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A Novel Tumor-Derived Mediator That Sensitizes Cytokine-Resistant Tumors to Tumor Necrosis Factor

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Therapeutic successes following treatment of murine tumors with tumor necrosis factor-α (TNF) have not been easily applied to clinical oncology because the concentrations of TNF required in humans induces systemic toxicity. This has led us to identify mediators which could sensitize tumors to the effects of TNF, permitting administration of lower doses and possible realization of the therapeutic potential of this cytokine. Our study reports the ability of a novel cytokine, endothelial-monocyte-activating polypeptide II (EMAP II), to sensitize initially resistant murine and human tumors to TNF-induced regression employing a murine model. Recombinant (r) EMAP II was purified from *Escherichia coli* transformed with a plasmid expressing mature EMAP II. The B16 melanoma, raised in C57BL/6 mice, or a human fibrosarcoma (HT-1080), grown in immunocompromised mice, was injected intratumorally with either vehicle or rEMAP II/heat-treated EMAP II (50–100 μg) followed by systemic TNF/heat-treated TNF (5 μg) and assessed for tumor volume, hemorrhage, and histologic appearance. Both the B16 melanoma and the HT-1080 human fibrosarcoma underwent thrombohemorrhagic and acute inflammatory changes concomitant with regression or significantly slowed growth after administration of intratumor EMAP II followed by systemic TNF. Omission or inactivation of either cytokine abrogated this effect. These results demonstrate that local treatment of certain tumors with EMAP II results in enhanced susceptibility to TNF-mediated induction of thrombohemorrhage and regression.

INTRODUCTION

The study of immunogenic murine tumors provides a means through which a host can mount an effective immune/inflammatory response resulting in destruction of the neoplastic lesion. Murine methylcholanthrene A (meth A)[1] fibrosarcomas are a well-studied example of such an experimental neoplasm; histologic appearance of the tumor indicates foci of host effector cells, zones of necrosis, and, at later times, spontaneous regression [1–4]. The striking response of meth A tumor vasculature following infusion of tumor necrosis factor-α (TNF) also distinguishes the neoplasm from normal host tissues. Intravenous administration of low amounts of TNF (3–5 μg) results in localized thrombosis, leukostasis, and hemorrhage in tumor neovessels leading to ischemic necrosis of the tumor [2–4]. These observations have led us to identify factors made by meth A tumor cells which have the capacity to induce activation of endothelial cells, monocytes, and polymorphonuclear leukocytes, which may explain the tumor thrombohemorrhage induced by TNF [5–8].

Our recent studies have led to the isolation and cloning of endothelial-monocyte-activating polypeptide (EMAP) II from conditioned media of meth A cells in culture [7, 8]. Analysis of the cDNA indicates that EMAP II is a novel polypeptide which is not a member of previous cytokine or growth factor families. EMAP II is synthesized as an ≈34-kDa precursor which is cleaved to an 18- to 22-kDa mature form, the latter released by meth A tumor cells. Our previous studies have shown that mature EMAP II activates endothelial cells (induction of tissue factor procoagulant activity, expression of leukocyte adherence molecules P-selectin and E-selectin), leukocytes (induction of chemotaxis and degranulation), and monocytes (generation of cytokines, tissue factor, and induction of cell migration) at picomolar concentrations. The presence of such a mediator in the bed of meth A tumors suggested that it could prime the vasculature for the exaggerated response to TNF displayed by these neoplasms. If this hypothesis was correct, we reasoned that local injection of EMAP II into the bed of a tumor initially not sensitive to TNF should prime the vasculature, resulting in subsequent sensitivity to low concentrations of TNF.

[1] Abbreviations used: meth A, murine methylcholanthrene A-induced fibrosarcoma; TNF, tumor necrosis factor-α; EMAP, endothelial-monocyte-activating polypeptide; r, recombinant; SCID, severe combined immunodeficiency.

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To test this concept required experiments with tumors that are relatively resistant to low doses of TNF, such as the B16 melanoma and HT-1080 human fibrosarcoma. This study reports the use of recombinant (r) EMAP II to sensitize tumors to the effects of subsequently administered TNF.

