ANTITUMOR EFFECT OF ENDOTHELIAL MONOCYTE-ACTIVATING POLYPEPTIDE-II ON HUMAN PROSTATE ADENOCARCINOMA IN MOUSE XENOGRAFT MODEL

A.G. Reznikov*, L.V. Chaykovskaya†, L.I. Polyakova*, A.I. Kornelyuk‡

1. V.P. Komisarenko Institute of Endocrinology and Metabolism, Academy of Medical Sciences of Ukraine, Kyiv, Ukraine
2. Institute of Molecular Biology and Genetics, National Academy of Sciences of Ukraine, Kyiv, Ukraine

Endothelial monocyte-activating polypeptide-II (EMAP-II) is a novel proinflammatory cytokine with anti-angiogenic properties. The aim of this work was to evaluate in vivo antitumor activity of EMAP-II in growing human prostate adenocarcinoma xenograft mouse model. Materials and Methods: Recombinant human EMAP-II was expressed in Escherichia coli and purified after cleavage with enterokinase (EMAP-II). EMAP-II preparations were injected to CBA mice bearing subrenal capsule xenografts of human prostate adenocarcinoma. After 3-days treatment, the xenografts were isolated and weighed, then the transplants exposed to EMAP II (100 or 200 µg/kg b. w.) were examined histologically. Results: EMAP-II administered daily at a dose of 100 or 200 µg/kg b. w. caused striking retardation of local tumor progression as compared to the controls. Low dose (10 µg/kg) was effective in some cases. Conclusion: EMAP II exhibits significant antitumor activity in vivo in human prostate adenocarcinoma xenografts in mouse model.

Key Words: endothelial monocyte-activating polypeptide-II, EMAP-II, prostate cancer, xenografts.

Targeting the key mechanisms of carcinogenesis is recognized to be an effective modality in therapeutic antitumor strategy. Besides oncogene-encoded proteins, various multifunctional proteins are produced in mammalian tumor cells due to alternative splicing and posttranslational mechanisms. They reveal remarkable impact on migration, differentiation, proliferation, apoptosis and other cellular events within local immune and vascular systems being involved in malignant transformation, and some of them exhibit antitumor activity. One family of these proteins is represented by tyrosyl-tRNA and tryptophanyl-tRNA synthetases, and the p43 auxiliary component of multisynthetase complex, which display cytokine-like activities upon their cleavage and release into intracellular space [7, 12, 23].

Endothelial monocyte-activating polypeptide-II (EMAP-II) is the C-terminal domain of p43 precursor protein that was isolated from methylcholantren induced mouse fibrosarcoma supernatant [8, 9]. EMAP II detected by its ability to stimulate endothelial-dependent coagulation in vitro apparently plays an important role in inflammation, apoptosis, and angiogenesis in tumor tissues [2–3]. It possesses proinflammatory, proapoptotic (only in proliferating and migrating endothelial cells) and antiangiogenic properties. These effects are realized through plenty mediators of cell adhesion, migration of endothelial cells, macrophages, neutrophils, and monocytes. EMAP II induces tumor host response in the actin cytoskeleton and fibronectin based focal adhesions of endothelial cells [17]. More adherent phenotype leads to the blockade of endothelial migratory signals [11], with the vascular thrombosis and the decreased expression of the vascular endothelial growth factor being involved [18] as well as induction of tissue factor in neovascularatory endothelium.

Obviously, EMAP-II is essential for tumor survival that is supported by the data on its constitutive secretion by tumor endothelial cells. Meanwhile, intracellular EMAP II/p43 level rises after treatment with TNF, demonstrating reciprocal interrelationship between those both polypeptides. EMAP-II makes tumor more sensitive to TNF-1 treatment by increasing TNF-1 receptor expression [3] or rather redistributing it from endothelial Golgi storage pool to cell membranes and in such a way facilitating TNF apoptotic signalling [20] and enhancing the effect of TNA on tumor-associated vasculature [10].

