Exp Oncol 2011
33, 4, 231–234

Experimental ONCOLOGY

COOPERATIVE ANTITUMOR EFFECT OF ENDOTHELIAL-MONOCYTE ACTIVATING POLYPEPTIDE II AND FLUTAMIDE ON HUMAN PROSTATE CANCER XENOGRAFTS

A.G. Reznikov[1,], L.V. Chaykovskaya[1], L.I. Polyakova[1], A.I. Kornelyuk[2], V.N. Grygorenko[3]*
[1]V.P. Komisarenko Institute of Endocrinology and Metabolism, National Academy of Medical Sciences of Ukraine, Kyiv, Ukraine
[2]Institute of Molecular Biology and Genetics, National Academy of Sciences of Ukraine, Kyiv, Ukraine
[3]Institute of Urology, National Academy of Medical Sciences of Ukraine, Kyiv, Ukraine

Recombinant cytokine-like endothelial monocyte-activating polypeptide II (EMAP II) and antiandrogen flutamide target different mechanisms of growth of androgen-dependent prostate cancer (PC). *The aim* of this study was to clarify whether combined treatment with EMAP II and flutamide is more effective than monotherapy with regard to retardation of PC progression. *Materials and Methods:* Antitumor effects of EMAP II (10 μg/kg b.w./d, s.c., 3d), or flutamide (10 mg/kg b.w./d, *per os*, 3d), or their combination were studied in CBA male mice bearing human androgen-dependent PC xenografts for 7 days. Androgen-dependent phenotype of the tumors was verified in preliminary castrated mice. The xenografts were weighed and underwent a histopathologic examination. The results were compared with those of non-treated mice. *Results:* EMAP II and flutamide used separately inhibited growth of the xenografts by 74% and 53% respectively. Both drugs caused destructive changes in malignant epithelial cells along with leukocyte infiltration of the tumor. Combined treatment inhibited tumor growth by 85%, and was more effective than monotherapy with regard to morphological changes. *Conclusions:* This study demonstrates cooperative inhibitory effect of EMAP II and flutamide on growth and morphology of human PC xenografts that could represent a new modality of palliative treatment of this disease.
Key Words: **prostate cancer, xenograft, CBA mice, EMAP II, flutamide.**

During the past few decades many countries registered a dramatic increase in the incidence of prostate cancer (PC) [1]. In Europe and North America, this life-threatening disease became the most common cause of tumor-related death in the elderly male population [2–4]. The risk of PC development depends on age, ethnic group, geography of living, family history, lifestyle, dietary pattern etc. The number of estimated new PC cases in the USA was 217 730 in 2010 [5]. Ukraine belongs to the group of countries with a relatively high rate of diagnosed PC: from 2001 to 2008, the number of patients increased from 17 223 to 28 353 (by 65%) [6].

According to clinical characteristics PC was proposed to divide into aggressive phenotype which prevails in 55–65 years old men, and so-called insignificant, e.g., a life-nonthreatening low-grade cancer, which is usually diagnosed in men around 75–80 years old [7]. Clinical management of an aggressive PC phenotype requires active treatment using surgery (radical prostatectomy, castration), radiation and/or drug therapy. Maximum androgen blockade provides effective palliative therapy of PC. Successful combined hormonal treatment of androgen-dependent PC includes administration of LH RH agonists and androgen receptor antagonists (cyproterone acetate, flutamide, bicalutamide), and it seems to be the most effective as compared to monotherapy, especially in cases of advanced PC and metastatic process [1, 8]. In the meantime, despite an initial transient positive response to androgen deprivation the disease relapses within a few years due to progression to androgen-independent phenotype of the tumor. This occurs because androgen ablation affects only androgen-dependent PC cells with no effect on androgen-independent cell clones. For this reason hormonal treatment of PC should be combined with drugs targeting oncogenes, growth factors, etc. With this regard some polypeptide components of aminoacyl-tRNA synthetase complexes, such as endothelial-monocyte activating polypeptide II (EMAP II), could provide new possibilities for antitumor therapy [9].

