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Expression of leptin and its receptor in female breast cancer in relation with selected apoptotic markers

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Abstract: Leptin and its receptor may be engaged in pathogenesis of breast cancer among various human tumors. In vitro investigations showed leptin-mediated escalation of estrogen synthesis and boosted activity of estrogen receptor ERα. Furthermore, leptin induced growth of malignant cells, counteracted apoptosis and stimulated cell migration as well as overexpression of angiogenic factors and degrading enzymes that split network of intercellular matrix. On the other side, leptin has been reported to favor apoptosis, lately. Proapoptotic effect of leptin action was revealed in interstitial cells of bone marrow and adipocytes. Our past reports provide evidences for overexpression of leptin and its receptor in breast cancer in comparison with benign mammary lesions. In current study we aimed at assessment of eventual relationships between leptin, leptin receptor and selected protein regulators of apoptosis in breast cancer. We applied immunohistochemistry for leptin, leptin receptor, anti-apoptotic Bcl-2 and Bcl-xL as well as pro-apoptotic Bak and Bax expression assessment in 106 cases of human breast cancers. The immunoreaction was graded and statistically evaluated. Expression of leptin was positively correlated with Bcl-xL, Bak and Bax ($p<0.001$, $r=0.614$; $p<0.001$, $r=0.518$; $p<0.001$, $r=0.511$, respectively). Statistical significances were noted between expression of leptin receptor and Bcl-xL or Bax ($p=0.011$, $r=0.210$; $p<0.001$, $r=0.313$, respectively). No correlation was encountered between leptin and Bcl-2, either leptin receptor and Bcl-2 or leptin receptor and Bak. On the basis of obtained results, leptin system could interfere in balance among expressions of pro- and anti-apoptotic proteins and regulate cell turnover and - by means of it - facilitate breast cancer progression.

Key words: Leptin - Leptin receptor - Bcl-2 - Bcl-xL - Bak - Bax - Apoptosis - Breast cancer

Introduction

In the process of breast cancer development and progression deregulated expression of growth factors, their receptors as well as apoptosis' disturbances play important role [~~17,19~~]. The main group of genes influencing apoptosis is the Bcl-2 family with inhibitors (Bcl-2, Bcl-xL, Mcl-1) and promotors (Bax, Bak, Bad, Bcl-xS) of this process [~~26~~]. Pro- and anti-apoptotic proteins may form homo- or heterodimers and and ratio between these proteins influence cell cycle and apoptosis. Accumulated Bax and Bak proteins could increase mitochondrial permeability, release cytochrome C and finally activate the downstream caspase cascade [~~26~~]. Anti-apoptotic proteins preserve mitochondrial structure and function.

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Leptin is a hormone produced mainly by the adipose tissue and plays roles in body weight homeostasis, neuroendocrine function, fertility, immune function and angiogenesis [~~1~~]. Leptin effects of action are thought largely mediated via hypothalamus, but in recent years it was widely discussed leptin action in peripheral tissues [~~32~~]. In the breast, leptin is required for normal mammary gland development and lactation [~~22~~]. Recent data revealed that leptin might be involved in breast cancer development and progression [~~30~~]. Breast cancer cells in culture express differ-

Abbreviations: ObR - leptin receptor; JAK - Janus kinase; STAT - signal transducer and activator of transcription; ERK - extracellular signal-regulated kinase; PKC - protein kinase C; Akt - protein kinase; GSK - Glycogen synthase kinase; cdk - cyclin-dependent kinase; ROS - reactive oxygen species; MAPK - mitogen-activated protein kinase; PI3-K - phosphoinositide 3-kinase; JNK - c-Jun NH2-terminal kinase; AP-1 - activator protein 1; HIF-1α - hypoxia-inducible factor 1α; HRE - hypoxia responsive element.

ent isoforms of the leptin receptor, including the long signaling form [8, 21]. We previously showed that leptin and its receptor are overexpressed in primary breast cancer and lymph node metastasis compared with non-cancerous tissues, possibly due to hypoxia and/or overexposure of cells to insulin, IGF-I and/or estradiol [7]. Leptin can stimulate cell growth, regulate apoptosis, induce migration and expression of matrix degrading enzymes and angiogenic factors (such as VEGF) [9]. It indicates that leptin system might be involved in the development and progression of neoplasms. Stimulation of the leptin receptor has been reported to activate multiple signalling pathways such as JAK2/STAT3, ERK1/2, PKC-alpha, and Akt/GSK3 [8,21,29]. Additionally, leptin upregulates cdk2 and cyclin D1 levels as well as inactivates of the cell cycle inhibitor, pRb and consequently induces cell cycle progression [24].