MATERIALS AND METHODS

Purification of rEMAP II. *Escherichia coli* (host HMS174[DE3]) transformed with a plasmid containing the coding sequence for mature EMAP II [8] were pelleted, frozen, and purified as described [7, 8]. EMAP II was then concentrated to the milligram range in phosphate-buffered saline. Endotoxin levels were estimated using the Limulus Amebocyte Lysate assay and found to be less than 0.15 EU/mg protein. SDS–PAGE [9] followed by Coomassie staining was employed to detect the purified protein and immunoblotting as described previously [10, 11]. Molecular weight markers (Bio-Rad) were electrophoresed in the gel simultaneously. Immunoreactive protein on blots was visualized by reacting the membrane sequentially with rabbit anti-mature EMAP II amino-terminal peptide IgG (0.1 μg/ml) followed by the alkaline phosphatase-conjugated anti-rabbit IgG (Sigma, St. Louis, MO).

Murine footpad model. Footpads of Balb/c mice (6–12 weeks) were injected with 30–40 μl of EMAP II in phosphate-buffered saline (50 μg), the same amount of EMAP II following heat inactivation by boiling for 30 min, or phosphate-buffered saline alone, as described [12–14]. After 6 hr, footpads were harvested, fixed in buffered formalin (10%), decalcified, and embedded in paraffin. Sections were stained with hematoxylin and eosin.

Treatment of tumors with EMAP II and TNF. B16/F01 melanoma cells, which form poorly immunogenic tumors [16], were used to produce tumors in C57BL/6 mice by intradermal injection of 1×10^6 cells into the dorsal skin of each animal. Human fibrosarcoma cells [17] were used to establish tumors in severe combined immunodeficiency (SCID) mice. When tumors reached 0.5–1.0 cm in diameter, they were directly injected using a 30-gauge needle with vehicle solution (phosphate-buffered saline with bovine serum albumin, 0.1%), EMAP II (50 and 100 μg in the vehicle, respectively, for B16 and human fibrosarcoma), or heat-treated EMAP II (50–100 μg). Alternatively, tumors were treated locally with EMAP II or heat-treated EMAP II followed by intravenous injection of TNF (5 μg) or heat-treated TNF (5 μg) 18 hr later. Purified, recombinant human TNF-α was generously provided by Knoll Pharmaceuticals (Whippany, NJ). Six hours after the injection, animals were sacrificed and tumors were excised for pathologic analysis after fixation in buffered formalin (10%). Where indicated, tumor volume was calculated from caliper measurements of length, width, and height, according to the formula for a spherical segment, $V = \pi h(h^2 + 3a^2)/6$, where h is the height of the segment, a is (length+width)/2, and V is the volume [17]. Tumor tissue was analyzed histologically following formaldehyde fixation and paraffin embedding by hematoxylin and eosin staining, and immunohistochemically using anti-rat fibrin antiserum [18] and alkaline phosphatase-conjugated secondary antibody to visualize sites of binding of the primary antibody.

Statistical analysis. A repeated measures ANOVA [37] was used to analyze the tumor-volume data. A logarithmic transformation on the tumor variable "volume" was used to normalize the data to satisfy ANOVA's assumption of normality. The null hypothesis that the group-over-time interaction (i.e., that the volume changes across time are the same for subjects in both groups) was tested by Hotelling's t^2 test—the multivariate analogue of Student's t test for two independent groups.

RESULTS

Characterization of rEMAP II. rEMAP II used in these studies was homogeneous on reduced and nonreduced SDS–PAGE (Fig. 1A), displaying $M_r \approx 20$ kDa. That this material with the expected apparent molecular weight on SDS–PAGE was EMAP II was further demonstrated by immunoblotting with antibody raised to EMAP II (Fig. 1B). Material derived from both reduced and nonreduced SDS–PAGE, following electrophoretic transfer to nitrocellulose membranes, showed a single immunoreactive band corresponding to migration of the major protein band.