EMAP II is the extracellular cytokine-like derivative of the auxiliary protein p43 which is associated with multisynthetase complex in mammals. This polypeptide is produced intracellulary during tissue lesion due to proteolytic cleavage of endogenous p43, and its aminoacid residue sequence corresponds to the C-terminal moiety of p43. EMAP II is known as a multifunctional protein showing various activities. Upon its cleavage and extracellular release, EMAP II becomes involved in the inflammation process, stimulates apoptosis, demonstrates antiangiogenic and pro-coagulation effects, and sensitizes tissues to the tumor necrosis factor-α, etc. [10–16].

EMAP II was shown to inhibit growth of glioma, human sarcoma, and pancreatic cancer in animal research [14, 15, 17–19]. Previously we have found an ability of recombinant EMAP II to suppress the growth of human prostate adenocarcinoma in mouse xenograft model [20]. Based on the absence of blood supply of the xenografts it was postulated that this effect was caused by stimulation of apoptosis and pro-inflammatory activity of the EMAP II. We hypothesized that its antitumor effect might be augmented by combined administration with flutamide,

Received: August 29, 2011.
*Correspondence: E-mail: reznikov39@gmail.com
Abbreviations used: EMAP II – endothelial monocyte-activating polypeptide II; PC – prostate cancer.

nonsteroidal androgen receptor antagonist. In this study, we compared the effects of separate and combined treatment with EMAP II and flutamide on the growth and morphology of androgen-dependent human PC xenografts.

MATERIALS AND METHODS

EMAP II preparation. Recombinant EMAP II was cloned and expressed in *E. coli* essentially as we described in details previously [21]. Briefly, the DNA fragment encoding human mature EMAP II (D146-K312) was cloned in pET30a plasmid (Novagen), and transformation of *E. coli* strain BL21(DE3) has been done. The presence of specific DNA insert was confirmed by restriction mapping and DNA sequencing. After 4 h induction of EMAP II overproduction with 1 mM isopropyl-β-D-thiogalactopyranosid (IPTG), bacterial cells were harvested by centrifugation and disrupted by ultrasonication in a buffer containing 0.1 mM phenylmethylsulfonyl fluoride (PMSF). EMAP II was isolated by metal chelate column chromatography on Ni-NTA agarose (Qiagen) and eluted with 200 mM imidazole in 50 mM NaH_2PO_4 buffer (pH 8.0) with 0.5 M NaCl and 0.05% Tween 20. The mature EMAP II (169 a.a., m.w.18 535 Da, pI 6.36) was obtained by specific cleavage with enterokinase and purified up to 98% purity grade as confirmed by SDS gel electrophoresis.

Animal experiments design. An animal research protocol was approved by the Animal Care Committee at the V.P. Komisarenko Institute of Endocrinology and Metabolism. Six-days the mouse renal subcapsular assay [22] was adapted to mature CBA male mice (20–25 g b.w.). They were bred and housed in the local animal care facility, and were supplied with standard diet and tape water *ad libitum*.

The PC tissue specimens were taken from four human patients (64–72 year old) during radical prostatectomy in the Oncourology Department, Institute of Urology (Kyev, Ukraine) after preliminarily obtaining their informed consent approved by Institutional Bioethics Committee. A patient's anonymity was carefully protected. The patients did not take neoadjuvant therapy before surgery. The tumors were identified by pathologist as adenocarcinomas (the Gleason scores ranged from 5 to 7). The tissue specimens were placed into Eagle's Minimum Essential Medium (Serva), containing Hanks salts, HEPES, calf embryonic serum, benzylpenicillinum (0.2 U/ml) and streptomycinum (100 μg/ml) at 4.0°C and delivered to the laboratory on ice. On the next day, they were dissected into 0.90–1.10 mg pieces, and two grafts were implanted under the left kidney capsule of normal or castrated immediately prior to transplantaion male mice using chloral hydrate for anaesthesia.

