Microvascular density and vascular endothelial growth factor expression in normal pituitary tissue and pituitary adenomas

P. Viacava*, M. Gasperi**, G. Acerbi***, L. Manetti**, E. Cecconi**, A.G. Bonadio*, A.G. Naccarato*, F. Acerbi***, G. Parenti***, I. Lupi**, M. Genovesi**, and E. Martino**

*Department of Oncology, Section of Pathology, **Department of Endocrinology and ***Department of Neuroscience, Section of Neurosurgery, University of Pisa, Pisa, Italy

ABSTRACT. Microvessel density (MVD) represents a measure of angiogenesis and may be used as an indicator of neoplastic aggressiveness. Vascular endothelial growth factor (VEGF) plays a pivotal role as angiogenic promoter by stimulating endothelial cell proliferation and migration and enhancing vascular permeability. The aim of this study was to investigate MVD and VEGF expression in human pituitary adenomas and normal pituitary gland tissues by immunohistochemistry, and to correlate data with clinical characteristics. Fragments from 46 pituitary adenomas (18 non-functioning, 12 ACTH-secreting, 12 GH-secreting, 4 PRL-secreting) and 19 specimens of normal anterior pituitary gland obtained at surgery were evaluated. MVD in normal anterior pituitary was significantly higher than in tumors (69.2±28.5 *vs* 29.3±19.7; $p<0.0001$). Within adenomas, no difference was found in MVD when different histotype, size, sex, age, rate of recurrence or medical pre-surgical treatment were considered. The degree of vascularity was somewhat related only to clinical invasiveness, as evaluated by pre-surgical MRI grading (grade 0 $p<0.05$ *vs* grade 1 and *vs* grade 2). No statistically significant difference in VEGF expression was found between normal tissue and adenomas and among tumors of different histotype ($p=0.3978$). Size, sex, age, rate of recurrence and medical pre-surgical treatment did not influence VEGF expression. No correlation was found between MVD and VEGF expression. In conclusion, MVD was reduced in pituitary adenomas with respect to normal gland. VEGF expression is however well preserved in adenomas and this might contribute to adequate tumoral vascular supply with complex mechanisms other than endothelial cells proliferation.

(J. Endocrinol. Invest. *26:* 23-28, 2003)

©2003, Editrice Kurtis

INTRODUCTION

Angiogenesis, the formation of neovessels from existent vascularization, is a fundamental event in the growth and metastatic capability of malignant neoplasia (1). In some tumoral models such as breast (2) and lung (3), angiogenesis, evaluated as microvessel density (MVD), is positively correlated with some negative prognostic parameters, namely reduced overall survival, metastases and recurrence rate.

Key-words: MVD, CD-34, VEGF, pituitary adenomas.

Correspondence: Dr. Maurizio Gasperi, Dipartimento di Endocrinologia, Ospedale di Cisanello, Via Paradisa 2, 56124 Pisa, Italy.

E-mail: mgasperi@endoc.med.unipi.it

Accepted June 10, 2002.

Angiogenesis is a complex process regulated by a series of promoting and/or inhibiting factors: the balance of these factors determines the degree of tumor vascularization.

A powerful angiogenic promoter is vascular endothelial growth factor (VEGF) that induces neovessel formation by increasing vascular permeability, stimulating endothelial cell proliferation and enhancing endothelial cell migration (4-6).

VEGF has been reported as an independent prognostic factor in patients with breast cancer (7), squamous cell lung cancer (8) and gastrointestinal cancer (9). However, despite the angiogenic action of VEGF, its expression is not always positively correlated with tumor vascular density.