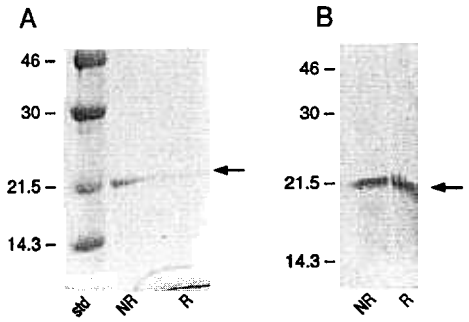
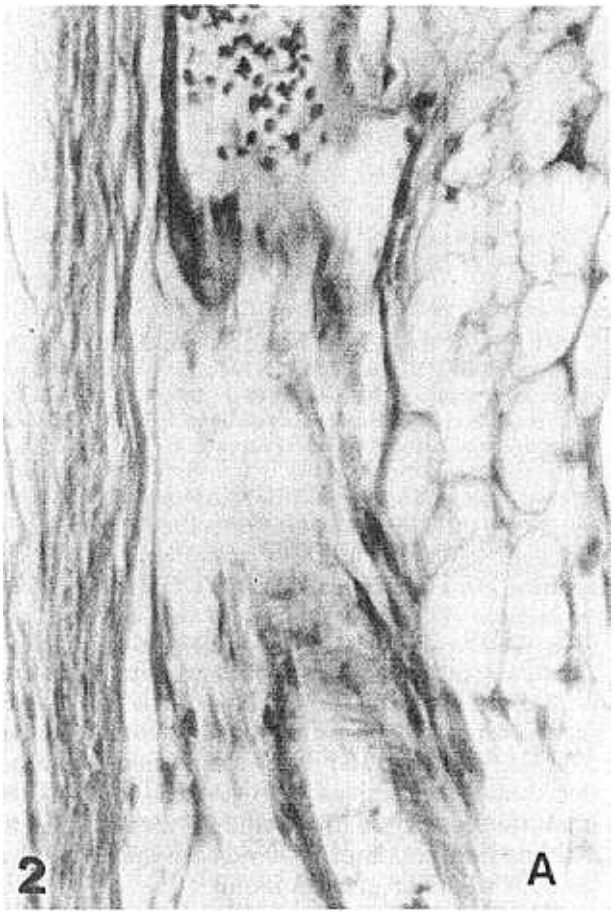


FIG. 1. (A) SDS–PAGE and (B) immunoblotting for characterization of rEMAP II. (A) rEMAP II (10 μg/lane) was subject to reduced (R) or nonreduced (NR) SDS–PAGE (12%), and protein in the gel was visualized by Coomassie blue staining. (B) rEMAP II (10 μg/lane) was subject to reduced or nonreduced SDS–PAGE (12%); material in the gel was electrophoretically transferred to nitrocellulose and immunoblotted with anti-EMAP II IgG. Migration of simultaneously run molecular weight markers is shown: lysozyme, 14.3 kDa; trypsin inhibitor, 21.5 kDa; carbonic anhydrase, 30 kDa; and ovalbumin, 46 kDa. The arrow indicates the band corresponding to EMAP II.

Host response elicited by rEMAP II in the murine footpad model. Murine footpads have been used as a model to characterize the inflammatory and edematous response to a range of inflammatory agents [12, 13]. In a previous study, we demonstrated that EMAP II derived from meth A-induced murine fibrosarcomas elicited swelling and inflammation in the murine footpad [14]. As an initial check on the *in vivo* activity of preparations of rEMAP II, its effects were tested in this model. Footpads injected with rEMAP II demonstrated vascular congestion with leukostasis, tissue infiltration by leukocytes, and edema (Fig. 2B) compared with controls receiving the same amount of heat-inactivated rEMAP II (Fig. 2A; appearance of these footpads is identical to untreated or phosphate-buffered saline-treated controls, data not shown). The inflammatory response appeared to last about 24 hr, was accompanied by considerable soft tissue swelling and erythema, and then resolved completely (data not shown).