Increased proliferation as well as deregulated apoptosis are important events in the process of carcinogenesis. Ogunwobi et Beales [23] described stimulation of proliferation and inhibition of apoptosis by leptin of human colon cancer cells and this effect involved JAK2, PI3-K and JNK activation. Despite proliferative and anti-apoptotic activities of leptin in different cells, there is increased number of evidences presenting opposing results of leptin action on apoptosis. Leptin was reported to be responsible for the induction of lipolysis and apoptosis of the adipose tissue and precursor cells of the osteoblast lineage [6,16,25]. The next possible proapoptotic mechanism of leptin action involves increase production of reactive oxygen species (ROS) [4, 34]. Thus data on association of leptin action with apoptosis are ambiguous and to our knowledge there is lack of studies demonstrating relationships between leptin system and proteins associated with apoptosis in breast cancer.

In current study we aimed at assessment of eventual relationships between leptin, leptin receptor and selected protein regulators of apoptosis in breast cancer: Bcl-2, Bcl-xL, Bax and Bak.

Materials end methods

Patients and tissue specimens. Tissue samples were obtained from 106 women who underwent partial or radical mastectomy because of primary breast cancer in years 2000-2002. The age of patients ranged from 30 to 80 years (mean 54.6 years). Tumor samples were collected immediately after tumor removal, fixed in 10% buffered formaldehyde solution for 48 h and then embedded in paraffin blocks at 56°C according to standard procedures. 5-µm sections were cut from the specimens and stained with hematoxylin-eosin. The diagnosis was based on the WHO and pTN classification of breast tumors [31].

Immunohistochemistry. The immunohistochemical analysis of leptin, leptin receptor, Bcl-2, Bcl-xL, Bak and Bax was investigated in representative tissue sections using antibodies: for leptin, rabbit polyclonal Ab (pAb) A-20 (Santa Cruz Biotechnology, Santa Cruz, USA), dilution 1:100; for leptin receptor, rabbit pAb H-300 (Santa Cruz, USA), dilution 1:75; for Bcl-2, mouse monoclonal antibody (Dako, Denmark), dilution 1:100; for Bcl-xL, goat pAb (Santa Cruz, USA), dilution 1:300; for Bak and Bax, goat pAb (Santa Cruz, USA), dilution 1:200. All primary Abs were diluted in PBS with 1.5% normal blocking serum. Immunohistochemistry was performed as described previously [17]. The studies were performed with avidin-biotin-peroxidase complex (ABC Staining System, SCBt, USA). Slides were counterstained with haematoxylin. In negative controls the primary antibody were omitted. The expression of leptin, ObR, Bcl-2, Bcl-xL, Bak and Bax was analyzed in 10 different tumor fields and the mean percentage of tumor cells with positive staining was evaluated. The expression of studied proteins in cancer samples was classified using a four-point scale: 0, <10% positive cells; 1+, 10-50% positive cells with weak staining; 2+, >50% positive cells with weak staining; 3+, >50% positive cells with strong staining.

Statistical analysis. The significance of the associations between studied proteins were determined using Spearman correlation analysis. Probabilities of $p<0.05$ were assumed as statistically significant.

Results

Immunohistochemical analysis of breast cancer sections revealed cytoplasmic localization and microgranular staining for leptin, leptin receptor, Bcl-2, Bcl-xL, Bak and Bax proteins. Immunostaining for leptin and leptin receptor was observed in 92/106 (86.8%) and 44/106 (41.5%) of the tumors; for anti-apoptotic proteins Bcl-2 and Bcl-xL in 79/106 (74.5%) and 77/106 (72.6%) of studied cases; for pro-apoptotic proteins Bak and Bax in 71/106 (66.9%) and 61/106 (57.5%) of the tumors, respectively.

Analysis of relationships between leptin and studied proteins showed statistically significant positive correlation between leptin and anti-apoptotic protein Bcl-xL ($p<0.001$, $r=0.614$) as well as between leptin and pro-apoptotic Bak ($p<0.001$, $r=0.518$) and Bax ($p<0.001$, $r=0.511$). On the other hand, leptin did not correlate with anti-apoptotic Bcl-2 protein ($p=0.789$, $r=0.028$).

We found statistically significant positive correlation between leptin receptor and anti-apoptotic Bcl-xL ($p=0.011$, $r=0.210$) as well as between leptin receptor and pro-apoptotic Bax ($p<0.001$, $r=0.313$). Similarly to leptin, we did not find significant association of leptin receptor with anti-apoptotic Bcl-2 ($p=0.738$, $r=0.032$). Additionally, correlation between leptin receptor and pro-apoptotic Bak was not found ($p=0.378$, $r=0.086$).