The role of angiogenesis and VEGF in the biology of pituitary tumors is poorly known. Pituitary adenomas

are a heterogeneous group of tumors: most of them are slow growing benign tumors, but some of them invade surrounding anatomical structures and, in very rare instances metastatize. In spite of the variable clinical behavior, these tumors are histologically similar and, at the moment, no specific prognostic marker allows the identification of the aggressive subgroups. The study of angiogenesis and VEGF expression in pituitary tumors may offer information not only of prognostic value but also of therapeutic significance, due to the future possibility of using anti-angiogenic and anti-VEGF drugs. Very few studies about MVD of pituitary adenomas have insofar been reported (10-13). Interestingly, most tumors showed a lower degree of vascularization in comparison with normal pituitary tissue, with the exception of invasive macroprolactinomas which are apparently characterized by an increased vascularization (12). To our knowledge, few data are known about VEGF expression in normal and pathologic human pituitary gland tissue (14-17).
The aim of the present study was to investigate MVD and VEGF expression in pituitary adenomas and normal pituitary gland tissue.

MATERIALS AND METHODS

Specimens

Tumor specimens were obtained at surgery from 46 patients (18 males and 28 females, age range 18-78 yr) affected with pituitary adenomas between 1995-2001. No patient had undergone radiotherapy. The tumors included: 18 non-functioning pituitary macroadenomas (NF); 12 (5 macroadenomas, 7 microadenomas) ACTH-secreting adenomas; 12 (10 macroadenomas, 2 microadenomas) GH-secreting adenomas and 4 (3 macroadenomas, 1 microadenoma) PRL-secreting adenomas. Nineteen fragments of normal anterior pituitary gland collateral to adenomas were also studied. Histological examination and immunohistochemistry for anterior pituitary hormones had been performed previously and were used to characterize each tumor type, together with clinical, endocrine and radiological data. In particular, pre-surgical MRI was used to assess 4 grades of invasiveness, defined as absence of radiological evidence (grade 0), intrasellar localization (grade 1), minimal extrasellar involvement (grade 2) and extrasellar spreading (grade 3).

Immunohistochemistry

Tissues were fixed in 10% formalin and embedded in paraffin. Five µm sections were stained with hematoxylin-eosin for histological evaluation. Five additional µm sections were used for immunohistochemistry.
The sections were incubated with the following primary antibodies: a) mouse anti-human CD34 (QB-END10, Dako dilution 1:100), b) rabbit anti-human VEGF (Oncogene Research Products, Cambridge, MA, USA, dilution 1:100). Incubation time was 12 h at 4 C. Five µm sections were deparaffinized in xylene and rehydrated in alcohol. Endogenous peroxide activity was blocked by incubating the slides in 1% hydrogen peroxide in methanol for 10 min. In order to unmask the antigens, the slides were microwave-treated in 10 mM citrate buffer, pH 6 for 10 min. The sections were incubated with primary antibodies and, then reacted with biotin-labelled secondary antibody (dilution 1/500) and avidin-biotin-complex (Vector Burlingame, CA, USA) for 30 min respectively. 3-3' diaminobenzidine tetrahydrochloride was used as chromogen. Finally, sections were counterstained with hematoxylin, dehydrated and mounted.
Vascular endothelial cells of breast carcinoma and squamous cell carcinoma of the neck were used as positive control for CD34 and VEGF, respectively (18, 19). Negative controls were obtained by omission of the incubation step with primary antibodies and substitution of a non-immune serum in place of the primary antibody. In order to test the specificity of the anti-VEGF antibody a pre-incubation step of this antibody with recombinant human $VEGF_{165}$ was performed before immunohistochemical study: VEGF peptide was added to aliquots of diluted primary antibody antiserum, and these aliquots were incubated overnight at 4 C. The supernatant obtained after centrifugation (2,000 g for 30 min at 4 C) was used for testing.

Evaluation of parameters

MVD

With regard to vascularization, the vessels were counted in 3 most vascularized areas ("hot spots") of the tumor and normal pituitary and a mean value was then considered. Microvessel counts were performed at x200 (x20 objective lens and x10 ocular lens; 0.74 mm^2 per field).
A single microvessel was defined as discrete clusters or single cells stained for CD34 and the presence of a lumen was not required for scoring as a microvessel.

