore examined the effects of conditioned medium from colorectal cancer cells on PBMC and Jurkat cell viability.

Tumor cell-derived soluble EMAP-II induces lymphocyte apoptosis in coculture

Fig. 6 shows a representative analysis of the viability of Jurkat cells exposed to medium conditioned by untreated DLD-1 tumor cells, or DLD-1 cells treated with combination TNF-α/IFN-γ, to enhance EMAP-II release. Conditioned medium from untreated DLD-1 cells has little or no effect on viability of Jurkat cells (Fig. 6*a*), whereas medium from cytokine-treated cells causes increased apoptosis (Fig. 6*c*). The cytokine combination alone induced some apoptosis of Jurkats (Fig. 6*b*), although much lower levels than conditioned medium, indicating that another soluble factor was responsible for the apoptosis. The effect of the conditioned medium could be partially reversed by prior addition of blocking Abs against EMAP-II (Fig. 6*d*), confirming that at least part of the apoptotic effect could be attributed to EMAP-II present in the DLD-1-conditioned medium. Exposure of Jurkat cells to conditioned medium from DLD-1 cells grown in hypoxia for 48 h also stimulated apoptosis (32% for hypoxia vs 3% for control), and this effect was partially reversed by the presence of Abs against EMAP-II. Conditioned medium had similar effects on PHA-activated PBMC, although no effects were seen with nonactivated PBMC (data not shown). Conditioned medium from HT29 cells, which do not express soluble EMAP-II (see Figs. 2 and 3*A*), had no effect on Jurkat cell or PBMC viability (data not shown).

Cell surface-associated EMAP-II induces apoptosis in Jurkat cells

As demonstrated above, HT29 cells express EMAP-II on the cell surface, although they do not release this protein into the medium. Surface expression is up-regulated by combination TNF-α/IFN-γ. Because the coculture model does not require previously conditioned medium, the HT29 cells can be exposed to cytokines to

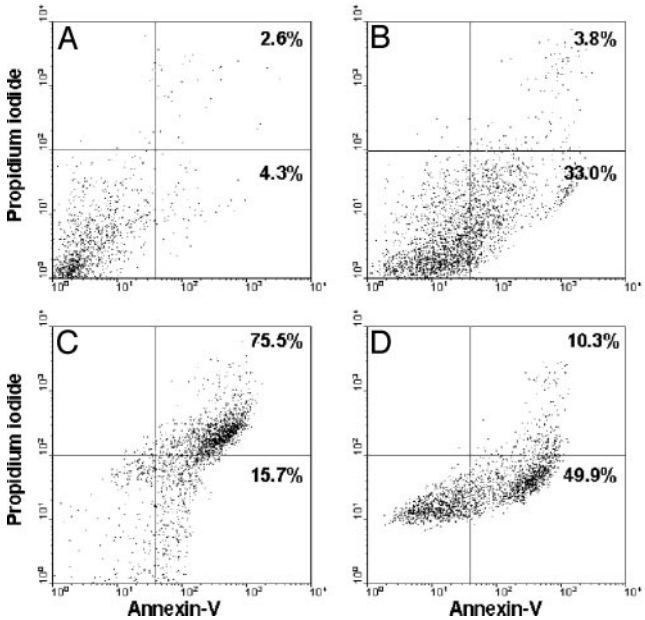

FIGURE 6. Medium conditioned by DLD-1 colorectal cancer cells induces apoptosis in Jurkat T cells. Jurkat cells were cultured for 24 h in untreated DLD-1 medium (*a*); untreated DLD-1 medium with TNF-α/IFN-γ (*b*), medium from DLD-1 cells pretreated with TNF-α/IFN-γ for 48 h (*c*), or medium from DLD-1 cells pretreated with TNF-α/IFN-γ and anti-EMAP-II for 48 h (*d*).

stimulate EMAP-II expression, and the medium exchanged before coculturing with PBMC or Jurkat cells. Therefore, at no time are the target Jurkat cells exposed to exogenous cytokines.

Fig. 7 shows a representative flow cytometric analysis of apoptosis in target Jurkat cells following 16-h coculture with control or cytokine-treated HT29 cells. There was induction of apoptosis in the Jurkat cells by treated HT29 cells (Fig. 7, *c* compared with *a*), and this effect was partially reversed by addition of blocking Abs against EMAP-II (Fig. 7*d*). These data suggest that, in addition to soluble EMAP-II present in conditioned medium, cell surface-associated EMAP-II induces apoptosis in activated lymphocytes.