Based on EMAP-II procoagulant activity, angiotoxic, antiproliferative and proinflammation properties, successful in vivo therapy of some malignant tumors with this polypeptide has been shown in animal research [13, 16].

Prostate cancer is one of the most prevalent malignant tumors within male populations all over the world. To the best of our knowledge, in vivo antitumor effect of EMAP II on prostate adenocarcinoma has not yet been studied. We hypothesized that in vivo administration of EMAP II might exert suppressive effect on growing prostate cancer. Here we demonstrate the retardation of the growth of human prostate adenocarcinoma xenografts in animal model.

MATERIALS AND METHODS

Bacterial expression and purification of EMAP II. Recombinant human EMAP-II was cloned and expressed in E. coli as described previously [5]. In brief, DNA fragment encoding human mature EMAP-II
(D146-K312) was cloned into NcoI and HindIII restriction sites of pET30a plasmid (Novagen). The presence of specific DNA insert was confirmed by restriction mapping and DNA sequencing. This construction designed as pET30-EMAP II was employed for production and purification of the 6His-tagged recombinant protein EMAP-II<sub>6h</sub>. E. coli strain BL21(DE3) harboring pET30-EMAP II plasmid after 4 h induction with 1 mM IPTG overproduced recombinant protein with corresponding molecular weight according to SDS gel-electrophoresis data. Bacterial cells were harvested by centrifugation and disrupted by sonication in a buffer containing 0.1 mM of PMSF. The recombinant EMAP-II protein was purified by metal-chelated chromatography on Ni-NTA agarose (Qiagen). The recombinant EMAP-II was completely eluted from Ni-NTA column with 60 mM of imidazole in 50 mM KH<sub>2</sub>PO<sub>4</sub>, pH 8.0 buffer with 500 mM NaCl, 0.05% Tween 20. The mature EMAP-II was obtained by specific cleavage with enterokinase and purified up to 98% homogeneity according to SDS gel-electrophoresis data. Physicochemical properties of these proteins have been analyzed by ProtParam program. The molecular weight of full-length recombinant EMAP-II<sub>211 aa</sub> (23173 Da, with isoelectric point pI = 6.19, and extinction coefficient of 8730 cm<sup>-1</sup>·mole<sup>-1</sup> (0.377 ml/mg) at 280 nm. The molecular weight of the mature enterokinase-cleaved EMAP-II<sub>169 aa</sub> is 18535 Da, with isoelectric point pI = 6.36, and extinction coefficient of 8730 cm<sup>-1</sup>·mole<sup>-1</sup> (0.471 ml/mg) at 280 nm.

**Animal experiments design.** Experiments were performed according to the protocols approved by the Animal Care Commission at the V.P. Komisarenko Institute of Endocrinology and Metabolism in accordance with the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (Strasbourg, 1986).

Adult CBA mice (20–22 g; n = 29) were housed in the local animal care facility, and were supplied with standard diet and tape water ad libitum.

The specimens of human prostate adenocarcinoma were taken from two patients subjected to radical prostatectomy at the Oncourology Department, Institute of Urology, Kyiv, Ukraine. The specimens were placed into MEM medium (Serva), containing Hank's salts, HEPES, calf embryonic serum, benzylpenicillinum (0.2 U/ml) and streptomycinum (100 µg/ml) at 4°C and delivered to the laboratory. Six-days subrenal capsule assay methodology [4] was applied to fresh surgical explants (1 ± 0.03 mg, two grafts under the left kidney capsule), inserted under diethyl ether anaesthesia. Tested preparations were diluted in isotonic saline and injected subcutaneously into the flank since 4th day over 3 following days at a daily dose ranging from 10 to 200 µg/kg b. w. Control animals were injected with a vehicle. On the 7th day of the assay, mice were euthanized with diethyl ether overdosing. The tumor transplants were isolated and weighed, then fixed in 4% paraformaldehyde. The xenografts exposed to EMAP II<sub>6h</sub> (100 or 200 µg/kg) were processed in the routine manner, paraffin-embedded, and 5 µm thick sections were cut. The sections were stained with haematoxylin-eosin or haematoxylin-Schiff reagent and examined with the light microscope.

