EFFECT OF LEPTIN ON PROLIFERATIVE ACTIVITY AND VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF) SECRETION FROM CULTURED ENDOTHELIAL CELLS HECA10 *IN VITRO*

BEATA MISZTAL-DETHLOFF, HENRYK STEPIEN, JAN KOMOROWSKI

Institute of Endocrinology, Medical University of Lodz, 91-425 Lodz, Poland
e-mail: komorowski.j@wp.pl

Objective. The aim of this study was to check if leptin influences the proliferative activity and vascular endothelial grofth factor (VEGF) release from cultured mouse endothelial cells *in vitro*.

Methods. The murine cell line HECa10 obtained from endothelial cells of mouse peripheral lymph nodes immortalised by transfection of plasmid with the gene for the large T antigen of Simian virus 40 was used in the experiments. The proliferative activity of HECa10 cells was studied by Mosmann and VEGF release by ELISA methods.

Results. Murine leptin in concentrations from 5 to 25 ng/ml stimulated the proliferative activity of 72 h endothelial cell cultures and in concentrations from 0.5 to 25 ng/ml augmented the release of VEGF into supernatants of 24 h and 72 h cultured cells.

Conclusion. Leptin stimulated proliferation and VEGF secretion of endothelial cells *in vitro*.

Key words: Angiogenesis - Leptin - Endothelial cells - Vascular endothelial growth factor

The discovery of leptin by ZHANG et al. (1994) created a new perspectives for further research of angiogenesis. Leptin is the product of the ob gene which is secreted by adipocytes and circulates as plasma protein. It plays a key role in the regulation of body weight by controlling food consumption, sympathetic nervous system activation and termogenesis (CARO et al. 1996). Leptin is a pleiotropic hormone which is involved in regulatory processes of immunity, inflammation, hematopoiesis (FANTUZZI et al. 2000) and angiogenesis (BOLOUMIERE et al. 1998; CAO et al. 2001). Numerous pro- and anti-angiogenic factors take a part in new vessels formation, vascular endothelial growth factor - VEGF being a major pro-angiogenic factor (TOI 1995). Among characteristic properties of this factor is e.g. endothelial specificity and a vascular permeability (DVORAK 1995). Stimulation of endothelial cells proliferation and angiogenesis was documented by administration of leptin *in vitro* and *in vivo*. Proliferation is one of stages which leads to the formation of new vessels by capillary sprouting from preexisting vessels. The vascular endothelial growth factor (VEGF) is necessary to trigger this process.

In fifties a genetic defect has been known which resulted in the identification of several obese phenotypes. Thus, obese mice ob/ob were unable to produce a satiety factor. Yet db/db mice produced the factor missed in those ob/ob mice, but could not respond to that factor because of receptor defect. As defined by Fantuzzi et al. (2000), these mice had ob and db gene defect. Leptin exerts its biological effect by binding to leptin receptor Ob-R, identified shortly after the discovery of leptin (TARTAGLIA et al. 1995). It was found to be the product of the db gene (LEE et al. 1996). There are at least six isoforms of leptin receptor (Ob-R) and they are found in different tissues throughout the body and with different levels of expression. The receptors are expressed in the hypothalamus, cerebral cortex, cerebellum, choroid plexus, lungs, kidneys, liver, skeletal muscles, pancreas, adipose tissue, adrenal medulla and in many others (REIDY AND WEBER 2000).

The Ob-Rb long isoform is associated with the activation of the JAK/STAT signalling pathways and is expressed in HUVECs, in microvascular and aortic bovine endothelial cells and human adipose or dermal microvascular ECs (Sierra-Honigmann et al., 1998).

