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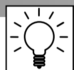

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Angiotensin peptides regulate angiogenic activity in rat anterior pituitary tumour cell cultures

Peptydy angiotensynowe regulują aktywność angiogenną w hodowlach komórkowych szczurzych guzów przedniego płata przysadki

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Abstract

Introduction: Angiogenesis has been shown to be necessary for the development and progression of solid tumours. VEGF is one of the crucial pro-angiogenic cytokines produced by the cells of many of the tumours examined, including various types of anterior pituitary adenomas. Angiotensin II (Ang II) is known to regulate the expression of VEGF in a variety of tissues both in the physiological and pathological conditions. Moreover, an association of the renin-angiotensin system (RAS) with oestrogen-induced vascular changes during the development of rat pituitary PRL-secreting adenoma has already been demonstrated.

The aim of the study was to determine the *in vitro* effects of angiotensin peptides (Ang II, Ang III and Ang IV) on the secretion of VEGF in two anterior pituitary adenoma cell cultures: the culture of the rat pituitary lactosomatotrope tumour cell line (GH3) and the primary culture of rat PRL-secreting tumour induced by diethylstilbestrol (DES).

Material and methods: GH3 and *prolactinoma* cells were cultured in an F-10 and an F-12 medium respectively and then placed into 24 multiwell plates (10^5 of GH3 cells/well and 10^6 of rat *prolactinoma* cells/well). After 12 hours of preincubation the cells underwent 24-hour treatment with Ang II, Ang III or Ang IV at final concentrations of 10^{-12}, 10^{-10}, 10^{-8} or 10^{-6}M and, in the case of the GH3 cells, combined treatment with Ang II (10^{-10}M) and specific AT1 or AT2 receptor antagonist (losartan or PD123319 respectively at a concentration of 10^{-8} or 10^{-7} M). The concentration of VEGF in the supernatant collected was determined using specific ELISA assay kits. Statistical evaluation was performed using Student's test and analysis of variance (ANOVA). Differences were considered significant if $p < 0.05$.

Results: The incubation of both GH3 cells and rat adenoma cells with Ang II, Ang III or Ang IV at concentrations of 10^{-12} – –10^{-8}M resulted in a significant increase in VEGF concentration in the culture medium. Exposure of GH3 cells to Ang III or Ang IV at concentrations of 10^{-6}M led to a significant inhibition of cytokine release, and Pearson's correlation curve showed a tendency for Ang II at concentrations of more than 10^{-6}M to inhibit VEGF secretion in primary *prolactinoma* cell culture. The stimulatory influence of Ang II on VEGF secretion in GH3 cell culture was negated by losartan or by PD123319 in both concentrations tested.

Conclusions: Ang II, Ang III and Ang IV affect the secretion of VEGF in cultures of the rat lactosomatotrope GH3 cell line and primary rat *prolactinoma* cells. Both AT1 and AT2 receptors mediate the stimulatory action of Ang II on the cytokine release in GH3 cell culture. The mechanism of the observed anti-angiogenic effects of angiotensin peptides remains unexplained.

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***Key words**: pituitary adenomas, angiotensins, VEGF, cell cultures*

