Effects of leptin on human breast cancer cell lines in relationship to estrogen receptor and HER2 status

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Abstract. Obesity is a risk factor for postmenopausal breast cancer and is associated with poor prognosis. Leptin, a cytokine synthesized in adipose tissue, has been implicated as a link between obesity and breast cancer. In the present study, the effects of leptin on cell proliferation and proteins associated with leptin signaling and/or breast cell growth were investigated in ER-positive, MCF-7, T47-D and MDA-MB-361, and ER-negative, MDA-MB-231 and SK-BR-3, breast cancer cell lines. MDA-MB-361 and SK-BR-3 also overexpress HER2/neu. For proliferation assays, 96-well plates were used and for protein determinations cells were synchronized in 6-well plates for 18-24 h in serum-free medium. Leptin was added at 0, 5, 10, 25, 50 and 100 ng/ml for 24 and 48 h. For Western blot analyses, protein extracts were probed for Ob-Rb, Ob-R, leptin, Jak2, PI3K, Stat3, p-Stat3, PCNA, cyclin D1, Cox-2, VEGF, Bcl-2, Bcl-x_L, Bax, insulin, IGF-I, IGFBP3, IGF-IRα, aromatase, CYP1A1 and CYP1B1. Overall, except for MCF-7 cells, leptin stimulated proliferation in all lines. MCF-7 cells expressed higher levels of Ob-Rb, Jak2, PI3K, Stat3 and p-Stat3 in a dose-dependent manner to 50 ng/ml at 24 h; and IGF-IRα increased at 24 h. Cyclin D1 and Cox-2 levels increased with leptin treatment. Higher CYP1B1 expression was observed at both 24 and 48 h. In MDA-MB-231 cells, p-Stat3 and Bcl-x_L were increased at 48 h; whereas PCNA and cyclin D1 expression increased in leptin-treated cells at 24 and 48 h. In T47-D cells, Jak2 and Stat3 were elevated at higher leptin concentrations at 24 and 48 h. However, p-Stat3 and PCNA demonstrated an increase only in 48-h leptin-treated cells. Furthermore, cyclin D1 exhibited higher expression at both 24 and 48 h, while Bcl-x_L expression was lower with increasing concentrations of leptin at 48 h. In MDA-MB-361 cells, Ob-Rb and VEGF increased at 24 and 48 h; whereas PI3K, Stat3, PCNA and insulin levels increased in leptin-treated MDA-MB-361 cells after 48 h. Bcl-2 and IGF-IRα were decreased at 24 h and a dose-dependent increase at 48 h was noted. Higher expression of CYP1B1 was observed with leptin for 24 h. In SK-BR-3 cells, Ob-R increased at both 24 and 48 h. A similar trend was found for IGF-I and IGFBP3 expression. Higher levels of Jak2 and PI3K were observed after 24 h. Interestingly, there was a gradual increase of leptin expression at 24 h, but a gradual decrease at 48 h in relation to the dose of leptin. In contrast, PCNA and IGF-IRα showed a decline at 24 h and an increase at 48 h. Elevated levels of cyclin D1, VEGF and Bax were detected at 48 h in cells and increased Cox-2 expression was observed at 24 h. These data indicate that leptin may influence breast cancer development in relation to ER status as well as to the presence or absence of HER2. Continued study on leptin may be helpful for a better understanding of breast cancer development in obese women.

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Introduction

Obesity is an important risk factor for breast cancer among postmenopausal women (1,2). Furthermore, obesity is associated with a poorer prognosis in both premenopausal and postmenopausal breast cancer patients (3,4). Additionally diet-induced and genetic obesities have been reported to shorten latency and/or increase incidence for transgenic and carcinogen-induced mammary tumors (5,6).

To explain the connection between postmenopausal obesity and breast cancer, various mechanisms have been suggested, which include increased biosynthesis of estrogens by adipose tissue aromatase (CYP19) and higher levels of insulin and insulin-like growth factors (IGFs) in association with the metabolic syndrome (7,8). Obviously, estrogens play a pivotal role in breast cancer probably by stimulating cellular proliferation and oxidative metabolism mediated by various cytochrome P450 (CYP) enzymes such as CYP1A1 and CYP1B1 (9). The function of estrogens is also associated with insulin (10,11), which increases the activity of IGF-I by enhancing its synthesis and by decreasing its binding proteins (IGFBPs). The metabolic signals induced by either insulin or IGF-I can promote tumor development (12). In fact, the IGF family, composed of ligands, receptors and binding proteins, is important in overall tissue homeostasis (10).

Body weight and/or body fat is closely related with circulating leptin concentrations (13,14). Thus, obesity is characterized by elevated leptin levels (15). Leptin is a protein with cytokine-like activity, secreted mainly by adipocytes, and

is thought to play an important role in the control of satiety, energy expenditure, food intake, and various reproduction-related functions (16,17). Leptin exerts its physiological functions through the leptin receptor (Ob-R), which has at least 6 alternatively spliced forms (Ob-Ra to Ob-Rf) with modifications mainly at the C-terminus; Ob-Rb, the long form of the leptin receptor, appears to be important for leptin's weight regulating effects. Leptin predominantly activates the Janus kinase (Jak)/signal transducer and activator of transcription (Stat) signal transduction pathway (18). The activation of Ob-Rb leads to Jak2 phosphorylation, which can then recruit Stat3 (tyrosine-phosphorylated), finally leading to nuclear translocation and stimulation of transcription (18,19). Further, leptin regulates a well-known insulin target, phosphatidylinositol 3-kinase (PI3K), this phenomenon perhaps indicating cross-talk between the leptin and insulin signaling pathways (20).

It has been suggested that leptin enhances aromatase activity (21), which is involved in the aromatization of androgen into estrogen and linked with postmenopausal breast cancer risk. However, there may be other potential pathways of interaction between leptin and estrogen activity. Several studies have proposed the involvement of leptin in the pathological process of breast cancer (22-24). For example, the leptin receptor (Ob-Rb) has been identified in breast tumor samples and in human breast cancer cell lines; and in addition, leptin has been shown to stimulate cell proliferation and tumor progression (25-27). Leptin has been reported to increase the expression of cyclin D1 in human breast cancer cells and vascular endothelial growth factor (VEGF) in mouse mammary cancer cells, probably through PI3K and/or Jak2/Stat3 signaling pathways (28,29), which are also associated with leptin signaling. Moreover, an association between Stat3 and the transcriptional regulation of human cyclin D1 has been demonstrated in breast cancer cells (30). Hsieh *et al* (31) observed elevated levels of phosphorylated Stat3 (p-Stat3) in invasive breast cancer tissues compared to normal breast tissue, and association of p-Stat3 with metastasis in regional lymph nodes. Their study also revealed that p-Stat3 levels were significantly associated with anti-apoptotic markers, VEGF and cyclooxygenase 2 (Cox-2), in invasive breast cancer tissues (31).