Net tumor progression during experiment was calculated by subtracting the final tumor graft weight from the original one. Antitumor activity was evaluated as a decrease of tumor growth calculated on the basis of differences in gain of the tumor weights between experimental and control groups. 25% or more growth retardation was considered as therapeutically effective modality. Student’s t-test was used for evaluation of the differences, and the results were considered significant at P < 0.05.

**RESULTS**

Within the dose range from 10 to 200 µg/kg b. w., EMAP-II had no visible toxic effects on mice over 3-day systemic administration.

The specimens of prostate cancer tissue that have been used for transplantation were identified as adenocarcinomas by the hospital pathologist. In control animal group, the xenografts exhibited extensive growth, and their mass values increased by 278% (experiment 1) or 155% (experiment 2) on average, ranging from 165% to 380% (experiment 1) or 25% to 130% (experiment 2). There was no complete cancer abrogation under EMAP-II therapy in this study, however, nevertheless the retardation of local tumor growth was significant (Fig. 1). Data obtained indicated that both tested polypeptide preparations at a dose of 100 µg/kg b. w. possessed striking antitumor activities against the first transplant generation of human prostate adenocarcinoma in mouse model (Table). Administered at this dose, EMAP-II inhibited tumor progression by 70–77% (experiments 1, 2), and full-length EMAP-II<sub>6h</sub> also demonstrates 79% retardation (experiment 2). In fact, in experiment 2 (animal groups 3 and 5), complete suppression of the tumor growth after administration of those preparations was observed (P > 0.05 in comparison with original xenograft weights). Surprisingly, EMAP-II, at a dose of 200 µg/kg had statistically significant, but less effect (44% retardation) in comparison with that of 100 µg/kg (P < 0.05, experiment 1). The lowest dose of EMAP-II<sub>6h</sub> (10 µg/kg), as well as that of EMAP-II<sub>6h</sub> did not affect the tumor progression while difference in average weight values was calculated (P > 0.05 in comparison with original xenograft weights). In the meantime, the effect varied within animal groups, and the tumor growth suppression was evident in some cases.

Histological sections of non-treated six-day tumor xenografts contained epithelial cells with malignant cytological features (Fig. 2, a). Active proliferation of the acinar epithelium with formation of multiple layers and the signs of cellular pleomorhoism and metaplasia were found. Acinar epithelial cells appeared spindle-shaped with oval nuclei stretched toward clear space of an acinus. There was enlargement of stromal tissue due to fibrosis and edema. Detritus and round-shaped
acinar epithelial cells were observed in some acinar clear spaces. Moderate leucocytes infiltration through stromal and epithelial tissues, and granulation at the xenograft margine that indicated an immune response were found.

**Fig. 1.** Appearance of human prostate tumor xenografts 6 days after transplantation in control mice (а) and those of treated with EMAP IIe, 100 μg/kg x 3 d (b) or 200 μg/kg x 3 d (c)