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Streszczenie
Wstęp: Angiogeneza to niezbędny etap w procesie formowania i progresji guzów litych. VEGF należy do kluczowych cytokin proangiogennych, syntetyzowanych przez komórki wielu badanych dotychczas guzów, włączając różne typy gruczolaków przysadki. Angiotensyna II należy do czynników regulujących ekspresję VEGF w różnych tkankach, zarówno w warunkach fizjologii, jak i patologii. Co więcej, udowodniono związek układu renina–angiotensyna (układu RA) z indukowanym estrogenami wzrostem naczyń w procesie formowania doświadczalnego guza prolaktynowego przysadki u szczura. Celem pracy było zbadanie wpływu peptydów angiotensynowych (ang II, ang III i ang IV) na wydzielanie VEGF w dwóch hodowlach komórkowych gruczolaka przysadki: w ciągłej hodowli linii guza laktosomatotropowego GH3 oraz w pierwotnej hodowli indukowanego dietylstilbestrolem (DES) guza prolaktynowego szczura.
Materiał i metody: Komórki GH3 oraz komórki *prolactinoma* hodowano odpowiednio w mediach F-10 i F-12, a następnie rozsiewano do studzienek 24-dołkowych płytek hodowlanych (10^5 komórek GH3 na studzienkę i 10^6 komórek gruczolaka na studzienkę). Po 12 godzinach preinkubacji komórki inkubowano przez 24 godziny w obecności ang II, ang III lub ang IV w stężeniach 10^{-12}, 10^{-10}, 10^{-8} lub 10^{-6}M lub, w przypadku komórek GH3, w obecności ang II (10^{-10}M) w połączeniu ze specyficznym antagonistą receptora AT1 losartanem lub receptora AT2 — PD123319 w stężeniach 10^{-8} lub 10^{-7}M. Stężenie VEGF w medium hodowlanym oznaczano, wykorzystując metodę ELISA. Analizy statystycznej dokonywano przy użyciu testu *t*-Studenta oraz na podstawie analizy wariancji (ANOVA), dla poziomu istotności $p < 0.05$.
Wyniki: Inkubacja komórek GH3 lub komórek pierwotnej hodowli guza prolaktynowego w obecności ang II, ang III lub ang IV w stężeniach 10^{-12}– 10^{-8}M prowadziła do wzrostu stężeń VEGF w medium hodowlanym. Przeciwnie, po ekspozycji komórek GH3 na działanie ang III lub ang IV w stężeniach 10^{-6}M dochodziło do zahamowania wydzielania cytokiny, a krzywa korelacji Persona wykazała dodatkowo tendencję do hamowania wydzielania VEGF w pierwotnej hodowli *prolactinoma* przez ang II w stężeniach przekraczających 10^{-6}M. Pobudzający wpływ ang II na sekrecję VEGF przez komórki GH3 znoszony był zarówno przez losartan, jak i PD123319.
Wnioski: Ang II, ang III i ang IV wpływają na wydzielanie VEGF przez komórki GH3 oraz komórki gruczolaka prolaktynowego szczura. Zarówno receptory AT1, jak i AT2 pośredniczą w proangiogennym efekcie ang II w hodowli GH3. Mechanizm obserwowanego, hamującego oddziaływania peptydów angiotensynowych na sekrecję VEGF pozostaje niewyjaśniony.

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***Słowa kluczowe**: gruczolaki przysadki, angiotensyny, VEGF, hodowle komórkowe*

Introduction

Angiogenesis is the process of formation of new blood vessels from pre-existing vasculature [1]. Vascular endothelial growth factor (VEGF) is one of the essential pro-angiogenic cytokines. This glycoprotein is known to be a strong specific mitogen for endothelial cells (EC) *in vitro* as well as a pro-angiogenic and vascular permeability-stimulating factor *in vivo* [2].

Neovascularisation has been shown to be crucial for the development of solid tumours beyond a few millimetres and for determining further tumour progression and spread [3]. VEGF mRNA has been shown to be upregulated in a variety of the tumours that have been examined, with invariable expression in tumour cells and, additionally, in the surrounding stroma and tumour vasculature [4–7]. Nevertheless, there is still controversy regarding the role of angiogenesis in pituitary tumour formation and growth [8]. In contrast to the results of studies on other tissues, the available data indicate lower vascular densities of pituitary adenomas compared to a normal non-tumorous gland [9–11]. However, the fundamental functional consequence of pituitary angiogenesis may be connected with the ingrowth of blood vessels from the systemic circulation [12–14]. The direct arterial blood supply uncouples the tumour from the portal blood vessel system, leading the cells to escape from hypothalamic control [15]. The importance of angiogenesis for pituitary tumour development is suggested by the growing number of experimental studies and clinical observations. A relationship has been reported between the degree of vascularisation and tumour invasiveness, particularly apparent in aggressive macroprolactinomas. Higher vascular densities were found in rare pituitary carcinomas compared to benign adenomas [8]. Increased vasculogenesis appeared during the development of oestrogen-induced rat *prolactinoma* [16]. Serum concentrations of pro-angiogenic factors, including VEGF, were elevated in the peripheral blood of patients with pituitary adenomas [17].

The involvement of the rennin–angiotensin system (RAS) in the regulation of angiogenesis has already been demonstrated by many authors. They observed angiotensin II (Ang II)-stimulated vessel growth in the mouse and rat sponge implant model of angiogenesis, developing chick chorioallantoic membrane, the cornea and rat cremaster muscle [18–21]. Ang II was shown to stimulate VEGF expression in human vascular smooth muscle cells (VSMCs), cardiac endothelial cells and renal mesangial cells, to promote VEGF receptor expres-

sion in bovine retinal microcapillary endothelial cells and retinal pericytes, and to potentiate VEGF-induced EC growth and tube formation [22–26]. This peptide has also been implicated in physiological angiogenesis induced by electrical stimulation or exercise [27, 28]. Experimental and clinical studies have also demonstrated the essential role of RAS in pathological angiogenesis and its potential association with such states as atherosclerosis, diabetic proliferative retinopathy and nephropathy. Treatment with angiotensin receptor blockers resulted in the suppression of retinal neovascularisation and reduction of VEGF expression by podocytes in diabetic rats [29, 30]. Feman et al. and Danser at al. revealed that the serum and intraviteral levels of prorenin, angiotensin-converting enzyme (ACE) and Ang II were increased in patients with proliferative diabetic retinopathy [31, 32]. Furthermore, patients with type 1 diabetes were less likely to develop proliferative diabetic retinopathy when treated with the ACE inhibitor lisinopril [33].