The aim of the present study was to expand the investigation of the *in vitro* effects of leptin concentration on cell proliferation and on proteins important in leptin signaling including leptin receptors (Ob-Rb and Ob-R) and Jak2, PI3K, Stat3, and p-Stat3 in estrogen receptor-positive MCF-7, T47-D and MDA-MB-361 and estrogen receptor-negative MDA-MB-231 and SK-BR-3 human breast cancer cell lines (MDA-MB-361 and SK-BR-3 are positive for HER2/neu, whereas others are negative). In addition, cell proliferation/tumor progression markers, e.g., proliferating cell nuclear antigen (PCNA), cyclin D1, Cox-2 and VEGF; and insulin and related parameters, e.g., IGF-I, IGFBP3, IGF-I receptor α (IGF-IRα), were determined. Also, Western blot analyses were performed to evaluate anti-apoptotic markers Bcl-2 and Bcl-x_L, pro-apoptotic marker Bax, aromatase (CYP19), CYP1A1 and CYP1B1 in the above-mentioned human breast cancer cell lines. Furthermore, the presence of leptin receptors was detected immunocytochemically in these cell lines.

Materials and methods

Cell culture. The following procedures were applied to all assays (i.e., cell proliferation, immunocytochemistry and the Western blot analyses). Estrogen-dependent breast cancer cell lines MCF-7, T47-D and MDA-MB-361, and estrogen-independent cell lines MDA-MB-231 and SK-BR-3 were cultured in serum-free medium for 18 h. For MCF-7 and T47-D, Eagle's minimum essential medium [American Type Culture Collection (ATCC), Manassas, VA] and RPMI-1640 (Mediatech, Herndon, VA) were used, respectively. Leibovitz's L-15 medium (ATCC) was used for MDA-MB-361 and MDA-MB-231. McCoy's 5A medium (ATCC) was used for SK-BR-3. All cell lines were purchased from ATCC. Then, along with the control (without any treatment), the following concentrations of leptin were added to the respective wells: 5, 10, 25, 50 and 100 ng/ml. The cells were incubated with leptin for 24 and 48 h.

Cell proliferation assay. All cells were cultured in T-75 culture flasks, up to 70-90% confluency. Cells were harvested and counted, and plated at 5000 cells per well in 96-well plates overnight. Afterwards, cells were grown in serum-free medium for 18 h, leptin was added according to the above-mentioned concentrations and incubated for 24 and 48 h. To every well, 10 μl of Cell Counting Kit-8 reagent (Dojindo, Japan) was added and incubated at 37˚C in a 5% CO_2 incubator for 3 h. Then, optical density (O.D.) was measured by Multiskan EX ELISA reader (Thermo Electron, Finland) at 450 nm (with 620-nm reference).

Cell proliferation assays were performed at least 3 times for each cell line (in replicates of 12 wells for each concentration of leptin in each experiment). Data are presented as means ± standard error for a representative experiment for each cell line.

Immunocytochemistry. Cover-slip preparations in 6-well plates were made in an aseptic condition. Cells were cultured in serum-free medium in 6-well plates for 18 h. Above-mentioned concentrations of leptin were added and cells were incubated for 24 and 48 h. Moreover, a separate experiment was conducted where cells were incubated with 50 ng/ml of leptin (along with control) for short durations, i.e., 30 min, 1 h, 1.5 h, and 2 h. After that, cells were fixed with chilled acetone:methanol (1:1). Subsequently, cells were washed with PBS and endogenous peroxidase blocking was performed using 1% hydrogen peroxide for 10 min. Cells were then treated with 0.2% Tween-20 with PBS for 20 min at room temperature and incubated with 3% non-fat milk in PBS for 1 h at room temperature to block the non-specific reacting sites, followed by incubation overnight at 4˚C with rabbit primary antibody against Ob-R optimally diluted in PBS with 5% milk. Afterwards, cells were rinsed in PBS and incubated for 30 min with biotinylated secondary antibody (anti-rabbit Igs, ABC Staining System, Santa Cruz, CA) at room temperature. Following the PBS wash, cells were incubated for 30 min with avidin-biotinylated horseradish peroxidase (HRP) complex at room temperature. After washing with PBS, the reaction was visualized by the peroxidase substrate diaminobenzidine (DAB). Subsequent to development of the proper color, the

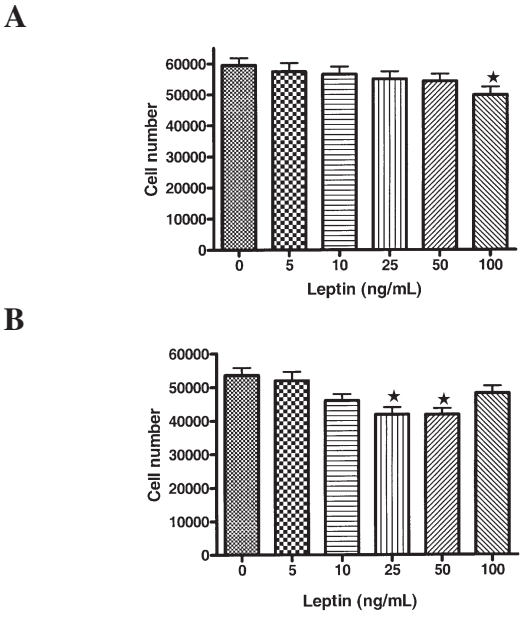

Figure 1. (A) MCF-7 cell proliferation assay after 24-h leptin treatment (ANOVA p=0.1171, Dunnett's multiple comparison posttest p<0.01 for 0 vs. 100 ng/ml). (B) MCF-7 cell proliferation assay after 48-h leptin treatment (ANOVA p=0.0002, Dunnett's multiple comparison posttest p<0.01 for 0 vs. 25 and 50 ng/ml).