Non-castrated mice prepared for the drug treatment were divided into four groups (each of 12–18 mice). In the first one, EMAP II solution was diluted with saline immediately before using and injected subcutaneously into the flank on the 4^{th}–6^{th} days after tumor transplantation at a daily dose of 10.0 μg/kg b.w. At the same terms, the second animal group was given flutamide as grinded tablets of Flutafarm (Farmak, Ukraine) suspended in Dorfman's gel (0.5% carboxymethyl cellulose sodium salt, 0.4% Tween 80, 0.9% benzyl alcohol in saline) *per os* using feeding tube at a daily dose of 10.0 mg/kg b.w. A combination of EMAP II and flutamide at the same doses was applied to the third animal group. Control mice were treated with vehicles according to mentioned treatment time-schedule. The next day after 3-day treatments, the mice were euthanized with overdosing diethyl ether, and the xenografts were removed and weighed. Net tumor growth during experiment was calculated by subtracting final xenograft weight from initial one. Antitumor activity of tested drugs was evaluated on the basis of differences in gain of the xenograft weights between experimental and control groups.

Histopathological studies. The xenografts were processed in the routine manner, that is they were fixed in 4% paraformaldehide, then paraffin-embedded, and 5 μm thick sections were prepared. The sections were stained with hematoxylin-eosin or hematoxylin-Schiff reagent and underwent histopathological examination using the microscopes Leika DME (Leika Microsystems, Germany).

Statistics. The results were compared with those of control (treated with vehicles) or castrated animals. Student's *t*-test was used for statistical analysis.

RESULTS

Tumor xenografts growth. During the observation period, all mice demonstrated good tolerance to EMAP II and flutamide treatments with no visible changes in behavior, food and water consumption, and body weight in comparison with control group. By the end of experiment, the average tumor xenograft weight in control group was doubled (Table). The xenografts harvested from castrated animals have demonstrated four-fold inhibition of the tumor growth. The same effect was observed after EMAP II injections, while flutamide at the selected dose inhibited transplant growth almost twice. Maximum effect regarding xenograft growth (85.2% inhibition) was obtained after 3-day combined treatment with EMAP II and flutamide.

Table. Effects of castration, and separate or combined administration of EMAP II and flutamide on growth of human PC xenografts in mouse renal subcapsular test (M ± m)

No	Animal group	Number of animals	Number of xenografts	Xenograft weight, mg		Weight gain, %	Weight gain *vs.* control, %
				initial	final		
1	Control	16	31	1.00±0.01	1,81±0.11	81.0	100
2	Castrated	12	24	1.03±0.02	1.23±0.09*	19.4	24.7
3	EMAP II, 10 μg/kg/d	18	36	1.02±0.01	1.23±0.05*	20.6	25.9
4	Flutamide, 10 μg/kg/d	13	26	1.01±0.01	1.39±0.07*	37.6	47.4
5	EMAP II, 10 μg/kg/d + flutamide, 10 μg/kg/d	12	24	1.01±0.01	1.13±0.08*	11.9	14.8

*$p < 0.05$ as compared with control.

Histopathological studies. Tissue sections of control xenografts allowed to grow for 7 days were characterized by heterogenic distribution of proliferating malignant epithelial tissue, which formed the taenias or acinar structures (Figure, *a*). The acinar epithelium formed multiple layers and was featured

by cellular polymorphism and metaplasia. Malignant epithelial cells appeared polygonal or spindle-shaped. Enlarged and cleared oval nuclei were stretched toward lumen and contained nucleoli. Detritus and round-shaped desquamated epithelial cells were present inside some acinar lumens. An enlargement of stromal tissue due to edema was observed. The xenografts from one patient contained a lot of non-functioning neogenic capillaries situated closer to the xenograft's margin. There were edema and destructive changes of stromal tissue, and fibrinoid swelling mainly in the central zone of some xenografts. Moderate leucocytes infiltration through stromal and some epithelial components, as well as marginal leukocyte accumulation around xenografts, were observed.

Histological sections of PC samples from castrated mice showed atrophy of malignant epithelium along with an increase of incidence of karyopyknotic cells that evidenced the tumor dependency on androgen stimulation.

Histopathological study revealed significant degenerative changes and necrotic death of many tumor cells, stimulation of apoptosis (significant increase in number of apoptotic bodies), an enlargement of necrotic areas and an enhancement of the leukocyte infiltration in the transplants exposed to EMAP II (Figure, *b*). There was a noticeable decrease in a number of acinar epithelial layers. Malignant epithelial cell volumes decreased, the majority of the nuclei did not contain the nucleoli. There was enhancement of leukocyte invasion into epithelial and stromal structures. Some epithelial cells developed round shape, plenty of them were separated from epithelial layer and translocated to a lumen. The stroma appeared similarly to the control.