Effect of rEMAP II on tumors resistant to TNF-induced thrombohemorrhage. EMAP II was first isolated from meth A tumors, which are well known for their elicitation of an inflammatory/immune response resulting in a heterogeneous appearance, with areas of inflammation and necrosis [4]. Administration of a low dose of systemic TNF to mice bearing meth A tumors results in thrombohemorrhage, which has been characterized in detail and begins with evidence of thrombosis and leukostasis in tumor vasculature [3, 4]. In view of the potent effect of EMAP II to activate endothelial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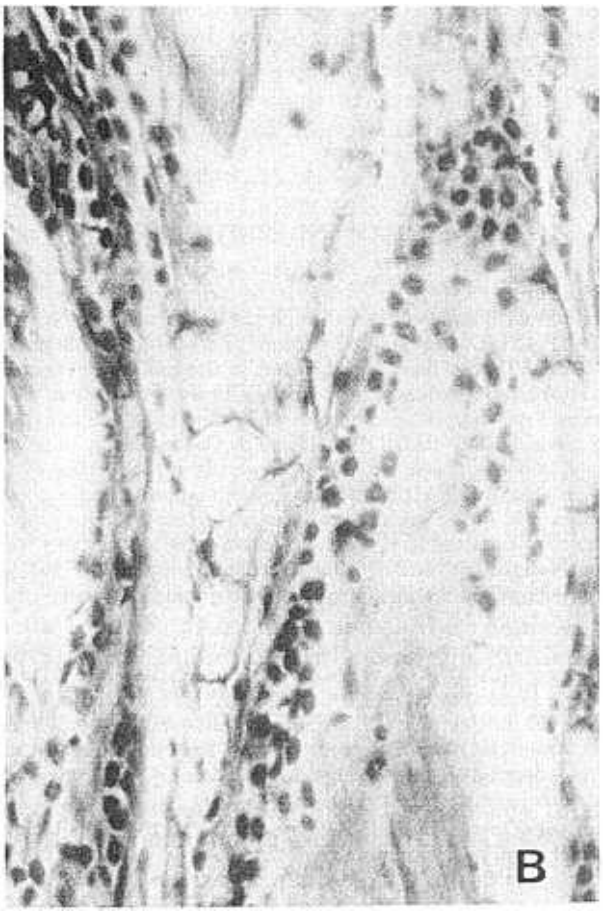
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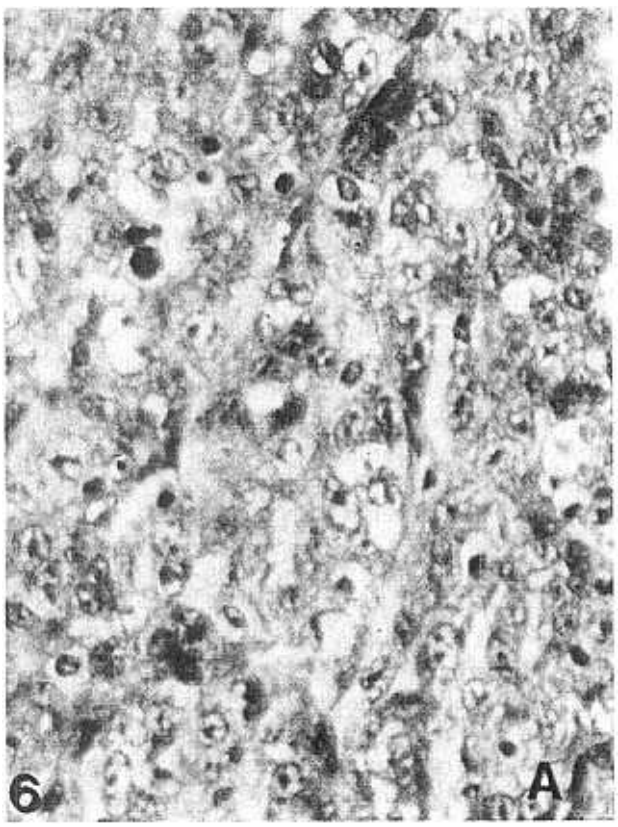
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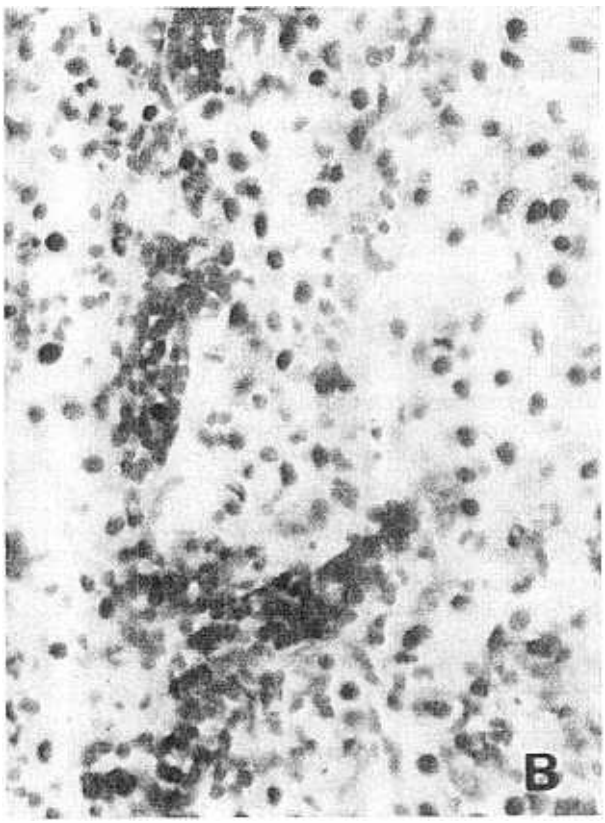
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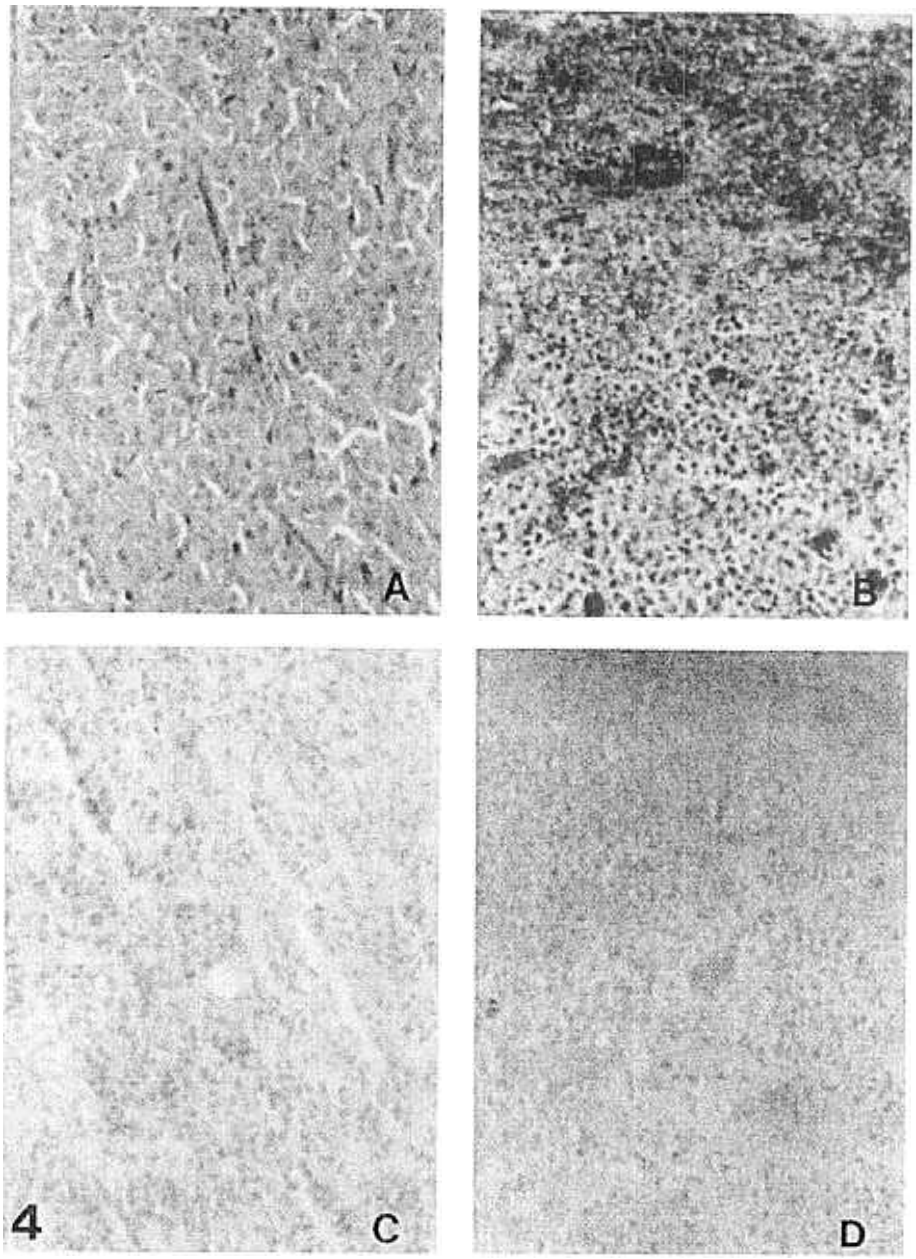