HT29-induced apoptosis of Jurkat cells is associated with activation of caspase 8

Caspase 8 activity was assayed in extracts of control Jurkat cells and Jurkats cocultured under a variety of conditions with HT29 cells (Fig. 8). Jurkat cells alone showed low levels of pNA product, presumably not due to caspase 8 cleavage, as this was not inhibited by Ac-IETD-CHO. Coculture of Jurkats for 24 h with HT29 cells caused a 4-fold increase in production of pNA, which was completely inhibited by Ac-IETD-CHO. Coculture with HT29 cells pretreated with TNF induced a slight, but not significant increase in caspase 8 activity. Addition of R2B2-blocking Abs against EMAP-II to the coculture completely inhibited the generation of caspase 8 activity in Jurkat cells.

Discussion

The role of EMAP-II in normal and neoplastic tissue is poorly understood. It may play a chemotactic role for phagocytic cells and potentiate the antivascular effects of TNF-α (5), and induce apoptosis in proliferating endothelial cells (7, 8), thereby inhibiting neovascularization. Given that a defining characteristic of tumors is their propensity to stimulate angiogenesis, it is unclear why release of EMAP-II would confer any biological advantage. In contrast, tumor angiogenesis is often characterized by high turnover and apoptosis of endothelial cells during remodeling, which might

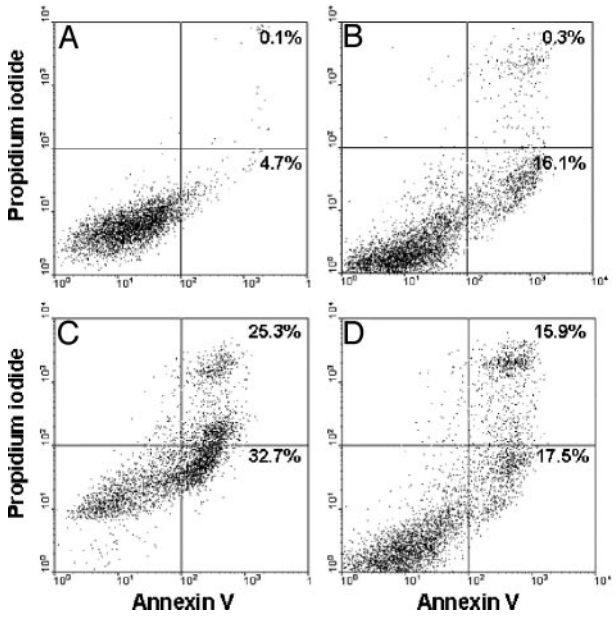

FIGURE 7. Tumor cell-associated EMAP-II induces apoptosis in Jurkat cells cocultured with HT29 tumor cells. Jurkat cells were cultured alone for 16 h (*a*), with untreated HT29 cells (*b*), with TNF-α/IFN-γ-treated HT29 cells (*c*), or with TNF-α/IFN-γ-treated HT29 cells and anti-EMAP-II (*d*).

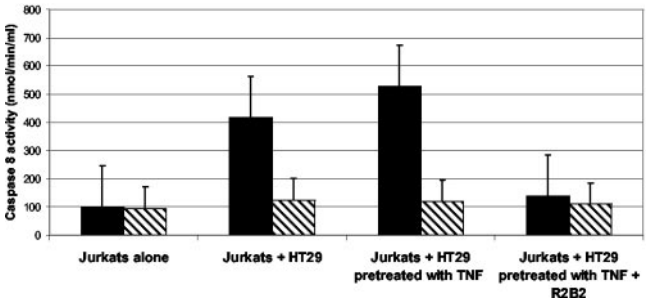

FIGURE 8. Caspase 8 is activated in Jurkat cells cocultured with HT29 tumor cells; activation is blocked by Abs against EMAP-II. Caspase 8 activity was measured in extracts of control Jurkat cells, cells cocultured for 24 h with HT29 tumor cells, and cells cocultured with HT29 cells pretreated with TNF-α in the presence or absence of R2B2 Abs (■). The caspase 8 inhibitor Ac-IETD-CHO was included in matched assay samples (▨) to demonstrate the specificity of the assay. Results represent the mean of three determinations + 1 SD.

suggest a role for EMAP-II. The data presented in this work provide evidence that physiologically relevant concentrations of EMAP-II also induce apoptosis in activated lymphocytes. Thus, a new and potentially important role for this protein may be to protect tumor cells from the activity of cytotoxic T cells.