Figure (*c*) illustrates the effect of flutamide treatment on histological appearance of the xenograft. Due to flutamide-induced atrophy and destruction of tumor cells, a number of epithelial layers decreased significantly. Spindle-shaped epithelial cells were laid on basal membrane, and they contained more hyperchromic and pyknotic nuclei as compared to control. Leukocyte infiltration was less evident in comparison with that of EMAP II. There were necrotic changes of the stroma in the central area of some xenografts.

A cooperative effect of combined EMAP II and flutamide treatment on the xenograft histological appearance was found, consistent with maximum tumor growth retardation. This treatment modality resulted in the most evident atrophy and destruction of tumor tissue. In many acinar structures, epithelial layer was quite thin. Oval-shaped epithelial cells, containing dense cytoplasm and pyknotic nucleus along with signs of fragmentation and formation of apoptopic bodies occurred more often than after separate administration of EMAP II or flutamide.

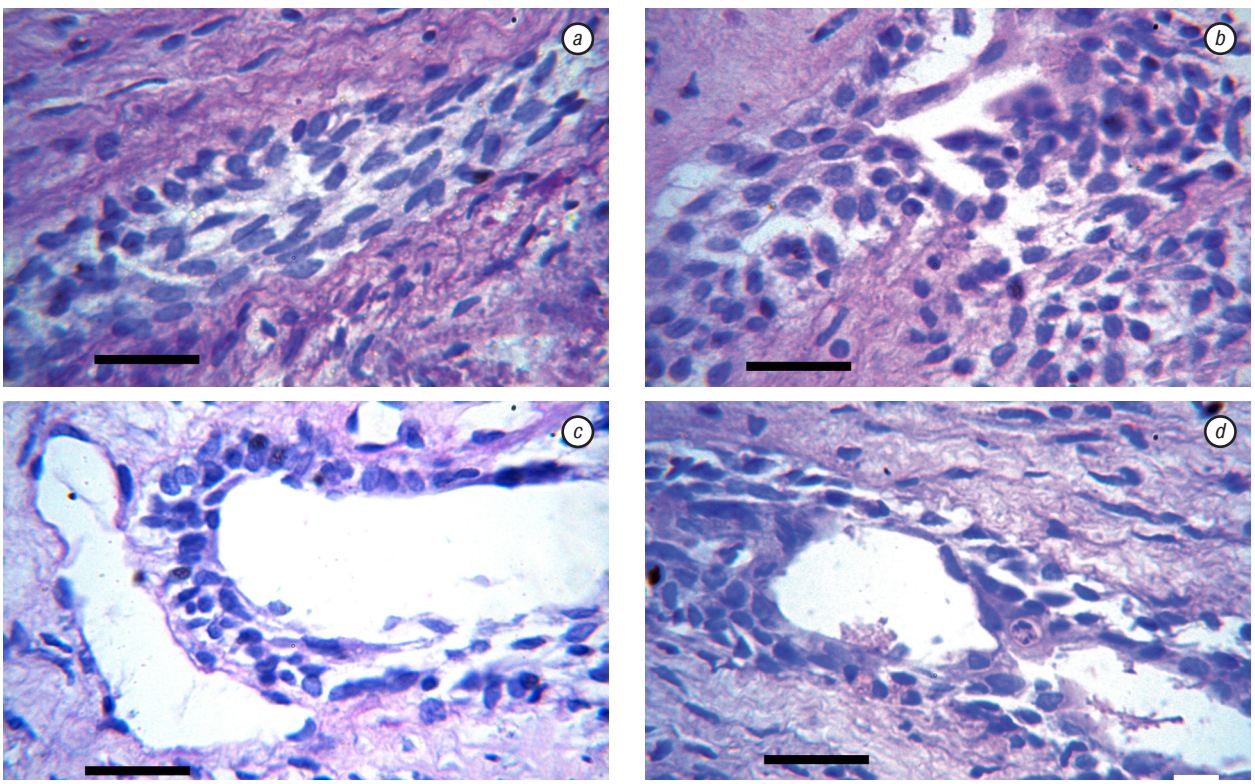

Figure. Histological sections of the human PC xenografts in a mouse model. The animals were sacrificed at the 7th day after tumor tissue transplantation under the CBA mouse kidney capsule. The PC tissue sections were stained with hematoxylin-Schiff reagent. Bar, 20 μ. *a*, The control xenograft. Malignant epithelial cells contain enlarged and cleared nuclei with nucleoli; *b*, The xenograft appearance after treatment with EMAP II, 10 μg/kg b.w./d, s.c., during 4th–6th days after transplantation. Note loosened epithelium, periacinar edema, reduction of malignant epithelial cells size, and nuclear hyperchromatism. The nucleoli are presented only in a few epithelial cells. There is an enhanced leukocyte invasion into epithelial and stromal structures; *c*, The xenograft appearance after treatment with flutamide, 10 mg/kg b.w./d, *per os*, during 4th–6th days after transplantation. Note significant malignant epithelium atrophy. 1, Lumen of the acinus; 2, dilated capillary; *d*, The effect of combined treatment with EMAP II and flutamide at the above mentioned doses and terms. There are sharp malignant epithelium atrophy, pericellular and periacinar edema, presence of hyperchromatic and pyknotic epithelium nuclei, an enhanced leukocyte invasion into epithelial and stromal structures.

DISCUSSION