FIG. 4. Effect of rEMAP II and TNF on B16 melanomas: Microscopic appearance of tumors after cytokine treatment. (A) Tissue from a representative tumor treated by local injection of vehicle (100 μl) followed by intravenous TNF (5 μg; 100 μl). (B) Tissue from tumor treated by local injection of rEMAP II (50 μg) followed by intravenous TNF (5 μg). (C and D) Samples from the same tumors, A and B, respectively, now stained with anti-fibrin antibody. Magnification, 100×.

FIG. 2. Proinflammatory properties of rEMAP II in the mouse footpad model. Heat-treated (A) EMAP II (100 μg) or (B) EMAP II (100 μg) was injected into the mouse footpad, and 5 hr later the tissue was harvested, fixed, and stained with hematoxylin and eosin. Magnification, 180×.

FIG. 6. Effect of rEMAP II and TNF on human fibrosarcomas: Microscopic appearance of human fibrosarcomas after cytokine treatment. (A) Tissue from a representative tumor treated by local injection of vehicle (100 μl) followed by intravenous TNF (5 μg; 100 μl). (B) Tissue from tumor treated by local injection of rEMAP II (100 μg) followed by intravenous TNF (5 μg). Magnification, 180×.

cells, mononuclear phagocytes, and polymorphonuclear leukocytes *in vitro* and its phlogogenic properties in the murine footpad (see above), we considered the hypothesis that EMAP II-induced inflammation might exert a negative effect on tumor growth. We reasoned that a tumor bed primed by the presence of EMAP II (with activated vasculature and inflammatory effector cells) might be tipped to overt thrombohemorrhage when systemic TNF was subsequently given. This hypothesis was tested in murine models using the murine B16 melanoma in C57BL/6 mice, and in the HT-1080 human fibrosarcoma in SCID mice.

Mice injected with B16 melanoma cells were allowed to establish tumors to a size of 0.5–1.0 cm in diameter. At this point, tumors were injected once with either rEMAP II (50–100 μg) or vehicle alone intratumorally,

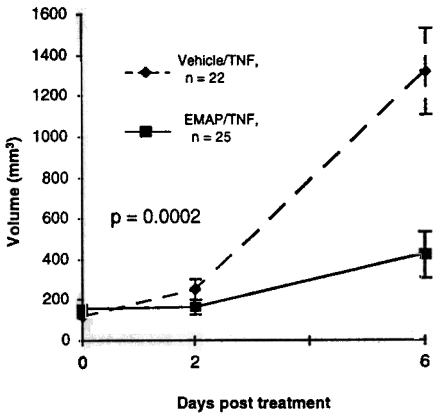

FIG. 3. Effect of rEMAP II and TNF on B16 melanomas: Changes in tumor volume. B16 melanomas were implanted into C57BL/6 mice and allowed to reach a size of 0.5–1.0 cm in diameter. Tumors were then injected locally with a single dose of either rEMAP II (50 μg; 100 μl, $n = 25$) or vehicle alone (100 μl, $n = 22$) followed by intravenous injection of TNF (5 μg; 100 μl). Tumor volume was determined at the indicated times. The P value was determined for the group-over-time interaction by repeated measures ANOVA (Hotelling's t^2 test).

and no effect on tumor volume was detectable. In order to test our hypothesis, tumor-bearing mice were primed for TNF-induced tumor regression by local administration of EMAP II using the following protocol: local injection of either rEMAP II (50–100 μg) or heat-treated rEMAP II (50–100 μg) followed 18 hr later by systemic TNF (5 μg) or heat-treated TNF (5 μg). Only animals receiving the combination of active rEMAP II and active TNF displayed significant suppression of tumor growth (Fig. 3). Note that control animals receiving only one dose of cytokine, heat-inactivated cytokine, or saline alone demonstrated tumor growth comparable to the group designated as "vehicle/TNF" in Fig. 3. No systemic toxicity was observed in mice receiving any of these regimens of rEMAP II and TNF. Histologic studies showed that the initially homogeneous B16 melanoma (Fig. 4A) developed extensive areas of thrombohemorrhage and an acute inflammatory infiltrate in tumor-bearing mice receiving EMAP II/TNF (Fig. 4B). Immunohistologic studies using an antibody selective for rodent fibrin displayed virtual lack of immunoreactivity in untreated tumors (Fig. 4C) and diffuse fibrin staining which colocalized with an area of thrombohemorrhage in treated tumors (Fig. 4, compare D and B).