Leptin has multiple autonomic and cardiovascular activity as sympathetic activation and increases endothelium nitric oxide. The pressor cardiovascular effect of chronic hyperleptinemia is mediated by the increased sympathetic activity and selective leptin resistance to the metabolic actions and may have potential cardiovascular implications in human obesity (Mark et al., 2002). This protein induced cell proliferation of varies cell lines e.g. a mouse embryonic cell line C3H10T1/2 (Takahashi et al. 1997), murine myelocytic and primitive hemopoietic progenitor cells (Umemoto et al. 1997) and endothelial cells HUVECs cells (Boloumiere et al. 1998), bovine endothelial cell (BCE) (Cao et al. 20014), and also leptin stimulates angiogenesis (Boloumiere et al. 1998; Cao et al. 2001). Angiogenesis is a complex process which leads to formation of new vessels by capillary sprouting from preexisting vessels. Formation of this capillary is fundamental to reproduction, development and repair (Folkman 1995, 1995). Angiogenesis takes place in physiological processes during the menstrual cycle, formation of the placenta in pregnancy and wound healing (Stępień et al. 2002). On the other hand, angiogenesis is required for patological processes such as: tumor growth and metastasis (Folkman 2002) or diabetic rethinopathy, rheumatoid arthritis, endometriosis (Risau 1997; Norrby 1997). Over 30 years ago, Judah Folkman put forward a hypothesis regarding tumor angiogenesis in the development and metastatic spread of tumors and a new means of treating cancer by inhibition of angiogenesis (Folkman, 1971, 1971, 1972). The discovery of the pro-angiogenic molecules such as basic fibroblast growth factor and vascular endothelial growth factor (VEGF) accelerate the research on forming of new blood vessels and clinical application of this process.

The most critical of this growth factors is VEGF-A, a central promotor of endothelial cell migration and proliferation, required for forming of new blood vessels (Ferrara 2002; Jain 2002). The other members of the VEGF family: VEGF-B, -C, -D and PIGF also play a role in angiogenesis but they are not so strongly pro-mitogenic factors as VEGF-A. This factor is required to initiate the formation of blood vessels during embryogenesis and in adult animals or people during plate formation and manstrual cycle (Hanahan 1997). Expression of VEGF-A is upregulated in many tumors and therefore can be a tumor marker (Dvorak et al. 1995; Dvorak et al. 1999; Ferrara 2000). It is proved that VEGF is responsible for an increase in vessel density, that leads to its faster growth (Ferrara and Alitalo 1999). In 1971 Folkmann et al. suggested a concept of ''anti- angiogenesis'' that inhibition of these growth factors may represent a novel treatment of cancer. The central role of VEGF-A in angiogenesis leads to the hypothesis that it can be inhibited in many diseases with pathologic angiogenesis. Preclinical studies showed that inhibition of VEGF with Bevacizumab (Avastin, rhuMAb-VEGF; Genentech, South San Francisco, CA) led to reduced tumor vessel permeability and caused vascular regression (Ferrara 2002; Jain 2002).

The aim of this study was to check if leptin influences the proliferative activity and VEGF release from cultured mouse endothelial cells *in vitro*.

Materials and Methods

Cell culture. The murine cell line HECa10 obtained from endothelial cells of mouse peripheral lymph nodes immortalised by transfection of plasmid with the gene for the large T antigen of Simian virus 40 (Bizouarne et al. 1993) was used in the experiments.

The continuous culture of endothelial cells was maintained in culture flasks (Nunc Eas Y flask 25 cm^2, NUNC). The cells were cultured in RPMI 1640 medium (Sigma) supplemented with 25 mM Hepes buffer (Sigma); 2 g/liter sodium bicarbonate (Sigma); 50 mM 2-mercaptoethanol (Serva); 4 mM l-glutamine (Sigma); 100 U/ml penicillin and 100 mg/ml streptomycin solution (Sigma); 0,1 mM MEM nonessential amino acid solution (Sigma); 1 mM sodium pyruvate (Sigma); 10 % heat-inactivated fetal calf serum (FCS, Hungarpol). Endothelial cells were incubated in so made complete RPMI at 37 °C in the humidified atmosphere of 95 % air and 5 % CO_2. The cells were confluenced twice a week and before this they were harvested after a 2-min incubation at room temperature in the presence of trypsin-EDTA (0.05 and 0.02% respectively) in Hanks-balanced salt solution (Sigma). The cells were washed three times in complete RPMI and after the last centrifugation seeded at 5 x 10^4 cells in 5 ml of fresh medium.