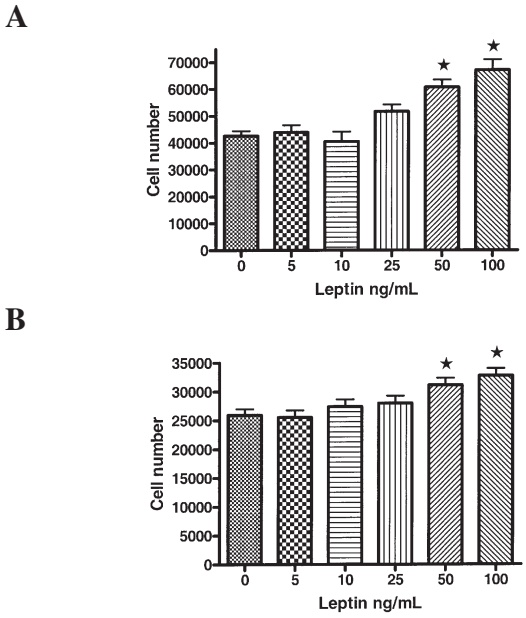

Figure 2. (A) MDA-MB-231 cell proliferation after 24-h leptin treatment (ANOVA p<0.0001, Dunnett's multiple comparison posttest p<0.01 for 0 vs. 50 and 100 ng/ml). (B) MDA-MB-231 cell proliferation after 48-h leptin treatment (ANOVA p=0.0002, Dunnett's multiple comparison posttest p<0.05 for 0 vs. 50 ng/ml, and p<0.01 for 0 vs. 100 ng/ml).

cells were washed with deionized water and counterstained with hematoxylin. Finally, the cells were washed and dehydrated gradually through graded alcohol and mounted on glass-slides.

Western blot analysis. Cells were cultured in 6-well plates in serum-free medium, then treated with leptin and incubated for 24 and 48 h following the above-mentioned protocol. For extraction of total soluble proteins from the cultured cells, the Mammalian Cell - PE LB™ kit (Genotech, St. Louis, MO) containing EDTA, DTT and protease inhibitor was used. Cell lysates were sonicated and subsequently centrifuged at 13,000 rpm for 10 min at 4˚C, and clear supernatants were collected. For the Western blot analysis, 10% SDS-PAGE was used and proteins were transferred to PVDF membranes (Immobilon - P, Millipore, Billerica, MA). Primary antibodies against the following proteins were used for probing: Ob-Rb (Linco, St. Louis, MO), Ob-R, leptin, Jak2, PI3K, Stat3, p-Stat3, PCNA (Abcam, Cambridge, MA), cyclin D1, Cox-2, VEGF, Bcl-2 (Alexis, AXXORA LLC, San Diego, CA), Bcl-x_L, Bax, insulin, IGF-I, IGFBP3, IGF-IRα, aromatase (Cell Signaling, Beverly, MA), CYP1A1 and CYP1B1 (except where indicated, all antibodies were from Santa Cruz, CA). Suitable secondary IgGs conjugated with alkaline phosphatase (AP) were used as secondary antibody. Proteins were detected by enhanced chemifluorescence (ECF substrate, Amersham, Newark, NJ) and developed in a Storm 840 system (Amersham, Piscataway, NJ).

Results

Cell proliferation assays. The following are representative results from multiple proliferation assays that were conducted for each cell line.

MCF-7. There was a dose-dependent decrease in cell numbers in this cell line following leptin treatment for 24 h in a serum-free system, with a statistically significant growth inhibitory effect observed at 100 ng/ml (Fig. 1A). A somewhat similar trend was observed after 48 h of leptin treatment, with 25- and 50-ng/ml doses exhibiting significantly inhibitory effects, but not the 100-ng/ml concentration (Fig. 1B). This appears contrary to some published reports on leptin's proliferative effects on this cell line (32).

MDA-MB-231. Fig. 2A and B illustrates the response of this cell line to leptin treatment for 24 and 48 h. At the 24-h time point, leptin concentrations of 50 and 100 ng/ml elicited proliferative responses that were significantly higher than untreated cells. This same effect was maintained at the 48-h time point. There appeared to be a dose-dependent effect of leptin in promoting MDA-MB-231 cell growth. Frankenberry and colleagues recently reported proliferative effects of a lower dose (40 ng/ml) of leptin treatment in this cell line at the 48-h time point (33).

T47-D. Leptin exhibited mitogenic effects in this cell line, and displayed dose dependency (Fig. 3A and B). Leptin concentrations of 25, 50 and 100 ng/ml were effective at promoting significantly greater cell growth compared to untreated cells at the 24-h time point, while only the 50- and 100-ng/ml concentrations maintained this effect at 48 h. Laud and colleagues reported that 50- and 100-ng/ml leptin concentrations were effective in maintaining significantly higher cell numbers than untreated control as determined by thymidine incorporation experiments and cell proliferation assays following 24 h of leptin treatment (26). Our proliferation assay results on this cell line concur with these observations.

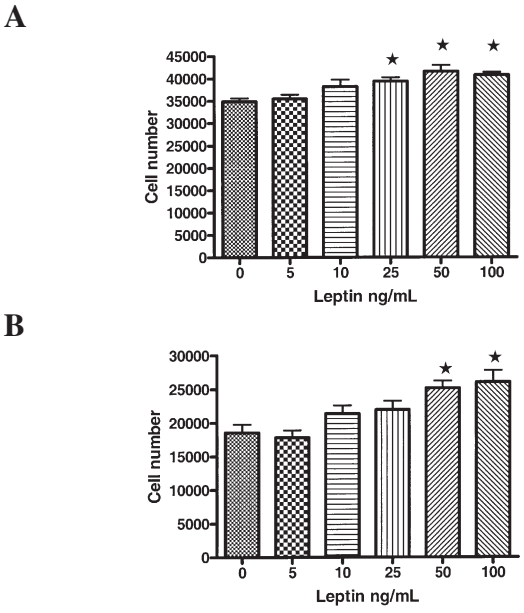

Figure 3. (A) T47-D cell proliferation after 24-h leptin treatment (ANOVA $p<0.0001$, Dunnett's multiple comparison posttest $p<0.05$ for 0 vs. 25 ng/ml, and $p<0.01$ for 0 vs. 50 and 100 ng/ml). (B) T47-D cell proliferation after 48-h leptin treatment (ANOVA $p<0.0001$, Dunnett's multiple comparison posttest $p<0.05$ for 0 vs. 25 ng/ml and $p<0.01$ for 0 vs. 50 and 100 ng/ml).