VEGF

VEGF expression was analyzed in adenohypophysial and endothelial cells. In particular, the degree of positivity was evaluated semiquantitatively by counting the total of cells of adenomas and normal anterior pituitary, and calculating the percentage of cells with cytoplasmic immunoreactivity for VEGF, regardless of intensity of staining.
All parameters were determined independently by two pathologists (P.V. and A.G.B.) and discordant cases were solved by simultaneous review.

Statistical analysis

MVD and VEGF expression were compared in normal pituitary and adenomas, in the different histotypes of adenomas and between macro- and microadenomas. SPSS software package was used. ANOVA with Student-Neuman-Keuls multiple comparison test and unpaired *t* test were used for analysis of data groups. The correlation between microvessel density and VEGF was also evaluated by Pearson correlation test.

RESULTS

MVD

MVD in normal anterior pituitary gland (Fig. 1A) was significantly higher than in tumors (mean±SD:

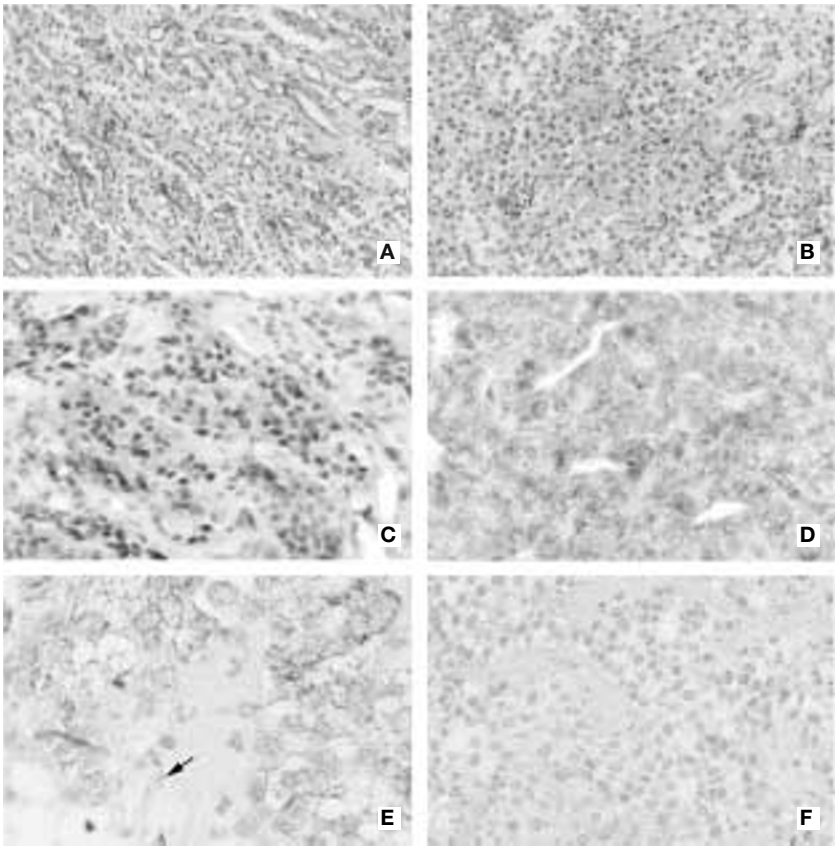

Fig. 1 - *A) Normal anterior pituitary with rich vascular network (immunohistochemistry, anti-CD34, x100). B) GH-secreting pituitary adenoma, showing reduced vascular density than normal tissue (immunohistochemistry, anti-CD34, x100). C) Normal anterior pituitary with diffuse cytoplasmic immunoreaction for vascular endothelial growth factor - VEGF (immunohistochemistry, anti-VEGF, x250). D) GH-secreting pituitary adenoma, showing VEGF expression similar to normal tissue (immunohistochemistry, anti-VEGF, x250). E) Pituitary adenoma. Vessel with endothelial cells, negative for VEGF (arrow, immunohistochemistry, anti-VEGF, x600). F) Pituitary adenoma. The section after immunoabsorption with VEGF peptide resulted negative (x 250).*

69.2±28.5 *vs* 29.3±19.7; $p<0.0001$) (Fig. 1B), regardless of histotype (Fig. 2).