Experiment 1. The cells was subjected to the trypsinization process as described above, suspended at 1 x 10^5 cells/ml in complete RPMI and 50 ml aliquots of

cell suspension (5 x 10^3 cells) were placed in the wells of culture chamber slides containing 130 ml of complete RPMI (Nunc TC Micro Well 96 F, NUNC). The cultures were incubated in 5 % CO_2 and at 37 °C for 24 hours and than 20 ml of leptin (R&D Systems USA) was added at the final doses 0.5, 5.0, 25.0 and 250 ng/ml to appropriate wells. The equal volume of RPMI medium was added to the control wells. After 24 and 72 h incubation of endothelial cells were labeled using a cells proliferation kit (EZ4Y, The 4th Generation Non Radioactive Cell proliferation & Cytotoxity Assay; BIOMEDICA). Colorimetric assay (EZ4U) based on the reduction of substrate [(3-(4,5-dimethyltiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] (MTT) to a formazan product by mitochondrial dehydrogenases in living cells was used to measure the proliferative activity of HECa10 cells. The optical density of each sample was measured in a microplate reader at 450 nm.

EXPERIMENT 2. After trypsinization process the cells were suspended at 3 x 10^5 cells/ml in complete RPMI and 50 ml aliquots of cell suspension (1,5 x 10^4 cells) were placed in the assay wells of culture dishes containing 850 ml (Nunclon Multidish 24-wells, NUNC). Culture dishes were divided into two groups. Leptin (100 ml) at the final doses 0.5, 5.0, 25.0 and 250 ng/ml was added to the suitable well of each group. The equal volume of culture medium was added to the control samples. The cultures were incubated (5 % CO_2, 37 °C) for 24 h (Group I) and 72 h (Group II) and then the supernatants were collected, centrifuged and stored at –20 °C. Mouse vascular endothelial growth factor kit (VEGF Immunoassay, Quantikine M, R&D Systems, USA) was used to measure VEGF concentration (sensitivity: 3 pg/ml; intraassay precision: CV<8 %)**.** The optical density of each sample was measured in microplate reader at 450 nm and the sample values of VEGF were read off the standard curve.

Statistical evaluation. The results are expressed as means ± SEM. The statistical significance of observed differences was tested with Student's t-test or analysis of variance followed by Snedecor F test. P values less than 0.05 were considered statistically significant.

Results

Experiment 1. Leptin in concentrations of 0.5, 5.0, 25.0, and 250.0 ng/ml did not change the proliferative activity of 24 h cultured cells compared with control (Fig. 1; p>0.05). However, leptin in increased concentrations of 5.0 and 25.0 mg/ml stimulated the proliferation of endothelial cells in 72 h cultures (Fig. 2; p<0.05).