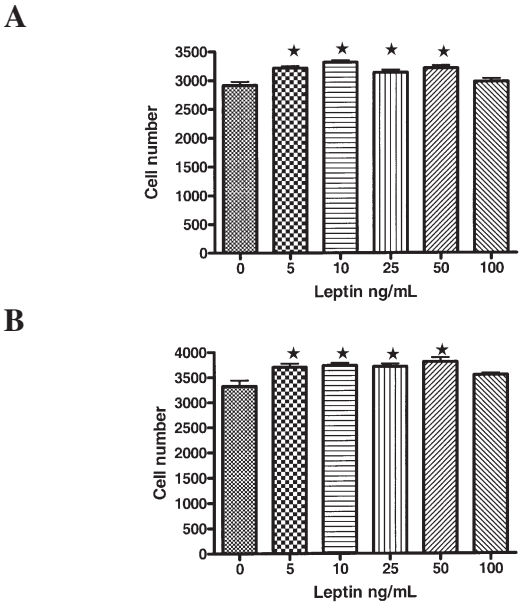

Figure 4. (A) SK-BR-3 cell proliferation after 24-h leptin treatment (ANOVA $p<0.0001$, Dunnett's multiple comparison posttest $p<0.01$ for 0 vs. 5, 10, 25 and 50 ng/ml). (B) SK-BR-3 cell proliferation after 48-h leptin treatment (ANOVA $p<0.0001$, Dunnett's multiple comparison posttest $p<0.01$ for 0 vs. 5, 10, 25 and 50 ng/ml).

SK-BR-3. Leptin treatment resulted in increased cell proliferation for this cell line at 5, 10, 25, and 50 ng/ml but not at 100 ng/ml at both 24- and 48-h time points. The effects of leptin at the mentioned doses were virtually identical at the two time points (Fig. 4A and B).

MDA-MB-361. Leptin also elicited increased cell proliferation for this cell line at 10, 25, 50 and 100 ng/ml in a dose-dependent manner at both 24- and 48-h time points (Fig. 5A and B).

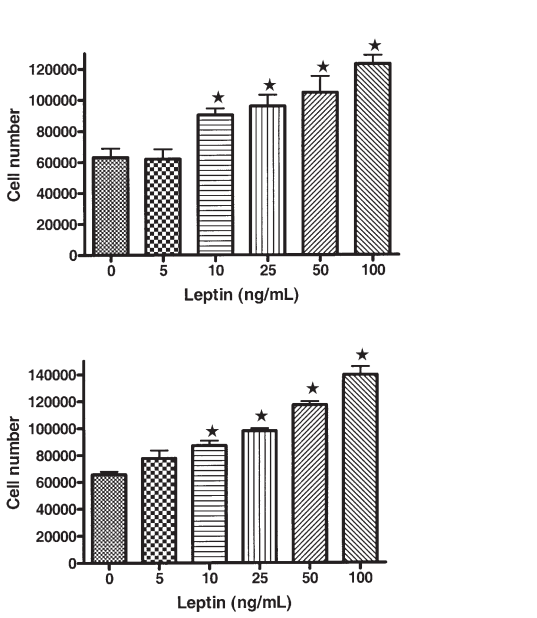

Figure 5. (A) MDA-MB-361 cell proliferation after 24-h leptin treatment (ANOVA $p<0.0001$, Dunnett's multiple comparison posttest $p<0.05$ for 0 vs. 10 ng/ml and $p<0.01$ for 0 vs. 25, 50 and 100 ng/ml). (B) MDA-MB-361 cell proliferation after 48-h leptin treatment (ANOVA $p<0.0001$, Dunnett's multiple comparison posttest $p<0.01$ for 0 vs. 10, 25, 50 and 100 ng/ml).

Immunocytochemical analysis. The immunocytochemical analyses showed the presence of Ob-R in all 5 examined cell lines. In general, the immunoreactivity for Ob-R was confined to the cell membrane and cytoplasm as observed in human breast tumors by Ishikawa *et al* (23). However, the distribution patterns of Ob-R were different among these cell lines. In MCF-7 and MDA-MB-231 cells treated with leptin for 24 and 48 h, four main types of Ob-R distribution were observed, namely, peripheral, crescent, bipolar and unipolar (Fig. 6A-D for MCF-7; Fig. 6E-H for MDA-MB-231). In the majority of MCF-7 cells treated for longer durations (i.e., 24 and 48 h), Ob-R was expressed around the periphery. On the contrary, in MCF-7 cells treated with leptin for short durations (i.e., 30 min to 2 h), the intensity of Ob-R immunostaining was usually weaker and no specific pattern of distribution was observed (Fig. 7A and B). The above-mentioned four distribution patterns were also observed in MDA-MB-231 cells treated for both short and long durations. However in the shorter time-course treatments Ob-R immunoexpression was primarily the crescent pattern in the majority of MDA-MB-231 cells; whereas a unipolar distribution was observed with longer leptin treatment times for this cell line. It also appeared that in MDA-MB-231 cells, the crescent pattern decreased while the unipolar pattern increased in relation with length of exposure to leptin. The expression of Ob-R in T47-D and SK-BR-3 cells did not show any specific staining pattern in response to either short- or long-term leptin exposure (Fig. 8A-E for T47-D; Fig. 9A-C for SK-BR-3). Similarly, no pattern of Ob-R expression was observed in MDA-MB-361 cells in the shorter time-course experiments (Fig. 9D). In contrast, many cells exhibited a unipolar distribution of