Discussion

Leptin system seems to play a dual role regarding apoptosis as it can act either as a pro- or an anti-apoptotic factor [3,4,6,16,23,25,34]. In the current study we found statistically significant positive correlations between leptin, its receptor and selected anti- and pro-

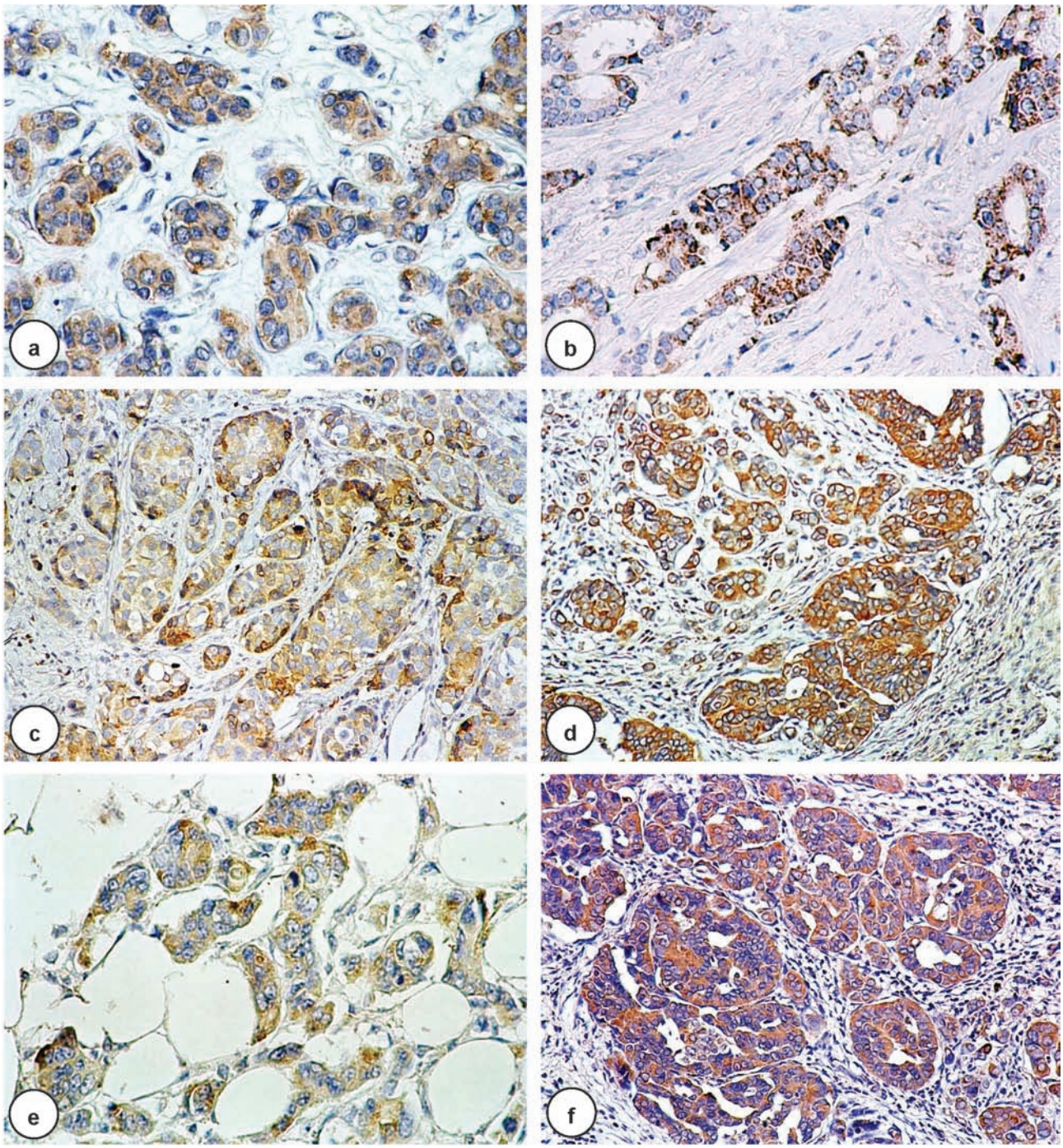

Fig 1. Immunohistochemical detection of leptin, leptin receptor, Bcl-2, Bcl-xL, Bak and Bax proteins. (**a**) The strong leptin staining is found in primary tumor in above 50% of cancer cells. (**b**) Coarsely granular immunostaining fo leptin receptor. (**c**) Cytoplasmic immunoreactivity to Bcl-2 in breast cancer. (**d**) Strong Bcl-xL immunostaining found in almost all cancer cells. (**e**) Moderate (assessed as 2+) Bak immunoreactivity in cancer cells. (**f**) Granular distribution of Bax in cytoplasm of breast cancer cells (original magnification ×200).

apoptotic proteins in human breast cancer. It suggests that leptin through auto- and paracrine mechanisms might activate breast cancer to increased tumor cell turnover and progression of the disease.

