Abstract. In 1990 Clauss et al. first reported on a 44-kDa polypeptide, later called Endothelial Monocyte Activating Polypeptide II (EMAP II). This protein was discovered in the supernatant of Meth-A fibrosarcoma cells and was shown to enhance the induction of the procoagulant Tissue Factor (TF) on endothelial cells. Besides up-regulation of TF mRNA, EMAP II increases cellular receptors for TNF on endothelial cells, which is likely to enhance the predisposition of tumors to undergo thrombosis and hemorrhagic necrosis, once challenged with TNF. This feature enables EMAP II to up-regulate the TNF sensitivity of TNF-resistant tumors, an observation of importance in developing new approaches aimed at improving the efficacy of TNF as an anticancer treatment. We describe the potential additional effects of EMAP II, when used in combination with TNF, with regards to antitumor activity in the Isolated Limb Perfusion (ILP) setting. In addition, we describe our experimental data in human sarcoma, which also supports this hypothesis.

Angiogenesis, the formation of new vessels from the endothelium of the preexisting vasculature, is a key element in a large number of normal and pathologic processes (1). When focusing attention on pathologic processes, angiogenesis can be demonstrated to play a crucial role in tumor development. Studies by Folkman (2) and other groups (3) have proven that production of proangiogenic factors by tumor cells is a prerequisite for tumor growth beyond a certain size, ranging from 0.2 mm diameter (in murine lung metastasis models) to a maximum of 2 mm diameter (in an a-vascular chondrosarcoma in the rat) (4). Further, it is known that tumors with greater capability of developing neovascularure are more lethal by means of growth velocity and metastatic potential (5-12). These observations have further strengthened the idea that antiangiogenic compounds may supply an additional treatment to slow down or inhibit the growth of primary solid tumors and their metastasis (13-15).

Neovascularisation of a tumor requires that the tumor switches to the angiogenic phenotype by changing the balance of angiogenesis inducers and countervailing inhibitors (16). At least two general mechanisms are recognized: (1) angiogenic activity arises from the tumor cells themselves through release of angiogenic molecules; (2) angiogenic activity arises from host cells recruited by the tumor (e.g. macrophages) through mobilization of the extracellular matrix, requiring concomitant loss of physiological inhibition of endothelial cell proliferation. The mechanism underlying shifts in the balance between all angiogenic regulators remain not fully understood.

Malignant cells communicate with their surrounding tissue and matrix by means of a large variety of signals which they produce. Interleukines, CAM’s (Cellular Adhesion Molecules), chemokines, integrins, proteases and receptors can all exert an effect on stroma, tumor matrix, tumor vasculature and pre-existing vasculature. This fast expanding list of pro- and antiangiogenic factors supports the hypothesis that the tumor itself is responsible for the process of angiogenesis. Cytokines from various sources are released in the vicinity of the tumor in response to hypoxia or ischaemia, and can rapidly initiate angiogenesis. Tumors can produce a large number of cytokines (17), all of them having different functions in the regulatory pathway of angiogenesis. Some have mitogenic effects on endothelial cells, others have chemotactic activity or induce tube formation or even have multiple functions. Some cytokines
are chemotactic for macrophages, infiltrate the tumor and stimulate secretion of more angiogenic cytokines. There is hope that these cytokines will be of major importance in the next decade in the field of antiangiogenic therapy. Therapies based on antiangiogenic strategies aimed at malignancies continue to evolve and clinical trials are being conducted (18). More than a decade has passed since Claus (19) first described a unique cytokine that was excreted by certain tumors, called EMAP II by later authors (20). Here we will discuss the role of this cytokine, its antiangiogenic properties and the possible co-existing impact on vascular damage when combined with TNF, leading to destruction of established tumors and inhibition of tumor growth.

**TNF and the isolated limb perfusion**

TNF is one of the cytokines currently used as an anticancer therapy. The systemic administration of TNF in cancer patients is associated with dose-liming toxicity already at low and ineffective doses without antitumor effects. The effective delivery of high concentrations of TNF in the clinic was pioneered by Lejeune and Lienard with the application of TNF in isolated limb perfusions in 1988, and led to a report of high complete responses in melanoma and sarcoma patients (21). In a multi-center European trial, ILP with TNF and melphalan (with (22) or without (22) IFN-gamma) resulted overall in a 76% response rate and a 71% limb salvage rate in patients with limb-threatening soft-tissue sarcomas (24). TNF-based ILP has been established as a highly effective new method of induction biocemotherapy in extremity soft tissue sarcomas with a 20-30% complete remission (CR) rate and an approximate 50% partial remission (PR) rate. On the basis of these results in the multi-center program, TNF was approved and registered in Europe in 1998. In the mean time, TNF-based ILP programs for limb salvage have been commenced in more than 30 cancer centers in Europe (22). High-dose TNF destroys tumor vasculature and it increases tumor-selective drug uptake (e.g. melphalan, doxorubicin) 3- to 6-fold (25,26). This latter observation makes it of critical importance to have as many TNF receptors as possible on the target tumor endothelium. Existing experimental data in animal studies suggest that EMAP II may add to this requirement.