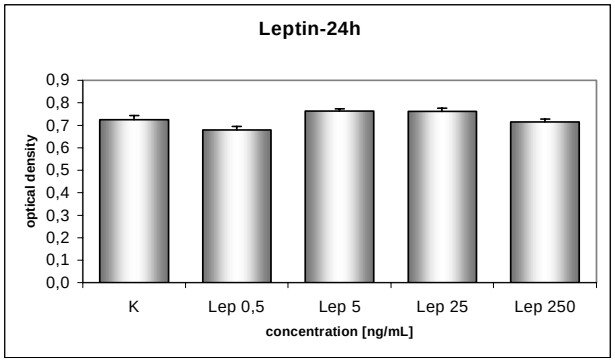

Fig. 1

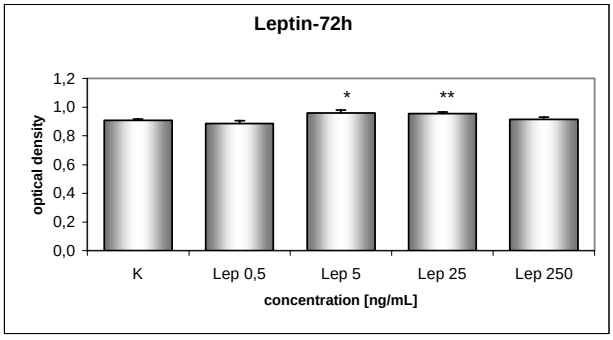

Fig. 2

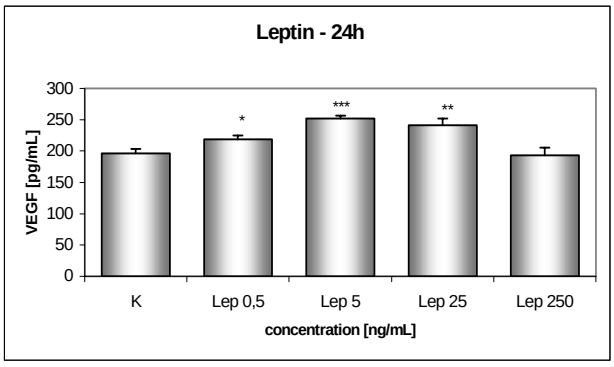

Fig. 3

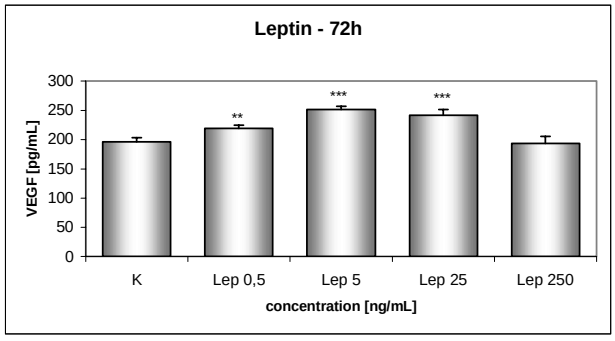

Fig. 4

Experiment 2. Leptin increased the release of VEGF to the supernatants of 24 and 72 h endothelial cells cultures. We have shown its stimulatory effect in concentrations of 0.5 ng/ml ($p<0.05$), 5.0 ng/ml ($p<0.001$), 25.0 ng/ml ($p<0.01$) and 0.5 ng/ml ($p<0.01$), 5.0 ng/ml ($p<0.001$), 25.0 ng/ml ($p<0.001$) in 24 and 72 h cultures, respectively. (Fig. 3 and 4).

Discussion

Human obesity is a complex, multifactorial disorder, with both genetic and environmental determinants, that affects approximately one-fourth of European. Obesity is an important public health problem associated with clear health risks, including hypertension, diabetes and dyslipidaemia (Haynes 2000). It has been also proven that abnormal accumulation of adipose tissue and leptin concentration are connected with cardiovascular diseases and cancer (Boloumie et al. 1998). The discovery of a hormon secreted by adipose tissue has raised the hope for a better understanding of the regulation of body weight. Leptin appears to play a major role in body weight regulation by promoting weight loss through reducing food intake acting in the hypothalamus and increasing energy expenditure (Zhang et al. 1994; Pelleymounter et al. 1995). This 16 kDa protein circulating as a 146 amino acid peptide is responsible for the obesity syndrome and has been encoded by the ob gene. Its circulating levels correlate directly with body weight and body adiposity (Ahren, 2000) and at the same time with the degree of hypertensive retinopathy (Artwohl et al. 2002). This hormone is primarily produced by adipose tissue, as well as by other cells/organs such as bone marrow adipocytes, placenta, stomach (Mix et al. 1999), and the central nerves system (Reichlin 1999). In the present study we have demonstrated the pro-proliferative effect of leptin on endothelial mouse cells. Leptin has stimulated proliferation of endothelial cells in 72-hour cultures althought it has not showed such activity in 24-hour cultures. It was indicated that leptin enhanced the proliferation of endothelial cells and the formation of new blood vessels *in vitro* (Boloumiere et al. 1998; Cao et al. 2001). We have demonstrated that leptin stimulated release of VEGF from the endothelial cells to a greater degree than its proliferation in the 24 and 72 h cultures. Leptin induces VEGF secretion from endothelium cells in 24 h cultures which needs longer than 24 h incubation period with a growth factor to stimulate proliferation of HECa10.