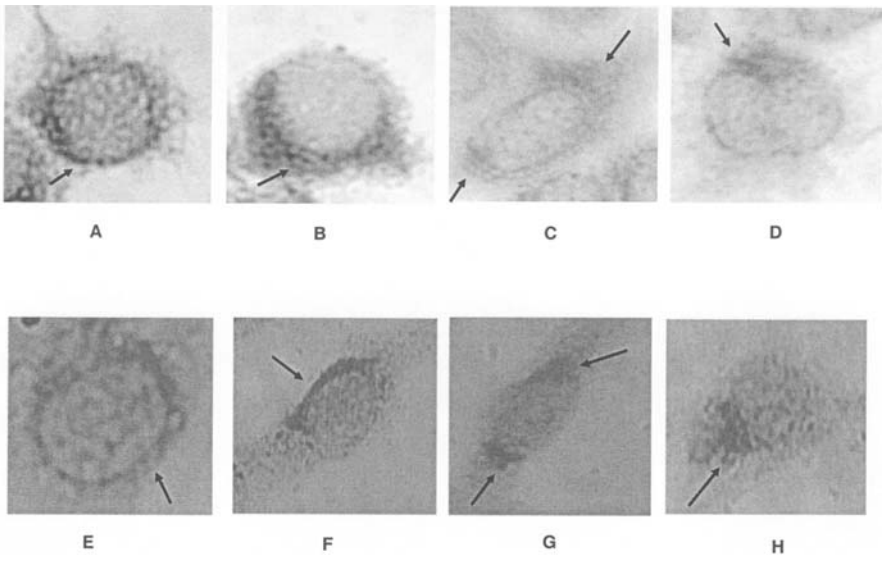

Figure 6. Expression of Ob-R in MCF-7 cells (A-D) and MDA-MB-231 cells (E-H). The cells were treated with 50 ng/ml of leptin for 24 h.

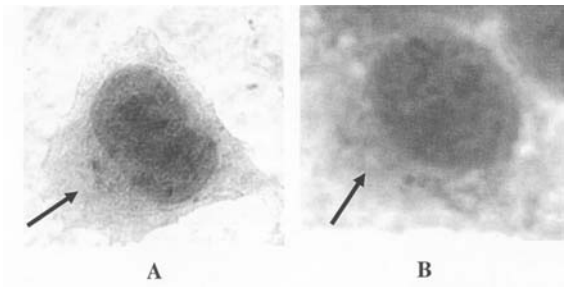

Figure 7. Expression of Ob-R in MCF-7 cells, which were incubated with 50 ng/ml of leptin for 30 min (A) and 2 h (B).

immunoreactivity for Ob-R in the longer term leptin treatments (Fig. 9E and F).

Western blot analysis. A summary of results for the Western blot analyses are presented in Table I. Details for individual cell lines are presented below.

MCF-7. MCF-7 cells treated with leptin for 24 h had increased expression of Ob-Rb, Jak2, PI3K, Stat3 and p-Stat3 in a dose-dependent manner up to 50 ng/ml. Interestingly, MCF-7 cells treated with 100 ng/ml of leptin for 24 h exhibited a decline of expression of all the above-mentioned proteins. In contrast, MCF-7 cells treated for 48 h exhibited no consistent results for these proteins. Inconsistent levels were observed in Ob-R and leptin expression. Also, PCNA showed inconsistent expression in response to leptin; whereas no change was noted in VEGF expression. However, cyclin D1 expression increased after 48 h of leptin exposure ranging in concentration from 25 to 100 ng/ml. Similarly, increases of Cox-2 levels were observed in 48 h in relation to leptin concentrations with a maximum response at 50 ng/ml. There was increased expression of Bax in MCF-7 cells treated with 10 and 25 ng/ml of leptin for both 24 and 48 h; but no definite increasing trends were recorded in the case of Bcl-2 and Bcl-x_L. On the other hand, IGF-IRα increased in a dose-dependent manner

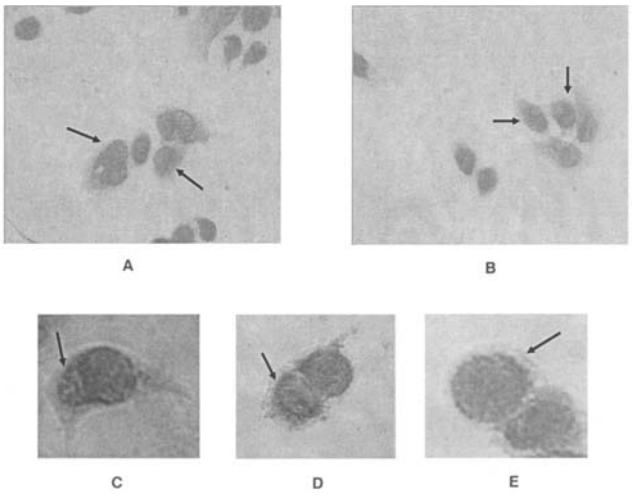

Figure 8. Expression of Ob-R in T47-D cells, treated with 50 ng/ml of leptin for 24 h (A-E).

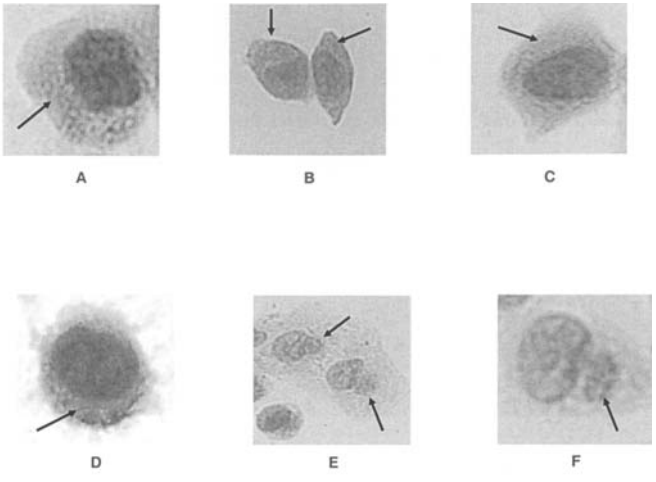

Figure 9. Expression of Ob-R in SK-BR-3 and MDA-MB-361 cells. (A-C) SK-BR-3 cells treated with 100 ng/ml of leptin for 48 h. (D) MDA-MB-361 cell treated with 50 ng/ml of leptin for 1 h. (E and F) MDA-MB-361 cells treated with 100 ng/ml of leptin for 48 h.

Table I. Summary of the findings for Western blot analyses for proteins related to leptin.