**EMAP II: Identification and background**

Endothelial Monocyte Activating Polypeptide II (EMAP II) is a cytokine first identified as a tumor-cell-secreted protein when it was isolated from the supernatant of a murine methylcholantrene A (methyl-A) fibrosarcoma (27,28).

Based on the diversity in responses of tumors to TNF, together with the fact that the TNF actions seem to be vasculature-mediated, the search for soluble mediators secreted by neoplastic cells was started. Kao et al. (27) focused on the Meth-A tumor cells, a tumor sensitive to TNF in vivo, but TNF-resistant in vitro (29). By directing a pilot study to possible factors influencing this effect, they found two polypeptides in the supernatant of Meth-A tumor cells that modulated monocyte function in vivo, leading to the acronym EMAP I and II. The latter soon proved to be the most potent in influencing TNF-mediated thrombosis in tumors (30).

EMAP II mRNA and the corresponding precursor protein, proEMAP, are constitutively expressed and produced by all cell types analyzed in vitro, whereas the mature cytokine is only present in the supernatant of apoptotic cells (31). EMAP II (or Meth-A factor) is an approximately 18 kDa pro-inflammatory cytokine, synthesized as a precursor protein lacking a conventional secretion signal peptide (28). Despite the cytokine activity of the mature EMAP II, no sequence homology is found based on amino-acid sequence with any other known cytokine (32). The sequence of several proEMAP-related proteins, however, suggests that the p43 component of the aminoacyl-tRNA multienzyme synthetases complex is the precursor of the active mature cytokine (33). Aminoacyl-tRNA synthetases are proteins that catalyze the activation of their cognate amino acids and transfer to the relevant tRNA. This complex consists of a subset of 9 synthases all specific for their own amino acid, together with three auxiliary proteins (34). One of those three auxiliary proteins turned out to be proEMAP (35). Wakasugi has shown (36) that human full-length Tyrosyl-tRNA synthetase (TyrRS), which normally resides in the cell cytoplasm, was secreted by a human hematopoietic cell line that had been triggered by serum deprivation. TyrRS is inactive as a cell-signaling molecule but intracellular TyrRS has enzymatic activity and, after cleavage into two fragments by extra cellular proteases, both fragments surprisingly appear to have retained their cytokine activity. The carboxyl-terminal fragment happens to show some homology with EMAP II, and is able to stimulate leukocyte and monocyte chemotaxis, to induce myeloperoxidase, and to excite tissue factor and tumor necrosis factor synthesis. The amino-terminal fragment behaves like cytokine IL-8, yet still retains complete TyrRS activity. This and other findings established the fact that the precursor of EMAP II is a multi-functional protein that assists in protein synthesis in normal cells. Upon apoptosis it is released as functional cytokine, linking cell viability to programmed cell death (37).

The mechanism of cleavage and secretion of EMAP II has been controversial. Knies et al. suggested that the coordinate program of cell death includes activation of a caspase-like activity that initiates the processing of a cytokine responsible for macrophage attraction to the sites
of apoptosis (38). This same group of researchers soon identified, with in vitro experiments, that caspase-7 and, to a lesser degree, caspase-3 is capable of cleaving proEMAP to a fragment corresponding to mature EMAP II (39). However Zhang and Schwarz, who could not manage to get the proEMAP cleaved by caspases-3 or -7, argue against these results (40). They suggest a more important function for EMAP II with regard to induction of apoptosis and are currently focusing on the protease that can be held responsible for the cleavage. The presence of apoptosis seems to correlate with posttranslational processing of EMAP II, which may explain apoptosis-induced influx and sequestration of leukocytes in the reperfused kidney (41). More research should be done to point out the exact relationship between EMAP II and apoptosis.

EMAP II: Actions and functions

Cells undergoing apoptosis are rapidly removed by monocyte-derived macrophages, suggesting a contemporaneous release of factors with leukocyte and monocyte chemotactic activity. EMAP II is a cytokine that recruits monocytes and macrophages in areas with apoptosis for scavenging apoptotic corpses. This tumor-derived cytokine has potent effects on endothelial cells (42).