Leptin levels are higher in female than in male mice and demonstrate diurnal rhythm with a nadir in the mornings (at 8 or 10 AM) and a nocturnal peak at 10 PM to 2 AM (Ahren 2000). Plasma levels of leptin between 8 and 10 AM in non- fasted mice is 6.2 ±0.3 and 6.4±0.5 for females and males mice respectively. This nocturnal rise in leptin correlates to the insulin response to meals (Ahren 2000), similarly as in human. There were no reports found that leptin can influence the secretion of VEGF stimulating this way of endothelial cells proliferation. However, Stallmeyer et al. (2001) proved that leptin takes part in reversing a disordered reparation process in ob/ob mice. As it is commonly known, angiogenesis is the main process responsible for correct course of a wound healing process. Application of leptin in those animals did not retreat highly reduced angiogenesis but caused an increase in VEGF expression. Within the wound it was even 40 % higher in ob./ob. mice than in wild ones.

Stimulatory effects of leptin have already been reported on human umbilical vein endothelial cells (HUVEC) and bovine capillary endothelium (BCE) in 72 hours incubation in vitro. Boloumiere at al.(1998) have proved that the maximal effect was elicited by a physiological plasma concentration of leptin (10 ng/ml) and was equivalent to that evoked by VEGF165 in the same concentration range. Similarly, in our study the maximal mitogen effect was caused by physiological concentrations in mouse.

Proliferation of endothelial cells constitutes one key event in the angiogenic process. Angiogenesis starts with cell-mediated degradation of basement membrane, followed by migration and proliferation of endothelial cells. Through stimulating own angiogenesis leptin can promote its own secretion into circulation and act as a endocrine hormone influencing the food intake and energy expenditure (Cao et al. 2001). The more the leptin is synthetised, the more leptin is released into the circulation and the greater is the endocrine effect in the organism. In such tissues as the placenta or fetal tissues as heart and bone (Trayhurn et al. 1999) leptin and VEGF are coexpressed and probably they can synergistically stimulate the angiogenesis (Cao et al. 2001).

Cao et al. (2001) investigated the influence of leptin and growth factors like VEGF and FGF-2 alone and together on the angiogenesis of corneal capillaries in the mouse model. They report that leptin modulates angiogenic responses induced by VEGF. It can be therefore stated that leptin is a significant pro-proliferative

and pro-angiogenic factor which role has not yet been appreciated enough. The mitogenic activity of leptin may be connected with its direct receptor activity or indirect activity: through the increase in density of the VEGFR-1 receptors, apoptosis inhibition and probably through stimulation of VEGF secretion. It was demonstrated that leptin in the concentration dependent manner reduced apoptosis in HAVECs (human adult vein endothelial cells) (Artwohl et al., 2002).

Because obesity in humans is associated with an elevation of leptin in the plasma as well as adipose tissue, we can speculate that leptin promotes angiogenesis which participates in the rise (increase) of the adipose mass. Nevertheless, further studies are needed to elucidate the interrelations in this field.

In conclusion, leptin stimulated proliferation and VEGF secretion of endothelial cells *in vitro.*

Acknowledgements

This work was supported by the Polish State Committee for Scientific Research, grant 5 11 P05A 035 20 (to J. K.).