Independently of histotype, no statistically significant difference was found in MVD between macroadenomas (34 cases: 28.2±19.2) and microadenomas (12 cases: 33.7±22.3). MVD was not correlated with age ($p=0.229$) and was not influenced by sex (female 29.7±17.4, male 33.3±22, p=NS). The least invasive (grade 0) pituitary adenomas showed higher values of MVD, significantly different ($p<0.05$) from grade 1 and 2 (Fig. 3). No difference was found between patients in remission after surgery (no.=21) and those (no.=25) with recurrence/persistence of disease, as assessed by clinical, hormonal and imaging criteria (26.5±11.6 *vs* 23.2±15.1). Only some acromegalic patients were treated before surgery, for 6 months, by somatostatin analogues: no difference was found between the 6 pretreated (26.5±11) and the 6 untreated ones (23.2±15.1).

VEGF expression

VEGF expression was found in adenohypophysial cells, while endothelial cells were negative (Fig. 1E). In normal pituitary the percentage of positive cells was between 10 and 80 (mean±SD: 40.0±18.8%), while in tumors the range was between 39.0 and 49.5 (mean 44.1±20.4%). No statistically significant difference ($p=0.3978$) in VEGF expression (Fig. 2) was found between normal tissue (Fig. 1C) and adenomas (Fig. 1D). The specimens after immunoabsorption test always resulted negative (Fig. 1F). VEGF expression was higher in ACTH and NF adenomas although this was not significantly different from other adenomas.

No statistically significant difference in VEGF expression between macroadenomas (34 cases: 44.3± 20.1%) and microadenomas (12 cases: 55.4±25.5%) was found. VEGF expression was not correlated with age ($p=0.2869$) and was not influenced by sex (male 41.9±16.5%, female 47.2±23.3%, p=NS). No difference was found among VEGF values of grades 0 to 3 of invasiveness (Fig. 3). VEGF values were not different between patients who recurred/persisted after surgery and those who were in remission (43.3±18.5 *vs* 46.8±23.2%). Finally, pre-treatment by somatostatin analogues in acromegalic patients did not influence VEGF values (40.0±23.7% in pre-treated *vs* 43.3±25.8% in untreated, p=NS).

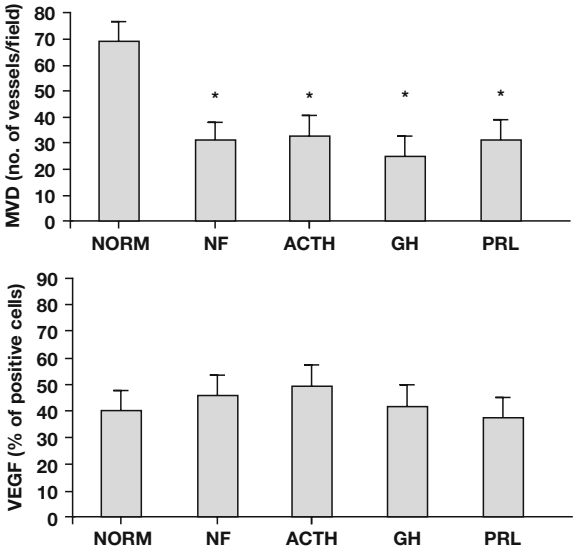

Fig. 2 - *Mean microvascular density (MVD) and vascular endothelial growth factor (VEGF) expression in normal pituitary and pituitary adenomas of different histotypes. MVD (mean±SE) is expressed as number of positive vessels/field (mean of 3 "hot spots") by CD34, VEGF (mean±SE) is expressed as percentage of positive cells. NORM: normal anterior pituitary gland, NF: non functioning tumor, ACTH: ACTH-secreting tumor, GH: GH-secreting tumor, PRL: PRL-secreting tumor. Statistical significance:* p*<0.001* vs *NORM.*

Correlation between MVD and VEGF

Both in normal tissue and in adenomas no significant correlation between MVD and VEGF expression was found (p=0.2 and p=0.136, respectively).