Flutamide is widely used clinically for palliative treatment of PC [1, 8]. Using gene expression-based bioluminescence imaging of the androgen receptor signaling in the prostate tumor xenografts it was shown that flutamide significantly inhibits androgen receptor function and its association with co-activators in mice [23]. In the present study, we tested whether flutamide is capable to enhance the inhibitory effect of recombinant cytokine-like polypeptide EMAP II on growth of human PC first generation transplants in the male mouse model. Effects of combined administration of these drugs on PC growth and morphology were not studied till now.

All tumors used in our study pertained to androgen-dependent phenotype that has been confirmed by striking retardation of the xenograft growth due to androgen deprivation caused by preliminarily castration of the animals. Leukocyte accumulation in the xenografts grown in non-castrated mice indicated immune response, which is natural because CBA mice represent immunocompetent strain. However, short-term xenograft test rules out the transplant rejection.

It is obvious that xenografts devoided blood circulation experienced hypoxia and nutritional deficiency. Histopathologic study was decisive for evaluation of the drug effects because leukocyte infiltration and hypoxia-induced oedema of the xenograft stroma could distort quantification of antitumor activity based on the xenograft weight changes.

The findings of this study demonstrate an additive effect of flutamide concerning antitumor activity of EMAP II in mice bearing androgen-dependent human PC transplants with regard to the xenograft growth and morphology. Combined treatment turned out even more effective than castration, probably due to blockade of the tumor androgen receptor which could be activated by adrenal androgens remained after castration.

CONCLUSIONS

A cooperative antitumor effect of recombinant cytokine-like polypeptide EMAP II and flutamide against androgen-dependent human PC xenografts in the mouse renal subcapsular test might be considered in the development of new therapeutic modality for prostate cancer based on combined administration of these drugs.

REFERENCES

1. Nelson WG, De Marzo AM, Isaacs WB. Prostate cancer. N Engl J Med 2003; **349**: 366–81.

2. Bosland MC. Sex steroids and prostate carcinogenesis: integrated, multifactorial working hypothesis. Ann N Y Acad Sci 2006; **1089**: 168–76.

3. Ferlay J, Shin H-R, Bray F, ***et al.*** Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. Int J Cancer 2010; **127**: 2893–17.

4. Jemal A, Siegel R, Xu J, Ward E. Cancer statistics, 2010. CA Cancer J Clin 2010; **60**: 277–300.

5. American Cancer Society: Cancer Facts and Figures 2010. Atlanta, GA: American Cancer Society 2010; 66–71.

6. Fedorenko ZP, Gulak LO, Gorokh EL, ***et al.*** Cancer in Ukraine, 2007–2008. Bull Natl Cancer Register (Ukraine) 2009; **10**: 105 p.