References

Ahren B. Diurnal variation in circulating leptin is dependent on gender, food intake and circulating insulin in mice. Acta Physiol Scand **169**, 325-331, 2000.

Artwohl M, Roden M, Holzenbein T, Freudenthaler A, Waldhausl W, Baumgartner-Parzer SM. Modulation by leptin of proliferation and apoptosis in vascular endothelial cells. Int J Obes Relat Metab Disord **26**, 577-580, 2002.

Bizouarne N, Denis V, Legrand A, Monsigny M, Kieda C. A SV-40 immortalized murine endothelial cell line from peripheral lymph node high endothelium expresses a new alpha-L-fucose binding protein. Biol Cell **79**, 209-218, 1993.

Bouloumie A, Drexler HC, Lafontan M, Busse R. Leptin, the product of Ob gene, promotes angiogenesis. Circ Res **83**, 1059-1066, 1998.

Cao R, Brakenhielm E, Wahlestedt C, Thyberg J, Cao Y. Leptin induces vascular permeability and synergistically stimulates angiogenesis with FGF-2 and VEGF. Proc Natl Acad Sci U S A **98**(11), 6390-5, 2001.

Toi M. Endothelial growth factors: a target fo antiangiogenesis The Cancer Journal **8**, 315-319, 1995.

Caro JF, Sinha MK, Kolaczynski JW, Zhang PL, Considine RV. Leptin: the tale of an obesity gene. Diabetes **45** 1455-1462, 1996.

Dvorak HF, Brown LF, Detmar M, Dvorak AM. Vascular permeability factor/vascular endothelial growth factor, microvascular hyperpermeability, and angiogenesis. Am J Pathol. **146**, 1029-1039, 1995.

Dvorak HF, Nagy JA, Feng D, Brown LF, Dvorak AM. Vascular permeability factor/vascular endothelial growth factor and the significance of microvascular hyperpermeability in angiogenesis. Curr Top Microbiol Immunol **237**, 97-132, 1999.

Fantuzzi G, Faggioni R. Leptin in the regulation of immunity, inflammation, and hematopoiesis. J Leukoc Biol **68**, 437-446, 2000.

Ferrara N.VEGF: an update on biological and therapeutic aspects. Curr Opin Biotechnol **11**, 617-624, 2000.

Ferrara N. Role of vascular endothelial growth factor in physiologic and pathologic angiogenesis: therapeutic implications. Semin Oncol **29** (Suppl 16), 10-14, 2002.

Ferrara N, Alitalo K . Clinical applications of angiogenic growth factors and their inhibitors. Nat Med. **5**, 1359-64, 1999.

Folkman J. Tumor angiogenesis: therapeutic implications. N Engl J Med. **285**, 1182-1186, 1971.

Folkman J. Anti-angiogenesis: new concept for therapy of solid tumors. Ann Surg **175**, 409-416, 1972.

Folkman J. Angiogenesis in cancer, vascular, rheumatoid and other disease. Nat Med. **1**, 27-31, 1995.

Folkman J. Seminars in Medicine of the Beth Israel Hospital, Boston. Clinical applications of research on angiogenesis. N Engl J Med **333**, 1757-1763, 1995.

Folkman J. Role of angiogenesis in tumor growth and metastasis. Semin Oncol **29** (Suppl 16), 15-18, 2002.

Folkman J, Merler E, Abernathy C, Williams G. Isolation of a tumor factor responsible or angiogenesis. J Exp Med **133**, 275-288, 1971.

Hanahan D. Signaling vascular morphogenesis and maintenance. Science **277**, 48-50, 1997.

Haynes WG. Interaction between leptin and sympathetic nervous system in hypertension. Curr Hypertens Rep **2**, 311-318, 2000.

JAIN RK. Tumor angiogenesis and accessibility: role of vascular endothelial growth factor. Semin Oncol **29** (Suppl 16), 3-9, 2002.