Tumors may evade immune surveillance, or fail to stimulate an effective cell-mediated response, or both. The ability to subvert the cellular arm of the immune response has been attributed to a number of mechanisms, among which is the elaboration of cytokines and other soluble mediators, including TGF-β (15), IL-10 (16), immunosuppressive peptides related to the p15E retroviral membrane protein (17), and virally encoded chemokines (18, 19). EMAP-II may represent yet another soluble mediator that suppresses cytotoxic lymphocyte activity in the immediate tumor environment, protecting tumors from cell-mediated immunity.

EMAP-II has no sequence homology with TGF-β or IL-10. The N-terminal region of 20-kDa EMAP-II, where much of the cytokine activity is believed to reside (20, 21), has minor sequence homology with IL-1β, IL-8, and von Willebrand factor Ag II, and some structural homology with the chemokines monocyte chemoattractant protein-1 and RANTES (22). The processed form of EMAP-II also has significant homology with tyrosyl-tRNA synthetase (tyrRS), an enzyme involved in protein synthesis. TyrRS is a 59-kDa polypeptide composed of two distinct domains: a 39-kDa N-terminal domain, which is responsible for catalytic activity, and a 20-kDa C-terminal portion (C-domain). The C-domain shows >60% amino acid homology with EMAP-II and shares a number of cytokine-like biological activities in vitro, including chemotactic and TNF-potentiating activity (21, 23). Our preliminary experiments indicate that C-domain of tyrRS also has similar activity to that of EMAP-II against lymphocytes (unpublished data). We have also conducted an amino acid sequence alignment that demonstrates minor homologies shared by EMAP-II, tyrRS, and an immunosuppressive sequence found in the retroviral transmembrane protein p15E, strengthening the case for the lymphocyte-inhibitory activity residing within the N-terminal region of the processed/cleaved forms of this protein.

Unlike the potent immunosuppressive protein TGF-β, both soluble and cell membrane-associated EMAP-II appear to stimulate apoptosis in activated lymphocytes. Under normal conditions, the colorectal tumor cell lines produce either no, or very low levels of, soluble EMAP-II. Following stimulation with TNF/IFN or hypoxia, maximal levels of ~0.5 nM were achieved in conditioned

medium, which proved to be potent inducers of apoptosis in activated PBLs and Jurkat T cells. Recombinant EMAP-II, in contrast, had an IC_{50} for inhibition of lymphocyte DNA synthesis of ~40 nM. Although not directly comparable, the data suggest that rEMAP-II may lack the potency of the naturally processed molecule. An alternative explanation may be that tumor-conditioned medium contains other unidentified factors that interact with EMAP-II. Although TNF-α is known to synergize with EMAP-II in the induction of endothelial tissue factor (5), in the coculture experiments exogenous TNF-α is removed after the initial incubation period and before addition of Jurkat cells to the culture, minimizing any direct role for this cytokine.

This is the first description of biological activity related to a membrane-associated form of EMAP-II, about which nothing is known. We previously demonstrated that recombinant 34-kDa precursor and 20-kDa EMAP-II are equally active in some bioassays (12), which suggests that cell surface activity could be associated with either molecular form. However, as Western blots of washed cell extracts only demonstrate the 34-kDa form, it would appear that this is likely to be the major form associated with the external membrane.

The mechanism by which the 34-kDa form is processed to smaller forms and subsequently found in the supernatants of cells such as DLD-1 is poorly understood. The EMAP-II precursor lacks a hydrophobic signal peptide necessary for membrane translocation, nor is it predicted to be GPI anchored. It has been suggested that the mature molecule is secreted via a novel pathway, in a similar manner to the leaderless precursor of IL-1β, which is now known to be shed initially in the form of membrane microvesicles, subsequently appearing in the vesicle-free supernatant in its processed form (24). There is conflicting evidence concerning the susceptibility of EMAP-II to proteolytic cleavage: one study (25) suggests pro-EMAP-II is cleaved at a critical aspartate residue by caspase 7, although another more recent study (26) could not confirm this, and our own data suggest broader susceptibility, in particular to serine proteases (13). It therefore seems possible that the precursor may be cleaved via a number of pathways. The discrepancy in size between the EMAP-II species seen in the DLD-1-conditioned medium (18–20 kDa) and the recombinant protein may also indicate that processing at the surface of these cells involves a cleavage site other than that thought to be susceptible to caspase 7. We are currently attempting to obtain N-terminal sequence data for the various species observed.