Increasing lines of evidence indicate an association between RAS and the neoangiogenesis and growth of solid tumour [34]. With respect to the pituitary, Pawlikowski et al. suggest an involvement of Ang II in the mechanism of estrogen-induced vascular changes during the development of experimental rat pituitary PRL-secreting adenoma [35]. However, the role of angiotensin peptides in the regulation of the angiogenic activity of rat pituitary adenoma cells *in vitro* has not been determined. The purpose of our study was to assess the influence of Ang II and its derivatives Ang III and Ang IV on the secretion of VEGF in two cell cultures, the culture of permanent rat lactosomatotrope tumour cell line GH3 and the primary culture of rat PRL-secreting adenoma. We also examined the potential association of the effects of Ang II with two main angiotensin receptor subtypes, the AT1 receptor and the AT2 receptor.

Material and methods

Cell cultures

GH3 cell culture

The rat lactosomatotrope cell line GH3 was obtained using ATCC, LGC Promochem. Continuous GH3 cell culture was maintained in culture flasks (Nunc Eas Y Flask 25 cm^2, NUNC). All the cells were grown in Ham's F-10 medium supplemented with 1.2 g/l Sodium Bicarbonate (Sigma), 100 U/ml Penicillin and 100 mg/ml Streptomycin Solution (Sigma), 15% heat-inactivated horse serum (Sigma) and 2.5% heat-inactivated foetal bovine serum (FBS, Biochrom, KG) at 37^{0}C and in a humidified atmosphere of 95% air and 5% CO_2. Every 7 days, the cells were harvested after a 2-min incubation at 37^{0}C in the presence of trypsin-EDTA (0.05 or 0.02% respectively) in Hanks balanced solution (Sigma). The cells were washed twice in complete F-10 medium and after the last centrifugation seeded at 1 x 10^5 cells in 5 ml of fresh medium.

Primary cell culture of rat pituitary adenoma

The pituitary tumours were grown in oestrogen-sensitive male four-week-old Fischer 344 rats. Silastic capsules containing 10 mg of diethylstilbestrol (DES, Sigma) were implanted subcutaneously in the lumbar region. Eight weeks after the DES implantation the animals were sacrificed and the pituitary tumours aseptically removed and placed in Ham's F-12 medium supplemented with 1.176 g/l sodium bicarbonate (Sigma), 100 U/ml penicillin and 100 mg/ml streptomycin solution (Sigma), 10% heat-inactivated horse serum (Sigma) and 3% heat-inactivated foetal bovine serum (FBS, Biochrom, KG). The suspension of the pituitary cells was obtained by mechanical dispersion and subsequent treatment for 30 minutes with collagenase (2 mg/ml) and hiluronidase (2 mg/ml) at 37^{0}C followed by mechanical agitation. The cells were then triple washed in complete F-10 medium and after the last centrifugation seeded in fresh medium. The procedure yielded a population of 95% viable cells.

Experiments

GH3 cells were subjected to the trypsynisation process, suspended at 10^6/ml in complete F-10 medium, and 100 ml aliquots of cell suspension (10^5 cells) were placed in the wells of the cell culture plates (24 Cell Culture Cluster Dish, Nuclon Multidishes, Nunc). All the plated cells were incubated for 12 h (5% CO_2, 37°C, 95% humidity), and after this preincubation period the cells in the appropriate wells were treated with the following substances diluted in fresh serum-free medium (100 ml/well): angiotensin II (Ang II, Sigma) at final concentrations of 10^{-12}, 10^{-10}, 10^{-8} and 10^{-6}M, angiotensin III (Ang III, Bachem) at final concentrations of 10^{-12}, 10^{-10}, 10^{-8} and 10^{-6}M, angiotensin IV (Ang IV, Bachem) at final concentrations of 10^{-12}, 10^{-10}, 10^{-8} and 10^{-6}M, 10^{-10}M Ang II + losartan (Merck) at a concentration of 10^{-8} or 10^{-7}M, 10^{-10}M Ang II + PD123319 (Sigma) at a concentration of 10^{-8} or 10^{-7}M, losartan at a final concentration of 10^{-8} or 10^{-7}M, and PD123319 at a final concentration of 10^{-8} or 10^{-7}M. The same volume of fresh serum-free culture medium was added to the control wells.