Kim *et al.* [16] observed that leptin induces apoptosis of the precursor cells of the osteoblast lineage via enhanced cytochrome c release into cytosol and the activation of caspase-9 and 3. Opposing leptin action reported Gordeladze *et al.* [10], who observed that leptin stimulates proliferation and inhibits cells from apoptosis. Qian *et al.* [25] found that intracerebroventricular administration of leptin induced adipose tissue apoptosis in addition to influencing lipid metabolism. This pro-apoptotic leptin action was related on Perox-

isome proliferator activated receptor γ (PPARγ) signaling pathway. Also Gullicksen *et al.* [12] observed that leptin treatment of rats led in different adipose tissues to increase in Bcl-2 or Bax proteins. It caused significantly reduction of adipose tissue cellularity by increased DNA fragmentation and upregulation of pro-apoptotic Bax protein. Proangiogenic leptin activity was widely accepted. Artwohl *et al.* [3] observed that leptin reduced apoptosis in human endothelial cells and this action correlated with increased expression of anti-apoptotic protein Bcl-2. On the other hand Cohen *et al.* [6] reported that leptin enhances apoptosis in adipose vascular endothelial cells.

Hoda *et al.* [13] reported mitogenic and anti-apoptotic leptin action for colon cancer cells, mediated via MAPK and PI3-K pathways. Also Ogunwobi et Beales [23] observed inhibition of apoptosis and stimulation of proliferation by leptin in colon cancer cells. This effect involved activation of JAK2, PI3-K, JNK, STAT3 and AP-1 pathways. Stimulation by leptin of multiple signaling pathways seems more complex problem in the light of evidence that activation of JNK could exert both anti- and pro-apoptotic action. Kim *et al.* [15] reported JNK-mediated Bax activation with stimulation of apoptosis. In our previous study [7] we noted negative association between leptin expression and proliferation marker Ki-67 in the group of lymph node metastases derived from ERα-positive, but not ERα-negative primary tumors. Also ObR negatively correlated with Ki-67 in lymph node metastases of breast cancer.

The next possible pro-apoptotic mechanism of leptin action involves increase production of reactive oxygen species (ROS). The increase of ROS was observed under the influence of leptin in endothelial cells and in polymorphonuclear neutrophils [4, 34]. ROS then mediate the formation of disulfide bridges between cytosolic pro-apoptotic Bax monomers, resulting in the formation of mitochondrial outer membrane channels. Membrane permeabilization leads to the release of apoptogenic proteins: cytochrome c, apoptosis-inducing factor, Smac/Diablo, HtrA2/Omi and endonuklease G. Cytochrome c initiates the proteolytic activation of caspases, which in turn cleave hundreds of proteins to produce the morphological and biochemical changes of apoptosis. The anti-apoptotic proteins, such as Bcl-2 and Bcl-xL, exerts protective role in apoptosis by preserving mitochondrial structure and function [14]. A ratio of anti- to pro-apoptotic proteins appears to determine the survival or death of cells following an apoptotic stimulus.

Hypoxia, which is associated with solid tumors, might stimulate, through hypoxia-inducible factor 1α (HIF-1α), expression of multiple genes, especially those encoding for glycolytic enzymes, growth factors, their receptors and vasoactive peptides [28]. It was found that hypoxia upregulates expression of leptin which increased together with accumulation of HIF-1α that enhance leptin expression via stimulation of Hypoxia Responsive Element (HRE) located in the promoter region of the leptin gene [2,11]. Previously, in endometrial and colorectal cancers we noted a positive correlations between HIF-1α and leptin as well as HIF-1α and ObR, which indicate that the expression of the leptin system could be HIF-1α - dependent [18, 20]. There is also growing evidence about HIF-1α involvement in the regulation of apoptosis [5,27]. In our previous study, we observed relationships between HIF-1α and Bax or Bcl-xL, which could suggest that expression of these apoptotic proteins in cancer cells, might be partially regulated by HIF-1α [33]. Observed in the current study associations between leptin system and apoptotic proteins could be also indirect and it's not out of the question that expression of studied proteins could depend on stimulatory influence of tumor hypoxia and HIF-1α.

In conclusion, it seems that leptin by stimulation of multiple intracellular signaling pathways could exert two actions related to apoptosis, both increase and inhibit this process. Consequently, leptin might increase tumor cell turnover and breast cancer progression via promotion of both processes mitogenesis and apoptosis, but functional relationships between leptin system and apoptotic proteins should be studied.

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