7. Drewa T, Jasinski M, Marszalek A, Chlosta P. Prostate cancer which affects an elderly man is a feature of senescence (cellular) — a biology phenomenon. Exp Oncol 2010; **32**: 228–32.

8. Vozianov A, Reznikov A, Klimenko I. Androgen deprivation strategy in prostate cancer. Naukova Dumka: Kyiv, Ukraine, 2001, 240 p.

9. Reznikov AG, Kornelyuk AI. Aminoacil-tRNA-synthetases — a new way for immune modulation, regeneration and antitumor therapy. Bull Pharmacol Pharmacy (Kiev) 2008**; 9**: 2–8.

10. Berger AC, Tang G, Alexander HR, Libutti SK. Endothelial monocyte-activating polypeptide II, a tumour-derived cytokine that plays an important role in inflammation, apoptosis, and angiogenesis. J Immunother 2000; **23**: 519–27.

11. Crippa L, Gasparri A, Sacchi A, ***et al.*** Synergistic damage of tumor vessels with ultra low-dose endothelial-monocyte activating polypeptide-II and neovasculature-targeted tumor necrosis factor-α. Cancer Res 2008; **68**: 1154–61.

12. Ivakhno SS, Kornelyuk AI. Cytokine-like activities of some aminoacyl-tRNA synthetases and auxiliary p43 cofactor of aminoacylation reaction and their role in oncogenesis. Exp Oncol 2004; **26**: 250–5.

13. Kornelyuk AI, Tas M, Dubrovsky A, Murray JC. Cytokine activity of the non-catalytic EMAP 2-like domain of mammalian tyrosyl-tRNA synthetase. Biopolymers and Cell (Kiev) 1999; **15**: 168–72.

14. Lans TE, van Horssen R, Eggermont AM, ten Hagen TL. Involvement of endothelial-monocyte activating polypeptide II in tumour necrosis factor-alpha-based anticancer therapy. Anticancer Res 2004; **24**: 2243–8.

15. Schwarz MA, Kandel J, Brett J, ***et al.*** Endothelial-monocyte activating polypeptide II, a novel antitumour cytokine that suppresses primary and metastatic tumour growth and induces apoptosis in growing endothelial cells. J Exp Med 1999; **190**: 341–54.

16. van Horssen R, Eggermont AM, ten Hagen TL. Endothelial monocyte-activating polypeptide-II and its functions in (patho)physiological processes. Cytokine Growth Factor Rev 2006; **17**: 339–48.

17. Lee YS, Han JM, Kang T, ***et al.*** Antitumour activity of the novel human cytokine AIMP1 in *in vivo* tumour model. Mol Cells 2006; **21**: 213–7.

18. Schwarz RE, Awasthi N, Konduri S, ***et al.*** EMAP II-based antiangiogenic-antiendothelial *in vivo* combination therapy of pancreatic cancer. Ann Surg Oncol 2010; **17**: 1442–52.

19. Schwarz RE, Schwarz MA. *In vivo* therapy of local tumour progression by targeting vascular endhothelium with EMAP II. J Surg Res 2004; **120**: 64–72.

20. Reznikov AG, Chaykovskaya LV, Polyakova LI, Kornelyuk AI. Antitumor effect of endothelial monocyte-activating polypeptide-II on human prostate adenocarcinoma in mouse xenograft model. Exp Oncol 2007; **29**: 267–71.

21. Dubrovsky AL, Brown J, Kornelyuk AI, ***et al.*** Bacterial expression of full-length and truncated forms of cytokine EMAP-2 cytokine-like domain of mammalian tyrosyl-tRNA synthetase. Biopolymers and Cell (Kiev) 2000; **16**: 229–35.

22. Bogden AE, Cobb WR, Lepage DJ, ***et al.*** Chemotherapy responsiveness of human tumours as first transplant generation in the normal mouse: six-day subrenal capsule assay. Cancer 1981; **48**: 10–20.

23. Ilagan R, Zhang LJ, Pottratz J, ***et al.*** Imaging androgen receptor function during flutamide treatment in the LAPC9 xenograft model. Mol Cancer Ther 2005; **4:** 1662–9.