Rat pituitary adenoma cells were plated in 24-well plates (24 Cell Culture Cluster Dish, Nuclon Multidishes, Nunc) at 2 × 10^6 cell/well and incubated for 12 h (5% CO_2, 37^{0}C, 95% humidity). The tested substances were then added to the appropriate wells: Ang II (Sigma) or Ang III (Bachem) or Ang IV (Bachem) at final concentrations of 10^{-12}, 10^{-10}, 10^{-8} and 10^{-6}M. The same

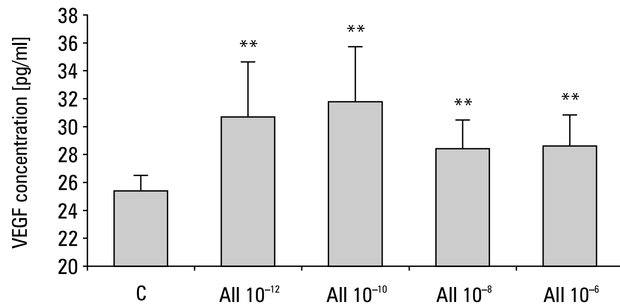

Figure 1. *The influence of angiotensin II (AII) on the secretion of VEGF in GH3 cell culture after 24 hours of incubation. X ± SEM; **p < 0.01* vs. *C (control)*

Rycina 1. *Wpływ angiotensyny II (AII) na wydzielanie VEGF w hodowli komórek GH3 po 24 godzinach inkubacji. X ± SEM; **p < 0,01* vs. *C (kontrola)*

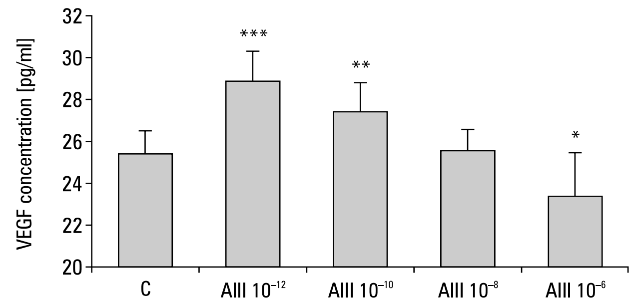

Figure 2. *The influence of angiotensin III (AIII) on the secretion of VEGF in GH3 cell culture after 24 hours of incubation. X ± SEM; *p < 0.05* vs. *C (control), **p < 0.01* vs. *C, ***p < 0.001* vs. *C*

Rycina 2. *Wpływ angiotensyny III (AIII) na wydzielanie VEGF w hodowli komórek GH3 po 24 godzinach inkubacji. X ± SEM; *p < 0,05* vs. *C (kontrola), **p < 0,01* vs. *C, ***p < 0,001* vs. *C*

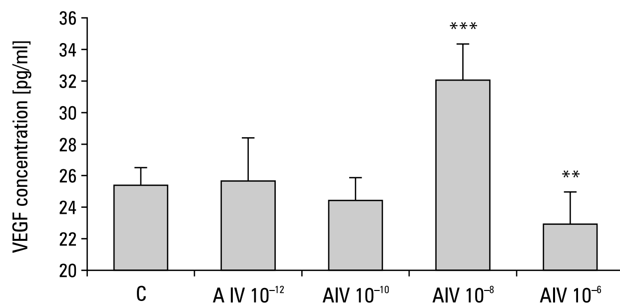

Figure 3. *The influence of angiotensin IV (AIV) on the secretion of VEGF in GH3 cell culture after 24 hours of incubation. X ± SEM; **p < 0.01* vs. *C (control), ***p < 0.001* vs. *C*

Rycina 3. *Wpływ angiotensyny IV (AIV) na wydzielanie VEGF w hodowli komórek GH3 po 24 godzinach inkubacji. X ± SEM; **p < 0,01* vs. *C (kontrola), ***p < 0,001* vs. *C*

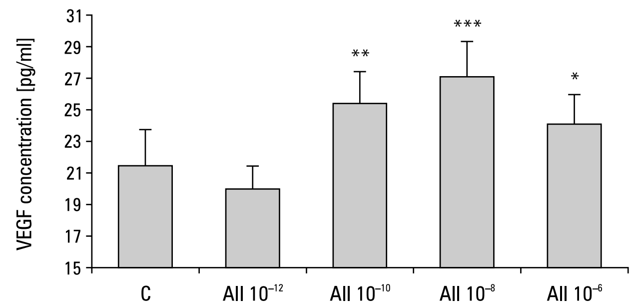

Figure 4. *The influence of angiotensin II (AII) on the secretion of VEGF in primary rat PRL-secreting adenoma cell culture after 24 hours of incubation. X ± SEM; *p < 0.05* vs. *C (control), **p < 0.01* vs. *C, ***p < 0.001* vs. *C*

Rycina 4. *Wpływ angiotensyny II (AII) na wydzielanie VEGF w pierwotnej hodowli komórkowej guza prolaktynowego szczura po 24 godzinach inkubacji. X ± SEM; *p < 0,05* vs. *C (kontrola), **p < 0,01* vs. *C, ***p < 0,001* vs. *C*

volume of fresh serum-free culture medium was added to the control wells.