	MCF-7	MDA-MB-231	T47-D	SK-BR-3	MDA-MB-361
Ob-Rb	Response ↑	Response ↑	Inconsistent	Inconsistent	Response ↑ ⇑
Ob-R	Inconsistent	Inconsistent	Inconsistent	Response ↑ ⇑	Inconsistent
Jak2	Response ↑	Inconsistent	Response ↑ ⇑	Response ↑	Inconsistent
PI3K	Response ↑	Inconsistent	Inconsistent	Response ↑	Response ⇑
Stat3	Response ↑	Inconsistent	Response ↑ ⇑	Inconsistent	Response ⇑
p-Stat3	Response ↑	Response ⇑	Response ⇑	No change	No change
Leptin	Inconsistent	Inconsistent	Inconsistent	Response ↑ ⇓	Inconsistent
Bcl-2	No change	Inconsistent	Inconsistent	Not detected	Response ↓ ⇑
Bcl-x_L	Inconsistent	Response ⇑	Response ⇓	Inconsistent	Inconsistent
Bax	Response ↑ ⇑	Inconsistent	Inconsistent	Response ⇑	Inconsistent
PCNA	Inconsistent	Response ↑ ⇑	Response ⇑	Response ↓ ⇑	Response ⇑
Cyclin D1	Response ⇑	Response ↑ ⇑	Response ↑ ⇑	Response ⇑	Inconsistent
Cox-2	Response ⇑	Inconsistent	Inconsistent	Response ↑	Response ↑ ⇑
VEGF	No change	Inconsistent	Inconsistent	Response ⇑	Response ↑ ⇑
Insulin	Not detected	Not detected	Not detected	Not detected	Response ⇑
IGF-I	Inconsistent	Not detected	Not detected	Response ↑ ⇑	Inconsistent
IGFBP3	Inconsistent	Inconsistent	Not detected	Response ↑ ⇑	Inconsistent
IGF-IRα	Response ↑	Inconsistent	No change	Response ↓ ⇑	Response ↓ ⇑
CYP19	Inconsistent	Inconsistent	Inconsistent	No change	Inconsistent
CYP1A1	Inconsistent	Inconsistent	Inconsistent	Inconsistent	Inconsistent
CYP1B1	Response ↑ ⇑	Inconsistent	Inconsistent	Inconsistent	Response ↑

(↑) An increase with 24-h incubation with increasing concentrations of leptin. (↓) A decrease with 24-h leptin incubation. (⇑) An increase after 48 h and (⇓) a decrease after 48-h incubation with leptin.

after 24 h; however, IGF-I and IGFBP3 expression was inconsistent, and we were unable to detect the expression of insulin. Further, an increase in CYP1B1 expression was observed at both 24 and 48 h with all concentrations of leptin, but aromatase and CYP1A1 levels were inconsistently affected by time and the addition of leptin.

MDA-MB-231. After 24-h exposure to leptin, there was a trend for Ob-Rb expression to increase with up to 25 ng/ml of leptin and then decline at higher leptin concentrations in MDA-MB-231 cells. On the other hand, no consistent changes were detected for Ob-R, leptin, Jak2, PI3K and Stat3 expression at both 24 and 48 h. Nevertheless, p-Stat3 exhibited an increased level after both 24- and 48-h incubation with leptin. It is interesting to note that PCNA and cyclin D1 expression was increased in cells treated with leptin for both 24 and 48 h; although Cox-2 and VEGF expression was inconsistent. Moreover, inconsistent expression levels were observed for Bcl-2 and Bax; whereas Bcl-x_L showed increased expression at the 48-h time point. We did not detect insulin and IGF-I protein expression in MDA-MB-231 cells. On the other hand, IGFBP3, IGF-IRα, and enzymes of estrogenic pathways such as aromatase, CYP1A1 and CYP1B1 revealed inconsistent levels in relation with different concentrations of leptin and time of incubation.

T47-D. There were no consistent patterns of expression in relation to leptin treatment in T47-D cells for Ob-Rb and Ob-R. In contrast, Jak2 and Stat3 expression increased with higher concentrations of leptin after both 24- and 48-h exposure to leptin. However, p-Stat3 showed increased expression only in 48-h leptin-treated cells from 25- to 100-ng/ml concentrations. Inconsistent results were found in the case of PI3K and leptin expression. Although there was no effect of exogenous leptin on PCNA after 24 h, increasing levels of PCNA were noted in cells incubated with 10-100 ng/ml of leptin for 48 h. Also, cyclin D1 levels showed higher expression at both 24 and 48 h with increasing leptin concentrations compared to controls. On the other hand, Cox-2, VEGF, Bcl-2 and Bax expression was inconsistently affected by time and leptin concentration. T47-D cells had lower expression of Bcl-x_L with increasing concentrations of leptin in comparison with controls following 24-h incubation and also Bcl-x_L expression was lower with increasing concentrations of leptin after 48 h of exposure. Expression of insulin, IGF-I and IGFBP-3 was not detected in T47-D cells. IGF-IRα expression was detected but no marked alterations were observed with leptin administration. Aromatase, CYP1A1 and CYP1B1 did not exhibit any consistent level of expression in relation with various leptin concentrations.

MDA-MB-361. In MDA-MB-361 cells, Ob-Rb expression increased at both 24 and 48 h, up to 50 ng/ml leptin. PI3K and Stat3 increased after 48 h in MDA-MB-361 cells incubated with leptin in a dose-dependent manner; however, no change was observed in p-Stat3 levels. Inconsistent levels of Ob-R,

Jak2 and leptin expression were recorded in leptin-administered MDA-MB-361 cells compared to controls. Similarly, Bcl-x_L and Bax revealed inconsistent levels of expression. For Bcl-2, there was a gradual decrease in expression as leptin increased at 24 h but there was a dose-dependent increase after 48 h. PCNA levels were also higher after 48 h at all leptin concentrations. Cox-2 exhibited higher expression levels at 5 and 10 ng/ml leptin in both 24- and 48-h cells; however, the levels declined afterwards. Further, VEGF expression increased at both the 24- and 48-h time periods, particularly at 100 ng/ml leptin, while cyclin D1, IGF-I and IGFBP3 exhibited inconsistent levels of expression in response to the addition of leptin regardless of the concentration used. In contrast to the three cell lines described above, insulin was expressed in MDA-MB-361 cells, and furthermore, an increase of insulin production was observed in 25, 50 and 100 ng/ml leptin-treated cells after 48-h incubation; but the levels were inconsistent in cells exposed to leptin for only 24 h. IGF-IRα levels declined at 24 h with increasing leptin concentrations; whereas there was an increase of IGF-IRα expression in a dose-dependent manner at 48 h. Higher expression of CYP1B1 with leptin treatment compared to no leptin was noted in MDA-MB-361 cells after 24 h; whereas a decline was found at 48 h. On the other hand, both aromatase and CYP1A1 levels were inconsistent.