DISCUSSION

The angiogenic events underlying the growth of human pituitary tumors are poorly understood. The few studies on this topic showed a reduced vascularization in pituitary adenomas with respect to normal tissue (11). In the group of adenomas no statistically significant difference of MVD was found between different histotypes, except for invasive tumors that resulted significantly more vascularized (11-13).

The role of angiogenic factors in vessel development of pituitary adenomas has been investigated even less. Rodent and human pituitary tumor cells *in vitro* can produce VEGF, a potent angiogenic factor, the production of which is inhibited by dexamethasone administration (14) and stimulated by estrogens (20). It is to be noted that, in a rat model, increased VEGF expression as determined by Western blotting in estrogen-induced pituitary tumors was accompanied by a decrease in the density of small blood vessels (<5 μ) and an increase in the density of large vessels (>12 μ) (21).

VEGF has been demonstrated to be expressed in the folliculostellate cells of normal human pituitary gland (22, 23) and in cells of the neural anterior lobes of rat pituitary (20). Moreover, VEGF expression was demonstrated in all types of pituitary adenomas by immunohistochemistry and *in situ* hybridization (17).

To our knowledge, no study has insofar analyzed the correlation between vascular density and VEGF expression in human pituitary adenomas.

Our data on MVD confirm previous reports (12) on reduced vascular density in pituitary adenomas compared to normal pituitary gland tissue. The group of adenomas did not show statistically significant differences in MVD, when different histotype, tumor size, sex, age, rate of recurrence or medical pre-surgical treatment were considered. Only clinical invasiveness, as evaluated by pre-surgical MRI grading, was somewhat related to the degree of vascularity, with the finding of higher vascularity in the least invasive tumors. These data are not in agreement with those of Turner *et al.* (11), who found higher MVD in invasive prolactinomas; this kind of tumors were however minimally represented in our series.

Overall, our results might be related to two pathogenetic mechanisms: a) adenomas have a different kind of vascularization with respect to normal tis-

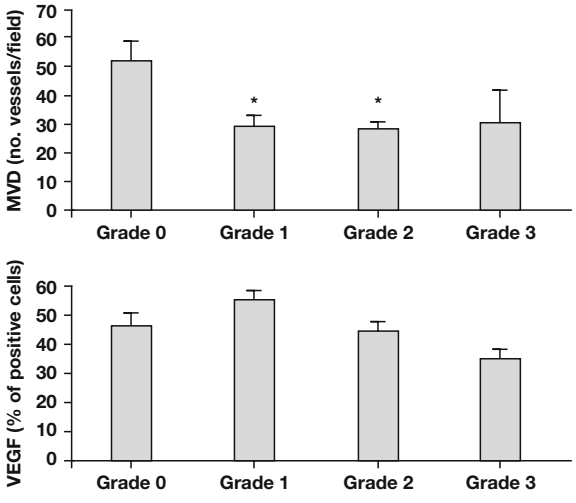

Fig. 3 - *Mean microvascular density (MVD) and vascular endothelial growth factor (VEGF), expression in normal pituitary and pituitary adenomas of different degrees of invasiveness. MVD (mean±SE) is expressed as number of positive vessels/field (mean of 3 "hot spots") by CD34, VEGF (mean±SE) is expressed as % of positive cells. Grade 0: not seen at RMI, Grade 1: intrasellar, Grade 2: mainly intrasellar with minor extrasellar involvement, Grade 3: extrasellar spreading. Statistical significance:* p*<0.001* vs *Grade 0.*