A major obstacle in extrapolation of murine data demonstrating TNF-induced tumor thrombohemorrhage and regression to humans has been that the higher concentrations of TNF required result in overt systemic toxicity [19, 20]. One approach to this problem has been development of an isolated limb perfusion system in which high concentrations of TNF can be administered without spilling into the systemic circulation [21, 22]. We hypothesized that another means of dealing with this problem would be sensitizing tumors to the effects of TNF by local pretreatment with rEMAP II. Our initial experimental approach was to raise the human fibrosarcoma (HT-1080) in SCID mice, and then to follow the same protocol as with the murine B16 melanoma above, i.e., intratumoral injection of EMAP II followed by infusion of systemic TNF (Fig. 5). Only tumors receiving active rEMAP II followed by TNF displayed suppression of tumor growth, in Fig. 5, compared with tumors receiving vehicle followed by TNF. The same results were observed in other control groups in which rEMAP II was replaced by heat-inactivated rEMAP II, tumors received only vehicle, or no treatment was administered. Histologic analysis of human fibrosarcomas treated with rEMAP II/TNF demonstrated extensive loss of tumor cells, hemorrhage, and an inflammatory infiltrate (Fig. 6B) compared with controls (Fig. 6A; the same group shown in Fig. 5). No systemic toxicity was observed with any of these treatment regiments using rEMAP II and/or TNF.

DISCUSSION

The murine methylcholanthrene A-induced fibrosarcoma and other immunogenic tumors have proven to be extremely useful in characterizing facets of host–tumor interactions. The consistent thrombohemorrhage, exaggerated response to agents such as TNF and flavone acetic acid, and eventual immunologic-mediated regression of these tumors indicate that meth A tumor cells prime host immune/inflammatory mechanisms in a manner quite different from neoplasms which successfully evade protective host effector pathways [1–4, 23–28]. This led us to speculate that mediators made by the meth A tumor might sensitize endothelium in the tumor to the action of other inflammatory and immune stimuli, the latter modulating properties of neutrophils, monocytes, and lymphocytes. Based on this hypothesis, we analyzed supernatants of

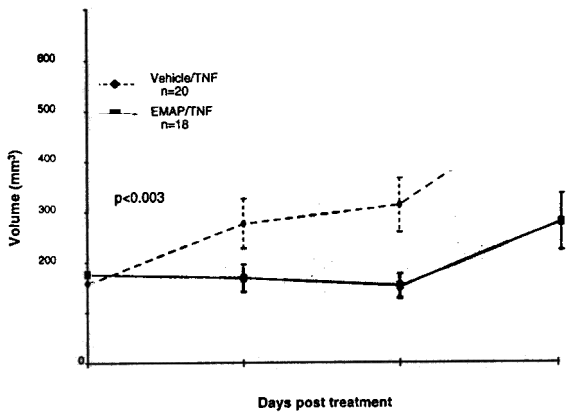

FIG. 5. Effect of rEMAP II and TNF on human fibrosarcomas: Effect on tumor volume. Human fibrosarcomas, raised from HT-1080 cells, were implanted into SCID mice and allowed to reach a size of 0.5–1 cm in diameter. Tumors were then injected locally with a single dose of either rEMAP II (100 μg; 100 μl, $n = 18$) or vehicle alone (100 μl, $n = 20$) followed by intravenous injection of TNF (5 μg; 100 μl). Tumor volume was determined at the indicated times. The P value was determined for the group-over-time interaction by repeated measures ANOVA (Hotelling's t^2 test).

meth A tumor cells for the presence of agents which would activate endothelium, inducing expression of the procoagulant cofactor tissue factor, increasing vascular permeability, and leading to expression of cell adherence molecules. Three polypeptides were characterized from meth A-conditioned medium: EMAP I, a 40-kDa polypeptide; the murine homolog of vascular endothelial growth factor; and EMAP II [5–7]. As EMAP II was a very potent perturbant of endothelial cell function and had an unique N-terminal sequence, our work has focused on its further characterization [7, 8].