SK-BR-3. In SK-BR-3 cells, there was a slight increase in Ob-R levels after 24 h with intermediate levels of leptin but a decrease at the 100 ng/ml level. After 48 h there was a dose-dependent increase of this protein. The expression levels of Ob-Rb were inconsistent, while there was a gradual increase of leptin expression at 24 h, but a gradual decrease at 48 h with increasing leptin concentration. Among the signaling proteins, Jak2 exhibited increased levels at 25, 50 and 100 ng/ml leptin after 24 h and higher expression of PI3K was observed in leptin-treated cells at the 24-h time point but at 48 h these proteins were not affected by leptin. No significant alterations or trends were recorded for either Stat3 or p-Stat3 expression. At 24 h, Bax levels increased initially at 5 ng/ml of leptin, but decreased afterwards. After 48 h, Bax expression increased to its highest level at 50 ng/ml leptin treatment. Interestingly, there was no detectable Bcl-2 expression in SK-BR-3 cells; while Bcl-x_L levels were inconsistent in response to the addition of leptin at both 24 and 48 h. In contrast, PCNA, cyclin D1 and VEGF increased in a dose-dependent manner at the 48-h time point. However, there was a decreasing trend for expression of PCNA in 24-h treated cells. On the other hand, increased Cox-2 expression was observed at 24 h. After 24 h, IGF-I expression increased in leptin-treated cells, and there was a gradual increase in a dose-dependent manner up to 50 ng/ml at 48 h. Moreover, there was an increase in IGFBP3 levels in a dose-dependent manner up to 50 ng/ml at both 24 and 48 h. On the other hand, IGF-IRα levels showed a decline at 24 h with increasing leptin concentrations, but at 48 h expression of IGF-IRα increased in a dose-dependent manner. As with three of the four other cell lines there was no detectable expression of insulin in the SK-BR-3 cell line. Furthermore, no changes in aromatase expression were observed in SK-BR-3 cells with varying concentrations of leptin. Additionally, CYP1A1 and CYP1B1 expression was not consistently affected by the addition of leptin at either time point.

Discussion

The incidences of both obesity and breast cancer in the United States are continuing to increase (34,35). Furthermore, the incidence of breast cancer is also increasing globally, and this trend is partly due to dietary and lifestyle changes including obesity and less physical activity (36). Obesity is a known risk factor for postmenopausal breast cancer (1). Furthermore, obesity may affect prognosis through numerous pathways including hormonal profiles, co-morbidities that can interfere with treatment, and other as yet unknown mechanisms (37). The relationship between obesity and breast cancer pathology is a complex phenomenon, and several factors, apart from estrogen, such as insulin, IGF-I and leptin may be involved in this complicated disease process (38,39).

A growing body of *in vitro* evidence indicates that proliferation of breast cancer cells is influenced by addition of leptin. Recently, Yin *et al* (40) observed that leptin stimulated the growth of MCF-7 and T47-D human breast cancer cells in a time- and dose-dependent fashion. Similar findings were previously recorded by Dieudonne *et al* (32) and Okumura *et al* (41) in MCF-7 cells as well as in T-47D cells by Hu *et al* (25) and Laud *et al* (26) and in ZR-75-1 cells by Somasundar *et al* (42) and Chen *et al* (28). In the present study we also noted an overall growth stimulation effect of leptin in the examined cell lines, i.e., T47-D, MDA-MB-361, MDA-MB-231 and SK-BR-3. However we were unable to find a positive effect in MCF-7 cells by leptin using a cell proliferation assay.

Similar to the observation of Dieudonne *et al* (32) in MCF-7 cells, the current study identified Ob-R expression by immunocytochemistry in the above-mentioned five cell lines. There appeared to be different patterns of cellular location for the protein, however, the significance (if any) with respect to leptin's action in mammary tumorigenesis is not clear at the present time. It appears that there are some trends of Ob-R localization within the individual breast cancer cell lines, particularly in MCF-7, MDA-MB-231 and MDA-MB-361 cell lines. Most probably, this phenomenon is associated with the progression of the cell cycle; and perhaps cytoskeleton elements are also involved in this process. Nevertheless, it has been documented in this study that Ob-R is not a static entity.

It appears that leptin induces multiple signaling pathways in breast cancer cells. Hu *et al* (25) observed increased levels of p-Stat3 in leptin-stimulated human breast epithelial cells (HBL100) and breast cancer cells (T47-D) compared to the corresponding untreated controls. The results of the present study pertaining to T47-D cells support these previous findings. Activation of signaling proteins following leptin exposure was clearly indicated in MCF-7 cells, another estrogen-dependent cell line. Several studies reported that stimulation of MCF-7 cells with leptin induced multiple signaling proteins including Stat3 (32,40,43). These earlier studies also showed an increase in p-Stat3 levels by leptin, as we do in the present study. Interestingly, our study indicated an enhanced expression of Jak-2 and PI3K in SK-BR-3 cells (estrogen-independent cells), similar to MCF-7 cells. However, activation of Stat-3 was not observed in SK-BR-3 cells.

Dieudonne *et al* (32) postulated that leptin, through Stat3 activation, may stimulate aromatase (CYP19) gene expression in MCF-7 cells. A strong association between CYP19 gene expression and the expression of the Cox-2 gene has been reported in breast cancer (44). Additionally, Catalano *et al* (21) reported a significant increase of aromatase enzymatic activity and protein content in MCF-7 cells upon leptin treatment. However, the present study did not demonstrate such effects of leptin in any of the examined cell lines. Regarding other estrogen metabolizing cytochrome P450 enzymes, we noticed an increased level of expression of CYP1B1 in estrogen-dependent MCF-7 and MDA-MB-361 cells, in response to leptin treatment. This observation may be of interest because CYP1B1 is known to be involved in the oxidative metabolism of estrogen (4-hydroxylation) (45) and activation of exogenous carcinogens as a phase I enzyme (46). Accumulating evidence suggests an association of CYP1B1 with risk and prognosis in breast cancer (47,48).