sue; b) angiogenesis is partially inhibited in pituitary adenomas. In the first case, it is possible that adenomas do not have the capability to reproduce the complex type of vascularization that characterizes normal tissue, *i.e.* portal venous system associated to arterial supply. Therefore, adenomas simply show a different vascular organization with respect to the normal tissue. In the second case, the MVD reduction in adenomas with respect to normal gland might be due to the expression of a partial inhibition of angiogenesis during the switch from normal to neoplastic growth. Interestingly even if the number of vessels in adenomas is lower than in normal gland, VEGF expression in adenomas is not different from normal counterpart. A possible explanation of this apparently contrasting feature is that inhibitory angiogenic factors could counterbalance the angiogenic stimulus of VEGF. Alternatively, in pituitary adenoma, VEGF might play a role in tumoral vascular growth not by increasing the number of vessels but by other mechanisms, such as an increase in vascular permeability that favors the abundant diffusion of nutrients. Analysis of VEGF receptors might help to unravel some of these complex hemodinamic and vascular events that accompany the growth of pituitary adenomas. In fact, VEGF may interact with two types of endothelial receptors, namely VEGFR-1 and VEGFR-2. Activation of VEGFR-1, at variance with VEGFR-2, does not induce endothelial cell proliferation (24). On the contrary activation of VEGFR-1 by VEGF in cooperation with placental growth factor increases vascular permeability (25). It cannot be excluded that this angiogenic mechanism is prevalent in pituitary adenomas.

In our study, VEGF expression was found in adenohypophysial and not in endothelial cells. It cannot however be excluded that some staining might be due to folliculostellate cells, that are very difficult to recognize on morphological basis. A certain heterogeneity of intensity in adenomatous cells was found at VEGF staining, with respect to normal counterparts. This feature, commonly observed with immunostaining analysis mostly in tumoral tissues, is very difficult to quantitate and not necessarily correlated to functional effects.

In conclusion, the angiogenetic phenotype of pituitary tumoral model is complex, and probably with multifactorial regulation. Vascularization is confirmed reduced in pituitary adenomas with respect to normal gland. There are no clear differences in vascular densities among different tumoral histotypes. There is no correlation between MVD and VEGF expression in normal and tumoral pituitary tissue. VEGF is nonetheless expressed in normal pituitary and pituitary adenomas. These data might be of importance in the development of future therapeutic strategies such as anti VEGF antibodies and drugs.

REFERENCES

1. Folkman J. What is the evidence that tumors are angiogenesis dependent? J. Nat. Cancer Inst. 1990, *82:* 4-6.
2. Horak E.R., Leek R., Klenck N. *et al.* Angiogenesis, assessed by pletelet/endothelial cell adhesion molecule antibodies, as indicator of node metastasis and survival in breast cancer. Lancet 1992, *340:* 1120-1124.
3. Macchiarini P., Fontanini G., Hardin M.J., Squartini F., Angeletti C.A. Relation of neovascularization to metastasis of non-small cell lung cancer. Lancet 1992, *340:* 145-146.
4. Ferrara N. Molecular and biological properties of vascular endothelial growth factor. J. Mol. Med. 1999, *77:* 527-543.
5. Brown L.F., Detmar M., Claffey K. *et al.* Vascular permeability factor/vascular endothelial growth factor: A multifunctional angiogenic cytokine. EXS 1997, *79:* 233-269.
6. Klagsbrun M., D'Amore P.A. Vascular endothelial growth factor and its receptor. Cytokine Growth Factor Rev. 1996, *7:* 259-270.
7. Brown L.F., Berse B., Jackman R.W. *et al.* Expression of vascular permeability factor (vascular endothelial growth factor) and its receptor in breast cancer. Hum. Pathol. 1995, *26:* 86-91.
8. Volm M., Koomagi R., Matter J. Prognostic value of vascular endothelial growth factor and its receptor flk-1 in squamous cell lung cancer. Int. J. Cancer 1997, *74:* 64-68.
9. Brown L.F., Berse B., Jackman R.W. *et al.* Expression of vascular permeability factor (vascular endothelial growth factor) and its receptor in adenocarcinomas of the gastrointestinal tract. Cancer Res. 1993, *53:* 4727-4735.
10. Turner H.E., Nagy Z., Gatter K., Esiri M.M., Wass J., Harris A.L. Proliferation, bcl-2 expression and angiogenesis in pituitary adenomas: relationship to tumor behaviour. Br. J. Cancer 2000, *82:* 1441-1445.
11. Turner H.E., Nagy Z., Gatter K., Esiri M.M., Harris A.L., Wass J. Angiogenesis in pituitary adenomas and the normal pituitary gland. J. Clin. Endocrinol. Metab. 2000, *85:* 1159-1162.
12. Turner H.E., Nagy Z., Gatter K., Esiri M.M., Harris A.L., Wass J. Angiogenesis in pituitary adenomas – relationship to endocrine function, treatment and outcome. J. Endocrinol. 2000, *165:* 475-481.
13. Vidal S., Kovacs K., Horvath E., Scheithauer B.W., Kuroki T., Lloyd R.V. Microvessel density in pituitary adenomas and carcinomas. Virchows Arch. 2001, *438:* 595-602.
14. Lohrer P., Gloddek J, Hopfner U. *et al.* Vascular endothelial growth factor production and regulation in rodent and human pituitary. Neuroendocrinology 2001, *74:* 95-105.
15. Berkman R.A., Merrill M.J., Reinhold W.C. *et al.* Expression of the vascular permeability factor/vascular endothelial