VEGF determination

All the cells were treated with the substances for 24 hours. The supernatant was than collected from the wells and the secreted VEGF isoforms, including the dominating mouse/rat VEGF164, were measured in terms of pg/ml using specific ELISA assay kits for mouse/rat VEGF (Mouse VEGF Immunoassay, Quantikine M, R&D System, USA).

Statistical evaluation

The results are expressed as means ± SD. The normality of the distribution of the results was examined by the Student test. Comparisons of individual groups were evaluated by analysis of variance (ANOVA). Differences were considered significant if $p < 0.05$.

Results

The results of the quantitative analysis and the statistical evaluation of these results are presented in Figures 1–9. The incubation of GH3 cells with Ang II at concentrations of 10^{-12}, 10^{-10}, 10^{-8} or 10^{-6}M, Ang III at concentrations of 10^{-12} or 10^{-10}, or Ang IV at a concentration of 10^{-8}M resulted in a significant increase in VEGF secretion, whereas exposure of cells to Ang III or Ang IV at concentrations of 10^{-6}M led to a significant inhibition of cytokine release (Fig. 1–3). The exposure of rat pituitary adenoma cells to Ang II at concentrations of 10^{-10}, 10^{-8} or 10^{-6}M, Ang III at a concentration of 10^{-8}, or Ang IV at a concentration 10^{-8}M resulted in a significant elevation of VEGF concentration in the culture medium (Fig. 4–6). However, Person's correlation curve showed a tendency

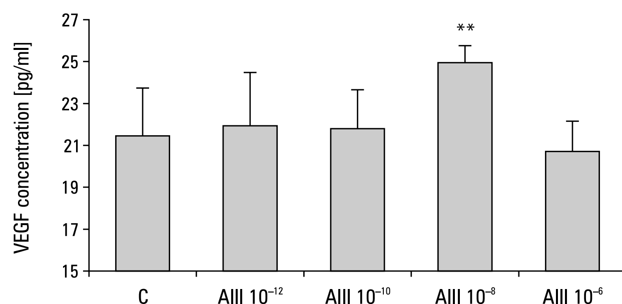

Figure 5. *The influence of angiotensin III (AIII) on the secretion of VEGF in primary rat PRL-secreting adenoma cell culture after 24 hours of incubation. X ± SEM;* $**p < 0.01$ vs. *C (control)*

Rycina 5. *Wpływ angiotensyny III (AIII) na wydzielanie VEGF w pierwotnej hodowli komórkowej guza prolaktynowego szczura po 24 godzinach inkubacji. X ± SEM;* $**p < 0,01$ vs. *C (kontrola)*

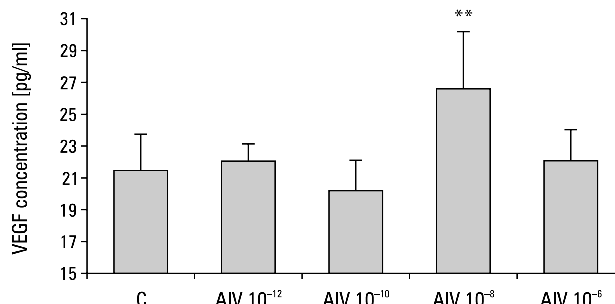

Figure 6. *The influence of angiotensin IV (AIV) on the secretion of VEGF in primary rat PRL-secreting adenoma cell culture after 24 hours of incubation. X ± SEM;* $**p<0.01$ vs. *C (control)*

Rycina 6. *Wpływ angiotensyny IV (AIV) na wydzielanie VEGF w pierwotnej hodowli komórkowej guza prolaktynowego szczura po 24 godzinach inkubacji. X ± SEM;* $**p<0,01$ vs. *C (kontrola)*

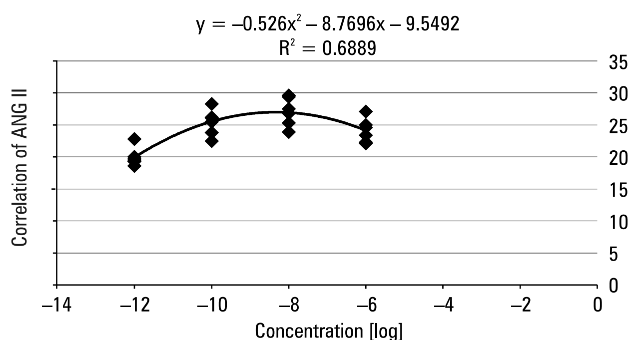

Figure 7. *Correlation between concentrations of angiotensin II (Ang II) and VEGF levels in primary rat prolactinoma cell culture after 24 hours of incubation with Ang II.* $y = ax + b$ *— simple regression equation, r — correlation coefficient,* $p < 0.05$