Tumorigenesis involves numerous pathological processes such as angiogenesis, proliferation and cell-cycle progression as well as evading apoptosis. All these phenomena are closely interrelated. Overall, the evidence suggests that the progressive inhibition of apoptosis and induction of angiogenesis may contribute to tumor initiation, growth and metastasis in the pathogenesis of breast cancer (49). VEGF, a key regulator of angiogenesis, was shown to induce the expression of the anti-apoptotic protein, Bcl-2, in human breast cancer cells (50). On the other hand, Cox-2 (the inducible form that converts arachidonic acid into prostaglandins and thromboxanes) expression is associated with tumor cell proliferation, resistance to apoptosis and induction of angiogenesis (51). In the study by Okumura *et al* (41), there were no significant effects of leptin on the expression of apoptotic markers including Bax, Bcl-2 and Bcl-x_L in MCF-7 cells indicating that leptin induced MCF-7 cell proliferation without altering apoptosis. However, their study recorded up-regulation of cell cycle-regulated proteins such as cyclin D1 in MCF-7 cells with leptin (41). Similarly, Chen *et al* (28) observed that leptin stimulated expression of cyclin D1 in cell cycle progression of ZR-75-1 breast cancer cells. In the present study we found no definitive trend for the expression of apoptotic markers in response to leptin as there were variations in expression of these markers among the different cell lines. However, in the majority of cell lines, an up-regulation of proliferation and cell-cycle progression markers such as PCNA and cyclin D1 was found following leptin exposure. Overall this suggests that leptin's primary effect concerning increasing breast cancer cell numbers is directly on proliferation.

An association between leptin and Cox-2 activity has been reported. For example, it has been demonstrated that increased leptin production in the presence of arachidonic acid was reduced by selective Cox-2 inhibitors, which also reversed the proliferation of estrogen-responsive and estrogen receptor-positive human breast cancer cells with leptin administration (52). Recently, a study on esophageal adenocarcinoma cells has shown that leptin stimulated cell proliferation and inhibited apoptosis through activation of Cox-2 and prostaglandin E2 production (53). In the current study, Cox-2 levels increased in MCF-7 cells following longer duration of incubation with leptin. Additionally, two HER2/neu-positive cell lines, i.e., SK-BR-3 and MDA-MB-361 cells, exhibited higher levels of Cox-2 and VEGF expression after leptin treatment. The angiogenic role of leptin has been documented in previous studies (54,55). It is worthy to mention that obesity, HER2/neu overexpression and tumor angiogenesis are all considered as poor prognostic factors in breast cancer (37,56-58); and an association between HER2/neu overexpression and angiogenesis has been recorded (59). Therefore, HER2/neu-positive cells in the tumor microenvironment may influence the disease course immensely under obese conditions.

Expression of the angiogenic stimulator VEGF is influenced by insulin, growth factors such as PDGF, EGF, TNF-α and TGF-ß, and several cytokines, as reviewed by Liekens *et al* (60) and Hausman and Richardson (61). On the other hand, tumor microvascular endothelial cells supply different growth factors including IGF-I (62). It has been hypothesized that leptin has an association with insulin and IGF-I (24). There are also supportive data from human subjects (63). However, the present study did not indicate a clear association between leptin and the insulin/IGF-I system at the intracellular level. It has been reported that higher circulating levels of IGF-I predict risk for premenopausal but not postmenopausal breast cancer (64). The mitogenic and survival effects of IGF-I on both normal and malignant breast cells are mediated primarily through the IGF-IR, which is overexpressed and highly activated in breast tumors (65). Some recent studies on primary breast tumor tissues reported approximately 50% positive immunostaining for IGF-IR (66,67). Nevertheless, expression of IGF-I and IGF-IR genes may undergo substantial change over the course of breast tumorigenesis (68). Moreover, Garofalo *et al* (24) proposed that factors such as insulin and IGF-I are possibly associated with the overexpression of leptin and its receptor in breast cancer.

Interestingly, Eisenberg *et al* (69) demonstrated that leptin might provoke the phosphorylation of HER2/neu/c-erbB-2 and thus enhance its oncogenic potential independently of HER family ligands or its overexpression. Furthermore, Menendez et al (70) observed that inhibition of fatty acid synthase suppressed HER2/neu expression in human breast cancer SK-BR-3 cells. However, in a recent study using MMTV-neu mice with diet-induced obesity and elevated serum leptin levels there was little impact on mammary tumor development compared to lean mice (71). However, it should be noted that the serum leptin levels were in the low range compared to *in vitro* studies and serum levels frequently documented in obese humans. In the present study we observed an influence of leptin on the SK-BR-3 cell line, which is estrogen receptor-negative and HER2/neu-positive, with reference to leptin production, related signaling proteins (Jak2 and PI3K), tumor growth markers (PCNA, cyclin D1, Cox-2 and VEGF) and IGF-I.

With respect to serum leptin levels a number of studies have addressed this issue. Several studies failed to demonstrate a relationship between circulating leptin levels and breast cancer risk (72-77). However, the majority of these studies were conducted on a small number of cases. Interestingly, an inverse association of blood leptin levels with breast cancer was recorded in two studies on premenopausal women (78,79). Also, in an earlier study, Mantzoros *et al* (80) did not find any significant correlation between serum leptin concentrations and premenopausal carcinoma *in situ* of the breast. Since obesity is

not a risk factor for premenopausal breast cancer, this finding is not unexpected. On the other hand, some studies observed higher blood levels of leptin among patients with breast cancer (81-84). Additionally, Goodwin *et al* (85) noticed that plasma leptin levels were strongly correlated with insulin and significantly higher with advanced tumor stage, higher tumor grade and estrogen receptor negativity; but no relation with lymph node metastasis was found. Recently, Miyoshi *et al* (86) demonstrated a significant association of high intratumoral leptin receptor mRNA levels with a poor prognosis which was observed only in the subset of the patients with high intratumoral leptin mRNA levels or high serum leptin levels. It is of interest to mention that inhibition of mammary tumor development has been demonstrated in leptin-deficient mice (22).

A large number of epidemiological studies indicate obesity as an important risk factor for postmenopausal breast cancer and a poor prognostic factor for all breast cancer cases. Further, a growing body of evidence shows that leptin may play a significant role in this complex pathological process. However, there is a need for detailed investigations on the estrogen-dependent and -independent status of breast cancer with reference to how leptin mediates its effects. Interestingly, the present study revealed that the impact of leptin is influenced by additional cell characteristics, not just estrogen receptor status. Because breast cancer cell lines represent tumor cells the present results may actually be a better reflection of breast cancer prognosis. It is known that treatment modalities are limited in patients with estrogen-independent tumors. Therefore, a proper understanding of the relationship between leptin and the estrogen-independent state may provide life-saving strategies for treatment. Overall, the results of this study suggest that leptin may contribute significantly to the pathology of breast cancer. However, the influence of leptin should be viewed in relation with other factors.

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