growth factor gene in central nervous system neoplasms. J. Clin. Invest. 1993, *91:* 153-159.

16. Nishikawa R., Cheng S.Y., Nagashima R., Huang H.J., Cavenee W.K., Matsutani M. Expression of vascular endothelial growth factor in human brain tumors. Acta Neuropathol. 1998, *96:* 453-462.
17. Lloyd R.V., Scheithauer B.W., Kuroki T., Vidal S., Kovacs K., Stefaneau L. Vascular Endothelial Growth Factor (VEGF) expression in human pituitary adenomas and carcinomas. Endocr. Pathol. 1999, *10:* 229-235.
18. Nakayama K., Kanzai A., Takebayashi Y. *et al.* Different features of angiogenesis between ovarian and breast carcinoma. Cancer Lett. 2001, *170:* 161-167.
19. Sauter E.R., Nesbit M., Watson J.C., Klein-Szanto A., Litwin S., Herlyn M. Vascular endothelial growth factor is a marker of tumor invasion and metastasis in squamous cell carcinomas of the head and neck. Clin. Cancer Res. 1999, *5:* 775-782.
20. Ochoa A.L., Mitchner N.A., Paynter C.D., Morris R.E., Ben-Johnathan N. Vascular endothelial growth factor rat pituitary: differential distribution and regulation by estrogen. J. Endocrinol. 2000, *165:* 483-492.
21. Banerjee S.K., Sarkar D.K., Weston A.P., Campbell D.R. Over expression of vascular endothelial growth factor and its receptor during the development of estrogen-induced rat pituitary tumors may mediate estrogen-initiated tumor angiogenesis. Carcinogenesis 1977, *18:* 1155-1161.
22. Gloddek J., Pagotto U., Paez Pereda M., Arzt E., Stalla G.K., Renner U. Pituitary adenylate cyclase-activating polypeptide, interleukin-6 and glucocorticoids regulate the release of vascular endothelial growth factor in pituitary folliculostellate cells. J. Endocrinol. 1999, *160:* 483-490.
23. Ferrara N., Leung D.W., Cachianes G., Winer J., Henzel W.J. Purification and cloning of vascular endothelial growth factor secreted by pituitary folliculostellate cells. Methods Enzymol. 1991, *198:* 391-405.
24. Neufeld G., Cohen T., Gengrinovitch S., Poltorak Z. Vascular endothelial growth factor (VEGF) and its receptor. FASEB J. 1999, *13:* 9-22.
25. Carmeliet P., Moons L., Luttun A. *et al.* Synergism between vascular endothelial growth factor and placental growth factor contributes to angiogenesis and plasma extravasation in pathological conditions. Nat. Med. 2001, *7:* 575-583.