The interest in our laboratory for EMAP II was sparked by observations of Wu et al. that up-regulation of EMAP II can render a previous non-responding tumor sensitive to TNF treatment (43). This research group demonstrated that, by over-expressing EMAP II in a TNF-resistant human melanoma line by retroviral-mediated transfer of EMAP II cDNA, the tumor becomes TNF-sensitive in vivo, but not in vitro. A possible explanation for this is that TNF acts on the vasculature, which, after being primed by EMAP II, eventuates in an up-regulation of TNF-R1 and TF expression on the endothelial cells. This mechanism will not be present in vitro cell cultures of tumor cells (44). TNF acts through two distinct cell-surface receptors of 55 kDa (TNF-R1) and 75 kDa (TNF-R2), which are ubiquitously expressed (45). The receptors utilize both dependent and shared intracellular signaling pathways to mediate a variety of effects on cells, some of which are receptor-specific. The cytotoxic effects of TNF are mediated by TNFR-1, whilst TNF-R2 has proliferative effects, as for example in lymphoid cells (46). TNF-R1 expression may be associated with the induction of endothelial cell apoptosis by TNF. Therefore, EMAP II produced by tumors may, at least in part, determine in vivo sensitivity to TNF by up-regulating TNF-R1 expression on endothelial cells, triggering cell death in the presence of TNF, leading to eventual ischaemic necrosis of the tumor. In vitro studies of Liu and Gottsch (47) show an EMAP II-like mediated cytotoxicity and apoptosis of corneal endothelial cells. This effect was even more profound after adding cycloheximide, a protein synthesis inhibitor.

An increase in secretion of active EMAP II by a tumor also leads to an up-regulation of TF on the surrounding endothelial cells, providing another link between EMAP II and TNF as used in an ILP, where it frequently results in hemorrhagic necrosis of the tumor. This up-regulation will

---

**Table I. Tumor volume (mm³) in rats on consecutive days after sham or TNF isolated perfusion.**

<table>
<thead>
<tr>
<th>Wild-type</th>
<th>0</th>
<th>2</th>
<th>4</th>
<th>6</th>
<th>8</th>
<th>10</th>
<th>12</th>
<th>14</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>622</td>
<td>1210</td>
<td>2156</td>
<td>3299</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNF</td>
<td>617</td>
<td>1134</td>
<td>1740</td>
<td>2967</td>
<td>3752</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Transfected</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham</td>
<td>713</td>
<td>846</td>
<td>1169</td>
<td>2941</td>
<td>5107</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNF</td>
<td>532</td>
<td>589</td>
<td>1184</td>
<td>1877</td>
<td>2159</td>
<td>2381</td>
<td>3322</td>
<td>5220</td>
</tr>
</tbody>
</table>

**Table II. Score staining intensity.**

<table>
<thead>
<tr>
<th>Score CR group (n=9)</th>
<th>Score NC group (n=9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF</td>
<td>2.8</td>
</tr>
<tr>
<td>EMAP II</td>
<td>3.2</td>
</tr>
<tr>
<td>MIB-1</td>
<td>2.0</td>
</tr>
<tr>
<td>CD34</td>
<td>3.6</td>
</tr>
</tbody>
</table>

CR= Complete response, NC = No response, VEGF = Vascular Endothelial Growth Factor, EMAP II = Endothelial Monocyte Activating Polypeptide II, MIB-1 = monoclonal antibody to the Ki-67 epitope, CD 34 = endothelial cell marker staining.
lead to an increased pro-coagulant activity and also a more permeable vasculature. Friedel et al. have found another explanation for the effects on tumor vasculature (48). They demonstrated that TNF only exerts its permeability and procoagulant activity when TF expression on cell surface is combined with extrinsic clotting factors in plasma.

Our data

We have demonstrated that in vitro stable transfection of rat sarcoma cells led to an up-regulation of endogenous pro-EMAP II in these tumor cells. This gene transfer induced sensitivity to subsequent regional TNF treatment by means of an isolated limb perfusion (ILP) in rats. To confirm that this was indeed an EMAP II effect, tumor-bearing rats were pre-treated with an intravenous injection of recombinant EMAP II followed by an ILP with TNF. These experiments resulted in a comparable tumor response as observed in the rats undergoing EMAP II gene therapy followed by TNF ILP (49).

Table I and Figure 1 show results of TNF ILP in rats bearing the transsected tumor compared to the wild-type tumor. Perfusion with Haemaccel alone resulted in progressive disease in all animals. Although ILP with TNF resulted in a slight inhibition of tumor growth of the wild-type BN tumor compared with the sham control, there was no significant difference in tumor response. As can be seen in this graph, the EMAP II-transfected tumor BN-E responded significantly better to ILP with 50 µg TNF. We saw macroscopically a more profound necrosis in the BN-E tumor that led to a significantly slower outgrowth of tumor after the perfusion. Even in animals with transsected tumors a small rim of viable tumor cells survived. However, as can be seen on day 9, the overall observed response rate still implied a decline in tumor dimension, while profound recurrent tumor growth was demonstrated in all animals from day eleven.