Molecular cloning indicated that the N-terminal sequence of EMAP II purified from meth A-conditioned medium actually arose from an internal portion of the molecule [8]. The deduced amino acid sequence predicted an ≈34-kDa proform of EMAP II which would be cleaved to yield an 18- to 22-kDa mature form with the predicted N-terminal sequence. This situation was reminiscent of interleukin 1β, especially in view of the presence of an aspartic acid residue at the P1 position in EMAP II which suggested it might be a substrate for interleukin 1β-converting enzyme (or a member of the emerging related family of sulfhydryl-dependent proteases) [8]. However, EMAP II displayed an unique sequence and only very limited homology to other cytokines. The current study, employing recombinant, mature EMAP II, demonstrates that this material has the capacity to stimulate an acute inflammatory response and to prepare a local site for a subsequent exaggerated response to systemic TNF. The histologic picture of the tumor bed after administration of EMAP II, followed by systemic TNF, indeed shares features with that observed in the Shwartzman reaction [28, 29]. This leads us to hypothesize that local production of EMAP II may serve as a preparatory stimulus, which then promotes the triggering of a destructive inflammatory response when TNF, the provocative stimulus, is administered. Such a two-hit model could explain the consistent localization of TNF effects to the tumor bed in meth A tumors; i.e., although intense thrombohemorrhage is occurring in the neoplasm, no evidence of thrombosis or other systemic toxicity is observed in normal tissues. This also suggests why other tumors may show apparent resistance to TNF at doses not affecting the normal organs; without local production of a mediator which primes the tumor bed, high concentrations of cytokine are required which would have an equal affect on normal and tumor tissues.

These data also suggest the importance of understanding the molecular basis of EMAP II's effects on cellular targets, especially in relation to TNF. In this context, we have exploited the biologic activity of N-terminal peptides from mature EMAP II to perform radioligand-binding studies [14]. Binding of ^{125}I-labeled EMAP II-derived peptides was dose-dependent and saturable. Cross-linking studies with disuccinimidyl suberate indicated that ^{125}I-EMAP II peptide specifically bound to monocytes became associated with an ≈73-kDa polypeptide. Competition studies demonstrated that unlabeled EMAP II-derived peptide and full-length EMAP II were effective competitors, though murine TNF-α and interleukin 1α were not. Taken together, these data suggest that the cell surface recognition site for EMAP II is likely to be distinct from that for TNF, and have led to ongoing studies with the goal of isolating the EMAP II receptor. Thus, at the present time we do not have a molecular explanation for EMAP II-induced enhancement of the TNF response of tumors, though it appears that the receptors for these two cytokines are distinct, and we have no knowledge of a direct interaction between these two molecules. One important possibility for such an interaction concerns the potential for EMAP II to induce changes in expression of TNF receptors, an issue currently being explored.

The exploitation of a local immune/inflammatory response has precedent in prior work with anti-ganglioside antibodies which show selective binding to melanomas [30]. Administration of the antibodies resulted in a local inflammatory reaction at the site of their binding in the tumor bed, speculated to underlie tumor destruction. Another approach might also be considered with EMAP II, especially in view of the biologic activity of a peptide from the N-terminus of mature EMAP II [14]. We have found that a fragment as short as a heptapeptide from the N-terminus of mature EMAP II activates polymorphonuclear leukocytes [14]. It is possible that conjugation of such a peptide to an appropriate immunogenic carrier, and its subsequent injection along with irradiated tumor cells, might stimulate an inflammatory response promoting tumor destruction, as has been observed in studies in which tumor cells were transfected to express immunostimulatory and inflammatory cytokines and administered locally to induce regression of the primary tumor [31–34].

One puzzling aspect of the biology of EMAP II concerns why a tumor would make such a molecule which does not appear to positively impact on tumor survival. The production of inflammatory cytokines by tumors is often balanced by expression of anti-inflammatory mediators, such as interleukin 10 [35] or soluble cytokine receptors [36], which prevent the cytokines from gaining access to cell-associated receptors and evoking pathologic responses. In contrast, EMAP II activity was easily detectable in supernatants of meth A cells (where it is present at very low levels), and these conditioned media effectively activated host effector cells. This leads us to speculate that EMAP II could be a potent agent in settings outside of tumor biology where it might underlie inflammatory changes in a variety of tissue beds. Alternatively, it is possible that EMAP II has other properties, yet to be identified, that do provide an advantage for the growth/spread of tumors.

The current studies in a murine model indicate that therapy of murine and human tumors with EMAP II has the potential to impact negatively on tumor growth. Further studies will be required under a range of conditions and with multiple tumors to determine if these effects can be extrapolated to cancer patients. An important issue to explore will be identification of

tumors and nonmalignant tissues which express EMAP II. In this context, we have not found expression of EMAP II at any significant level in normal murine tissues and are exploring what stimuli might bring about its expression. The studies presented in this article provide a next step in understanding the biology of EMAP II and suggest the importance of future work to establish the basis of its cellular interactions, especially to map its cellular receptors, in order to exploit its properties for potential future therapeutic use.

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