**Table.** Effect of EMAP II on growth of human prostate adenocarcinoma xenografts in mice

<table>
<thead>
<tr>
<th>Animal group number</th>
<th>Treatment</th>
<th>Number of xenografts/Number of recipients</th>
<th>Xenograft weight, (M ± m) mg original</th>
<th>after treatment</th>
<th>difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control (vehicle)</td>
<td>6/3</td>
<td>1.0 ± 0.02</td>
<td>3.78 ± 0.39</td>
<td>2.78 ± 0.39</td>
</tr>
<tr>
<td>2</td>
<td>EMAP IIe, 100 μg/kg</td>
<td>8/4</td>
<td>1.0 ± 0.03</td>
<td>1.64 ± 0.11*</td>
<td>0.64 ± 0.11*</td>
</tr>
<tr>
<td>3</td>
<td>EMAP IIe, 200 μg/kg</td>
<td>8/4</td>
<td>1.0 ± 0.03</td>
<td>2.55 ± 0.31*</td>
<td>1.55 ± 0.29*</td>
</tr>
<tr>
<td>1</td>
<td>Control (vehicle)</td>
<td>6/3</td>
<td>1.0 ± 0.02</td>
<td>1.60 ± 0.16</td>
<td>0.60 ± 0.16</td>
</tr>
<tr>
<td>2</td>
<td>EMAP IIe, 10 μg/kg</td>
<td>8/4</td>
<td>1.0 ± 0.01</td>
<td>1.34 ± 0.10</td>
<td>0.34 ± 0.09</td>
</tr>
<tr>
<td>3</td>
<td>EMAP IIe, 100 μg/kg</td>
<td>6/3</td>
<td>1.0 ± 0.02</td>
<td>1.18 ± 0.08*</td>
<td>0.18 ± 0.08*</td>
</tr>
<tr>
<td>4</td>
<td>EMAP III, 10 μg/kg</td>
<td>8/4</td>
<td>1.0 ± 0.03</td>
<td>1.59 ± 0.15</td>
<td>0.59 ± 0.14</td>
</tr>
<tr>
<td>5</td>
<td>EMAP III, 100 μg/kg</td>
<td>8/4</td>
<td>1.0 ± 0.01</td>
<td>1.13 ± 0.09*</td>
<td>0.13 ± 0.08*</td>
</tr>
</tbody>
</table>

*P < 0.001; **P < 0.05 as compared to control.

A decrease in number of the xenograft acinar epithelial layers after exposure to EMAP-IIe at a dose of 100 μg/kg was evident (Fig. 2, b). Epithelial cells became more differentiated, more flattened and adherent to basal membrane. Some malignant cells after treatment became rounded, plenty of them were translocated to the acinar clear space. The stroma appeared similarly to the control. In the tumor xenografts exposed to EMAP-IIe at a dose of 200 μg/kg, the acini were flattened, and distinct destructive changes of acinar epithelium with the signs of necrosis were found (Fig. 2, c). Acinar basal membrane was thickened and developed hyalinosis. The xenograft tissues were represented mostly by
stroma, which was edematous and revealed fibrinoid swelling in the central site of the transplant.

**DISCUSSION**

Because of the rapidly growing proportion of elderly human male population worldwide, prostate cancer has become one of the major causes of death at that age in many countries. Usually, it is diagnosed at histopathological examination as adenocarcinoma demonstrating androgen-dependent growth in 80% of cases and a clear trend for early metastasizing. Pharmacological antagonists of the androgen receptor and other antiandrogens are known as effective tools for androgen deprivation strategy in palliative therapy of the patients with advanced and metastatic prostate cancer [22]. Meanwhile, androgen ablation leads to formation of the hormone resistance, and prescription of chemotherapy and/or brachytherapy or external beam radiotherapy become the treatment of choice.

A variety of factors with tumor and its surrounding environment derived cytokines and other peptides are involved into tumor growth as well as its prevention. Expression of a novel antiangiogenic, proapoptotic and proinflammatory polypeptide EMAP-II was found in the prostate adenocarcinoma cells. LNCaP and DU-145 human prostate adenocarcinoma cells constitutively express a Mr 34 000 form of EMAP II that is retained intracellularly. In the presence of agents that induce necrosis or apoptosis, as well as at the condition of hypoxia, these cells release the Mr 34 000 form followed by processing of the protein to biologically active forms, Mr 27 000 and Mr 22 000 [1]. The authors suggested that the release of EMAP-II might potentiate the effects of those agents on the tumor through the local activation of host effector mechanisms.

In the present study, the strong antitumor activities of EMAP-II preparations tested for prostate cancer xenografts have been shown. Importantly that at the doses administered in in vivo experiments (10–200 μg/kg b. w.), EMAP-II did not exhibit obvious systemic toxicity in mice. As far as EMAP II is concerned, there were no visible systemic toxic effects either in this study, or in related research by other authors [18].