Rycina 7. *Korelacja stężeń VEGF i dawek ang II w pierwotnej hodowli komórkowej guza prolaktynowego szczura poddanej 24-godzinnej inkubacji z ang II.* $y = ax + b$ *— równanie prostej regresji, r — współczynnik korelacji,* $p < 0,05$

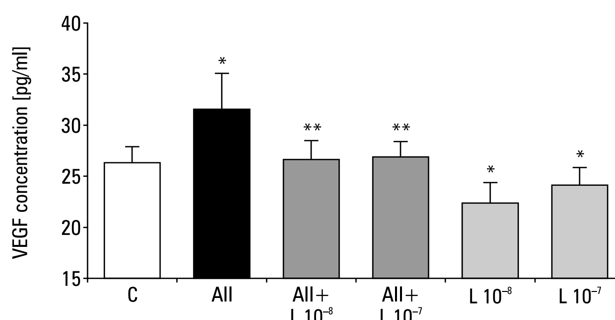

Figure 8. *The influence of 24 hours of treatment of GH3 cells with Ang II (AII,* 10^{-10}*M), alone or with the combination of Ang II and specific AT1 receptor antagonist losartan (L), on VEGF secretion in culture. X ± SEM;* $*p < 0.05$ vs. *C (control),* $**p < 0.05$ vs. *AII*

Rycina 8. *Wpływ dodania losartanu (L) do angiotensyny II (AII,* 10^{-10}*M) na wydzielanie VEGF w hodowli komórkowej linii GH3 po 24 godzinach inkubacji, X ± SEM;* $*p < 0,05$ vs. *C (kontrola),* $**p < 0,05$ vs. *AII*

for Ang II at concentrations of more than 10^{-6}M to inhibit the VEGF secretion (Fig. 7).

The stimulatory effect of Ang II (10^{-10}M) on VEGF secretion in GH3 cell culture was cancelled by losartan or PD123319 in both the concentrations tested. Furthermore, incubation of cells with losartan (10^{-8} or 10^{-7}M) or PD123319 (10^{-7}M) alone was followed by a decrease in VEGF concentration in the culture medium (Fig. 8 and 9).

Discussion

VEGF expression has been shown in the rat, ovine, mouse and human pituitary. In a normal gland this glycoprotein is thought to be predominantly produced by folliculostellate cells, but its presence in epithelial cells and co-localisation with anterior pituitary hormones have also been demonstrated [36]. The studies on animal models revealed overexpression of VEGF and upregulation of VEGF receptor (VEGFR-2) during oestrogen-induced pituitary tumour angiogenesis in Fischer 344 rats [37, 38]. In human pituitary adenoma a positive correlation between cytokine expression and tumour behaviour has been suggested by some authors [39].

Increasing evidence suggests that a pivotal role is played by endocrine pituitary tumour cells in the regulation of the angiogenic process essential for tumour progression. *In vitro* VEGF was basally secreted by rodent pituitary tumour cells, including folliculostellate TtT/GF, corticotrope AtT20, gonadotrope αT3-1 and lactosomatotrope GH3, as well as in hormone-inactive, corticotrope, somatotrope and lactotrope human pituitary adenoma cell cultures [40]. The presence of VEGF

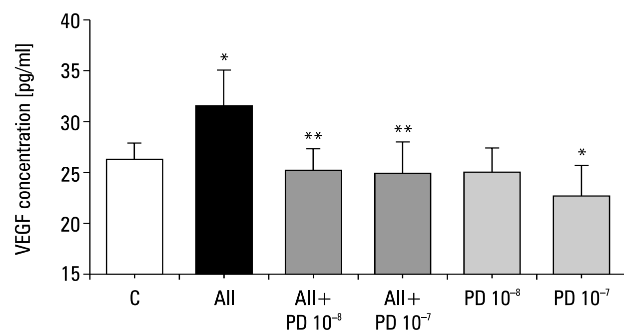

Figure 9. *The influence of 24 hours of treatment of GH3 cells with Ang II (AII, 10^{-10}M), alone or with the combination of Ang II and specific AT2 receptor antagonist PD123319 (PD), on VEGF secretion in culture. X ± SEM; *p < 0.05* vs. *C (control), **p < 0.05* vs. *AII*