EMAP II: Potential clinical implications

Pharmacological administration of EMAP II as a neoadjuvant therapy prior to TNF will not be instituted before complete elucidation of the mechanism of activation of the mature protein. Since the relative expression of EMAP II may correlate with a tumor’s sensitivity to TNF based on a dose-dependent increase of TNF receptor on endothelial cells, high EMAP II levels must be obtained before treatment with TNF. The intended effects will be the up-regulation of TF on tumor endothelial cells leading to massive thrombosis at the place of vascular damage. This combined with an increased amount of TNF receptors will intensify the action of administered TNF and, as a result of this necrosis of tumor vasculature, an increased port d’entrée for melphalan or other chemokines.

At this moment the approved use of TNF in Europe is restricted to the Isolated Limb Perfusion setting where TNF is combined with melphalan for unresectable soft-tissue sarcomas to facilitate limb salvage (50). Furthermore TNF is used successfully in this setting, treating a great variety of tumors such as melanoma, drug-resistant recurrences of bony sarcomas, squamous cell carcinomas, merkel cell carcinomas and others (50).

All these studies have greatly increased the understanding of the way in which TNF works. As a consequence of its toxicity when administered systemically, techniques like the isolation of limbs and organs have been developed. At present we hope that EMAP II may be valuable in potentiating the effect of TNF. By hypothesizing that a higher EMAP II concentration in the surroundings of a tumor, either by systemic injection, gene therapy, or other modalities, will lead to a profound response to TNF treatment, perhaps a diminished dose of TNF might be enough to induce the same clinical responses. So far this hypothesis can not be validated by clinical data.

We have used immunohistochemistry to analyze the expression of EMAP II in sarcoma of 18 patients who had been treated with TNF and melphalan in an ILP, in order to estimate the predictive value of EMAP II expression. From a large database of patients treated with ILP with TNF and melphalan, we selected two patient groups with identical demographic characteristics and equal grade and histological classification of their soft tissue sarcomas. Patients in the first group responded with a complete response or partial response, with over 50% tumor necrosis; patients in the second group responded with progressive disease, no change, or a partial response with less than 25% tumor necrosis. Biopsies from defined morphological areas of paraffin-embedded tumors were analyzed by immunohistochemistry with EMAP II antibody, vascular endothelial growth factor (VEGF) antibody, CD34 antibody as a vascular marker and MIB-1 antibody to determine proliferative indices; these characteristics were compared to patient outcome concerning response rates. A promising good correlation was found between the extent of EMAP II expression in the tumors and the response of the patient. Table II demonstrates a great diversity between the tumors regarding EMAP II expression, with a preference to a high intensity in the group of patients with a CR, and a much lower expression of EMAP II in the NC group. However, throughout these experiments data was published that immunohistochemistry with EMAP II would mark pro-EMAP as well as the mature and active form of EMAP II. This data implied that our initial conclusion could no longer be sustained. Currently we are conducting a prospective study to determine EMAP II expression in tumors by means of Western blot. By this method we will be able to distinguish between pro- and mature EMAP II and we will be in the position to answer the question.

2246
Currently studies are directed to the exact cleavage proteases, the profile of the receptor on endothelium, effects of EMAP II on normal cells and expression patterns of EMAP II in the tissue of patients treated with TNF. Also more research needs to be done in order to define possible side-effects of EMAP II, since cytokines have shown to provoke numerous undesirable effects.

**Summary**

The action of EMAP II in cancer is complex, just as is the case for cytokines such as IL-1β and TNF-α. Endogenous EMAP II chronically produced in the tumor micro-environment enhances the antitumor properties of TNF in an ILP. Moreover EMAP II is a crucial effector molecule in apoptosis, it shows chemotactic properties and is capable of up-regulation of proteins on endothelial cells like TF and TNF-R1. It seems that EMAP II plays a role in stabilizing and some controversies about EMAP II are elucidated and some controversies about EMAP II are cleared up, future studies are justified where EMAP II will be administered as a neoadjuvant or primary therapy, concurrent with or prior to TNF administration.

By exploring these routes of multiple-therapy, an increased understanding of the balance between proangiogenic and antiangiogenic agents will be obtained. Therapies that have proven to be effective in animal studies will, in future studies, have to demonstrate whether this holds true in man.

**References**


25 de Wilt JH, ten Hagen TL, de Boeck G, van Tiel ST, de Bruijn.