LEE GH, PROENCA R, MONTEZ JM, CARROLL KM, DARVISHZADEH JG, LEE JI, FRIEDMAN JM. Abnormal splicing of the leptin receptor in diabetic mice. Nature **379**, 632-635, 1996.

MARK AL, CORREIA ML, RAHMOUNI K, HAYNES WG. Selective leptin resistance: a new concept in leptin physiology with cardiovascular implications. J Hypertens **20**, 1245-1250, 2002.

MIX H, MANNS MP, WAGNER S, WIDJAJA A, BRABANT G. Expression of leptin and its receptor in the human stomach. Gastroenterology **117**, 509, 1999.

NORRBY K. Angiogenesis: new aspects relating to its initiation and control. APMIS **105**, 417-37, 1997.

PELLEYMOUNTER MA, CULLEN MJ, BAKER MB, HECHT R, WINTERS D, BOONE T, COLLINS F. Effects of the obese gene product on body weight regulation in ob/ob mice. Science **269**, 540-543, 1995.

REIDY SP, WEBER J. Leptin: an essential regulator of lipid metabolism. Comp Biochem Physiol A Mol Integr Physiol **125**, 285-298, 2000.

RISAU W. Mechanisms of angiogenesis. Nature **386**, 671-674, 1997.

SIERRA-HONIGMANN MR, NATH AK, MURAKAMI C, GARCIA-CARDENA G, PAPAPETROPOULOS A, SESSA WC, MADGE LA, SCHECHNER JS, SCHWABB MB, POLVERINI PJ, FLORES-RIVEROS JR. Biological action of leptin as an angiogenic factor. Science **281**, 1683-1686, 1998.

STĘPIEŃ HM, KOŁOMECKI K, PASIEKA Z, KOMOROWSKI J, STĘPIEŃ T, KUZDAK K. Angiogenesis of endocrine gland tumours—new molecular targets in diagnostics and therapy. Eur J Endocrinol **146**, 143-451, 2002.

TAKAHASHI Y, OKIMURA Y, MIZUNO I, IIDA K, TAKAHASHI T, KAJI H, ABE H, CHIHARA K. Leptin induces mitogen-activated protein kinase-dependent proliferation of C3H10T1/2 cells. J Biol Chem. **272**, 12897-12900, 1997.

TARTAGLIA LA, DEMBSKI M, WENG X, DENG N, CULPEPPER J, DEVOS R, RICHARDS GJ, CAMPFIELD LA, CLARK FT, DEEDS J, ET AL. Identification and expression cloning of a leptin receptor, OB-R. Cell, **83**, 1263-1267, 1995.

UMEMOTO Y, TSUJI K, YANG FC, EBIHARA Y, KANEKO A, FURUKAWA S, NAKAHATA T. Leptin stimulates the proliferation of murine myelocytic and primitive hematopoietic progenitor cells. Blood **90**, 3438-3443, 1997.

REICHLIN S. Is leptin a secretion of the brain? J Clin Endocrinol Metab **84**, 2267-2269, 1999.

STALLMEYER B, PFEILSCHIFTER J, FRANK S. Systemically and topically supplemented leptin fails to reconstitute a normal angiogenic response during skin repair in diabetic ob/ob mice. Diabetologia **44**, 471-479, 2001.

TRAYHURN P, HOGGARD N, MERCER JG, RAYNER DV. Leptin: fundamental aspects. Int J Obes Relat Metab Disord **23** (Suppl 1), 22-28, 1999.

ZHANG Y, PROENCA R, MAFFEI M, BARONE M, LEOPOLD L, FRIEDMAN JM. Positional cloning of the mouse obese gene and its human homologue. Nature **372**, 425-432, 1994.

Corresponding author: Prof. Jan Komorowski, M. D.
Institute of Endocrinology
Medical University of Lodz
91-425 Lodz
Poland
e-mail: komorowski.j@wp.pl