Although short-time xenografts have no developed vascular system, one can suppose, that another antitumor mechanisms but antiangiogenic ones might be responsible for profound suppression of prostate adenocarcinoma transplant growth in EMAP-II treated mice. Taking into account the lymphoid infiltration through the prostate adenocarcinoma xenografts treated with EMAP-II preparations found at histopathological examination, the inflammatory processes could also contribute into therapeutic effect of this polypeptide. In the studies of the developmental lung-mesenchimal-epithelial relationship, a marked increase in cellular apoptosis in contact region in the presence of exogenous mature EMAP-II was demonstrated [25]. Therefore, an increase of apoptosis resulting from the direct impact of EMAP-II on the tumor tissues could not be excluded.

As it was mentioned, EMAP-II administered at a dose of 200 μg/kg did not exceed an effect of 100 μg/kg dosage judging by tumor size, which is surprising enough. Meanwhile, histopathological features of the tumor growth suppression were well exhibited at both therapies. This is a preliminary result, and more experiments need to be done with an aim to evaluate dose-dependent therapeutic effects of EMAP-II.

The results of our study are in concordance with antitumor activity of EMAP II/p43 preparations alone or combined with TNF demonstrated in glioma [18], human sarcoma [13], stomach cancer [14] and several experimental tumors [16].

Beside of possible direct proinflammatory and proapoptotic effects, the antitumor potency of EMAP-II is mediated with TNFα. It was shown that a single intratumoral injection of EMAP-II into Meth A sarcomas induced acute thrombohemorrhage and partial tumor regression [8]. The same treatment with EMAP-II of MC-2 mammary carcinoma induced the sensitivity to systemic treatment with TNF [8]. Similar effects were observed in mouse melanoma B16 and human fibrosarcoma HT-1080 treated with EMAP-II [15]. In the meantime, presence of EMAP II is obligate for activating TNFα effects on the tumor. It was shown, that transfer of the EMAP-II gene into established TNFα-resistant human melanomas render these tumors sensitive to subsequent systemic TNFs treatment [6]. The enhancing effect of EMAP-II on TNF-anti-cancer therapy was suggested to expand the clinical use of TNF [21]. This therapeutic approach, as well as EMAP II systemic administration, seems to be promising with regard to overcoming primary unresponsiveness of the tumor toward TNFα because of reducing the dose necessary for antitumor responses and thus reducing TNFα toxicity in clinical treatment strategies.

In conclusion, we speculate on the possible therapeutic applications of a novel anti-angiogenic EMAP-II cytokine for prostate cancer monotherapy or combined with TNFα or androgen receptor antagonists.

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ПРОТИВООПУХОЛЕВОЕ ДЕЙСТВИЕ ПОЛИПЕПТИДА EMAP II НА КСЕНОТРАНСПЛАНТАТЫ АДЕНОКАРЦИНОМЫ ПРЕДСТАТЕЛЬНОЙ ЖЕЛЕЗЫ ЧЕЛОВЕКА У МЫШЕЙ

Эндоотелий-моноцитактивирующий полипептид-II (EMAP-II) — новый провоспалительный цитокин с антитumorными свойствами. Цель данной работы — исследование in vivo противоопухолевого действия EMAP-II на рост ксенотрансплантатов аденокарциномы предстательной железы человека у мышей. Материалы и методы: рекомбинантный EMAP-II человека экскрессировали в Escherichia coli и очищали после его расщепления энтерокиназой (EMAP-II). Препараты EMAP-II инъецировали мышам СВА с трансплантированными под капсулу почки фрагментами аденокарциномы предстательной железы человека. После трехдневного введения препаратов трансплантаты извлекали и взвешивали, затем проводили гистологическое исследование ксенографов, подвергнутых воздействию EMAP-II в дозе 100 или 200 мкг/кг массы тела вызывал выраженноe торможение роста опухоли в сравнении с контролем. Отмечали эффективность низкой дозы (10 мкг/кг) в отдельных случаях. Выводы: EMAP-II проявляет in vivo выраженную противоопухолевую активность в отношении ксенотрансплантатов аденокарциномы предстательной железы человека у мышей. Ключевые слова: эндотелиальный полипептид, активирующий моноциты, EMAP II, рак предстательной железы, ксенотрансплантаты.