Rycina 9. *Wpływ dodania PD123319 (PD) do angiotensyny II (AII, 10^{-10}M) na wydzielanie VEGF w hodowli komórkowej linii GH3 po 24 godzinach inkubacji, X ± SEM; *p < 0,05* vs. *C (kontrola), **p < 0,05* vs. *AII*

in secretory granules, the Golgi apparatus and rough endoplasmic reticulum of GH3 cells has been confirmed by immunoelectromicroscopy [41]. Lohrer et al. revealed, that VEGF production by TtT/GF cells was augmented in response to pituitary adenylate cyclase polypeptide-38 (PACAP-38), interleukine-6 (IL-6), transforming growth factor-α (TGF-α), insulin-like growth factor-I (IGF-I) and somatostatin analogue octreotide, whereas in GH3, AtT20 and αT3-1 cultures basal cytokine levels were not enhanced by any of the stimuli tested. TGF-α, PACAP-38 and 17β-oestradiol increased VEGF release in human hormone-inactive, somatotrope and PRL-secreting tumour cell cultures respectively [40]. Since all the factors examined are known to be produced by pituitary adenoma cells, these results possibly indicate complex auto and paracrine regulation of VEGF-dependent angiogenesis during pituitary tumorigenesis.

The present study demonstrates, for the first time to our knowledge, the involvement of angiotensin peptides in the regulation of the angiogenic activities of the rat anterior pituitary tumour cells. We revealed that Ang II, Ang III and Ang IV, at concentrations of 10^{-12}–10^{-8}M, were able to stimulate VEGF release by both GH3 cells and by the cultured rat prolactinoma cells. These results are not surprising in the context of previous observations. Ang II was found to enhance *in vitro* VEGF secretion in many cell cultures, including human VSMCs, human mesangial cells, rat heart endothelial cells and bovine retinal microcapillary pericytes [22–24, 26]. The Ang II derivative Ang IV induced DNA synthesis and lung endothelial cell proliferation [42]. The pro-angiogenic effect of exogenous Ang II in pituitary lactosomatotrope tumour cell culture appears to be dependent on both AT1 and AT2 receptors. As we demonstrated, the specific antagonist of the AT1 receptor losartan or the specific AT2 receptor blocker PD123319 cancelled the stimulatory action of the peptide on VEGF secretion by GH3 cells. Furthermore, we also noticed the inhibitory influences of both AT1 and AT2 receptor antagonist on the basal VEGF secretion in GH3 cell culture. We speculate that this phenomenon reflects the auto-paracrine regulation of the pro-angiogenic activities of GH3 cells by endogenous angiotensin peptides. Losartan and PD123319 possibly enable locally produced angiotensin peptides to stimulate VEGF secretion via the AT1 and AT2 receptors. Such an explanation is highly relevant, especially with respect to immunohistochemical and molecular studies demonstrating the presence of RAS components in GH3 cells [43]. It is noteworthy that our results are consistent with the results of a previous *in vivo* study. Pawlikowski et al. [35] noticed that an oestrogen-induced increase in vessel area in the anterior pituitary of DES-treated rats was blocked by the AT1 receptor antagonist losartan and, to a lesser degree, by the AT2 receptor antagonist PD123319.

The essential role of the AT1 receptor in vascular growth-promoting effects has already been demonstrated by many authors. This receptor subtype mediated the induction of VEGF receptor KDR expression and the potentiation of VEGF mitogenic effects in Ang II-stimulated bovine retinal microcapillary endothelial cells [25]. The AT1 antagonist losartan negated the stimulatory influences of Ang II on VEGF expression in human VSMCs, cardiac endothelial cells and renal mesangium [22–24]. *In vivo* it was demonstrated that the pathway involving the AT1 receptor was engaged in an increase in vessel density in the cremaster muscle of rats infused continuously with Ang II and in VEGF-dependent electrically stimulated angiogenesis in skeletal muscle [21, 28]. AT1 receptor blockade prevented retinal neovascularisation in an animal model of retinopathy of prematurity and suppressed hind limb ischemia-induced angiogenesis in mice [44, 45]. In contrast, it has frequently been suggested that the AT2 receptor exerts an influence that is antagonistic to the AT1 receptor. The anti-angiogenic activity of the AT2 receptor in hypoxia-induced angiogenesis has been reported [46]. Stoll et al. observed an AT2-dependent anti-proliferative effect which offsets the growth-promoting action mediated by AT1 in the coronary endothelial cells [47]. Fujiyama et al. found that the AT1 receptor mediates endothelial growth factor receptor (EGFR) transactivation, heparin binding EGF-like growth factor (HB-EGF) release and subsequent induction of VEGF and angiopoietin expression in cardiac microvascular endothelial cells. The AT2 receptor was shown to halt AT1-induced

EGFR phosphorylation and to attenuate angiogenic activity by AT1 [48]. Furthermore, AT2 receptor stimulation counteracted VEGF-stimulated endothelial cell migration and *in vitro* tube formation in three cell cultures, including human coronary artery, human dermal microvascular endothelial cells and the permanent human endothelial cell line EA.hy926 [49]. This receptor subtype was also responsible for inhibition of AT1--induced smooth muscle cell (SMC) migration in AT2 transfected SMCs [50]. AT2 receptor blockade enhanced the pro-angiogenic effect of Ang II in the rat subcutaneous sponge granuloma [18].