In the DLD-1 cell line, and in a previous study of prostate cancer cell lines (12), we have shown that low oxygen levels can enhance EMAP-II release and conversion. Hypoxia is known to up-regulate the expression and release of matrix metalloproteinases and plasminogen activator-1 from tumor cells (27, 28), and these enzymes could potentially be involved in EMAP-II processing at the cell surface. EMAP-II was shown by flow cytometry to be present on the surface of the colorectal tumor cells in these experiments, and low levels of apoptosis were seen when these cells were cocultured with Jurkat cells. However, when the HT29 (but not DLD-1) colorectal tumor cells were pretreated with cytokines, there was a significant increase in surface expression of EMAP-II, without shedding of Ag, and a concomitant increase in apoptosis of Jurkat cells in the coculture. These data suggest that surface expression alone, without release into the medium, and subsequent cell-cell contact may be sufficient to render EMAP-II active against lymphocytes.

Another protein known to induce lymphocyte apoptosis is FasL. Both membrane bound (29, 30) and soluble forms (31) of FasL exist, and hypoxia has been shown to stimulate the metalloproteinase-dependent release of FasL from the cell surface (32). Furthermore, both forms of FasL have been implicated in the counterattack hypothesis (29), according to which protection against cell-mediated immunity is conferred on colorectal and other tumor cell lines by the induction of Fas-mediated cell death in lymphocytes. This hypothesis has proven highly controversial (33–35), and there are several reports questioning both the expression of FasL by tumor cells and its ability to stimulate apoptosis in lymphocytes (36, 37). A very recent study (38) suggests that lymphocyte apoptosis can be induced by FasL-containing microvesicles secreted by melanoma cells, and these authors suggest that this novel mechanism explains how FasL may be undetected on the surface of tumor cells, yet still be biologically active against lymphocytes. We did not detect soluble FasL by ELISA in conditioned medium from our colorectal tumor cell lines, nor have we been able to detect FasL in extracts of the same cells by Western blotting (data not shown), which would appear to rule out a role for tumor cell-derived FasL in our experiments. However, using cDNA expression arrays, we have recently observed enhanced *FasL* transcription in anti-CD3/anti-CD28-activated T cells treated with EMAP-II (unpublished data), raising the possibility that EMAP-II stimulates activation-induced cell death by a FasL/Fas-dependent autocrine or paracrine mechanism (33).

Berger et al. (39) have shown that EMAP-II up-regulates the expression of TNF-R1 (p55) on endothelial cells in vitro. TNF-R1 and Fas, both members of the TNF receptor family, are believed to be primary receptors for the initiation of death signals in lymphocytes (40). It is therefore possible that either or both may be involved in the initiation of apoptosis in lymphocytes by EMAP-II. Gastman et al. (41) have described a coculture model in which cells derived from head and neck cancers induce apoptosis in Jurkat cells, partly mediated by an unknown, non-FasL-dependent mechanism. Our data demonstrating activation of caspase 8 in Jurkat cells following coculture with HT29, which can be blocked by anti-EMAP Abs, strongly implicate a death receptor pathway in apoptosis initiated by EMAP-II.

In conclusion, EMAP-II can trigger apoptosis in activated lymphocytes. This protein could act directly through death receptors, which would group it with TNF, FasL, and TNF-related apoptosis-inducing ligand, or indirectly, inducing or potentiating one of these death ligand/receptor interactions. Furthermore, it appears that EMAP-II can induce apoptosis either through direct cell-cell contact, conferring a high degree of selectivity on the process, or by acting as a soluble mediator signaling cell death. Although the mechanism of EMAP-II-induced apoptosis remains unclear, it is apparent that this pathway is restricted to a limited number of target cells, including endothelial cells and activated lymphocytes. Elucidation of the receptor(s) for EMAP-II may facilitate a better understanding of the biological significance of this pathway. In the meantime, our results would appear to define a novel pathway by which tumor cells may eliminate attacking T cells.

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