However, there is increasing evidence that the AT2 receptor may exert growth-promoting and pro-angiogenic effects. Hence this receptor mediated cardiac and aortic hypertrophy resulting from pressure overload and Ang II treatment respectively [51, 52]. Rizkalla at al. revealed that blockade of both AT1 and AT2 receptors led to a significant decrease in Ang II-induced VEGF and its receptor KDR expression in the rat kidney and to concomitant retardation of Ang II-stimulated glomerular cell proliferation [53]. In addition the AT1 and AT2 receptors were responsible for increased retinal expression of VEGF in experimental diabetic rats and in Ang II treated rats [54]. Our results extend these findings to effects on VEGF secretion in GH3 cell culture, providing evidence that both AT1 and AT2 receptor subtypes may mediate the stimulatory effect of exogenous and possibly endogenous angiotensin peptides on VEGF release by rat pituitary tumour cells. The documented pro-angiogenic and anti-angiogenic AT2-associated effects both indicate that the so-called cell-type-dependent angiotensin activities are instead determined by post-receptor events induced in a particular tissue. A number of recent reports suggest that AT2 and AT1 share, at least in part, common signalling pathways and may thus participate positively in the regulation of cellular proliferation and angiogenesis [46].

Nevertheles, in addition to the pro-angiogenic effects of peptides, we noticed that incubation of GH3 cells with Ang III or Ang IV at a concentration of 10^{-6}M led to a significant decrease in VEGF levels in the culture medium. Furthermore, Pearson's correlation curve showed a tendency for Ang II at concentrations of more than 10^{-6}M to inhibit VEGF secretion in primary rat prolactinoma culture. These stimulatory and inhibitory effects on VEGF release may reflect the biphasic influence of some angiotensin peptides on angiogenesis. A similar phenomenon was described by Banerjee et al. with respect to oestrogens. The authors observed an increase in endothelial cell adhesion and proliferation after chronic exposure to nanomolar concentrations of oestradiol (E2), whereas both cellular adhesion and proliferation were inhibited by micromolar concentrations of E2 [55]. The present study has not defined the mechanisms whereby angiotensins may inhibit VEGF release. The possible activation of non-specific mechanisms by supraphysiological doses of exogenous peptides should be taken into consideration. It can also be supposed that the inhibitory effect is exerted by Ang II-derived peptides via non-AT1, non-AT2 receptors such as AT4 or Ang 1–7. Such a possibility is supported by the observation that the effect discussed is exerted at a lower concentration by Ang III and Ang IV in comparison with Ang II. However, this presumption needs to be confirmed in further studies.

The results reported here strongly suggest that an important role is played by RAS in pituitary tumour angiogenesis, as the overexpression of VEGF seems to reflect a switch to an angiogenic phenotype, crucial for the initiation of angiogenesis [56]. Moreover, VEGF may also stimulate the growth of tumoral cells directly, as was demonstrated recently in human pituitary adenoma [57, 58] and glioma [59] cells in culture. It is possible that in the early stage of pituitary tumorigenesis, oestrogens or other tumorigenic factors stimulate local or systemic angiotensin peptides to induce VEGF expression via the AT1 and AT2 receptors. Nevertheless, the same peptides, overproduced by pituitary adenoma cells, may lead to suppression of VEGF production and thus contribute to the inhibition of angiogenesis and limitation of further tumour progression. Such a hypothesis is possible in relation to the observed biphasic influence of angiotensins on VEGF release by rat pituitary tumour cells in culture. This is also probable in the context of the low vascular density and the usually slow growth rate of most anterior pituitary tumours.

In summary, Ang II, Ang III and Ang IV affect VEGF secretion in GH3 and primary rat *prolactinoma* cell cultures. Both AT1 and AT2 receptors mediate the stimulatory actions of exogenous and possibly endogenous peptides on VEGF release. However, this *in vitro* study must be interpreted with caution when extrapolating these findings to an *in vivo* context. Further studies, including *in vivo* study and clinical trials, are necessary to strengthen the hypothesis of the pro-angiogenic effects of angiotensin peptides in the anterior pituitary and to establish the potentially beneficial role of AT1 receptor antagonists in complementary treatment for patients with PRL-secreting tumours.

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