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Leptin Promotes Cell Proliferation and Survival of Trophoblastic Cells[1]

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ABSTRACT

Leptin, the 16-kDa protein product of the obese gene, was originally considered as an adipocyte-derived signaling molecule for the central control of metabolism. However, leptin has been suggested to be involved in other functions during pregnancy, particularly in placenta. In the present work, we studied a possible effect of leptin on trophoblastic cell proliferation, survival, and apoptosis. Recombinant human leptin added to JEG-3 and BeWo choriocarcinoma cell lines showed a stimulatory effect on cell proliferation up to 3 and 2.4 times, respectively, measured by ^{3}H-thymidine incorporation and cell counting. These effects were time and dose dependent. Maximal effect was achieved at 250 ng leptin/ml for JEG-3 cells and 50 ng leptin/ml for BeWo cells. Moreover, by inhibiting endogenous leptin expression with 2 μM of an antisense oligonucleotide (AS), cell proliferation was diminished. We analyzed cell population distribution during the different stages of cell cycle by fluorescence-activated cell sorting, and we found that leptin treatment displaced the cells towards a G2/M phase. We also found that leptin upregulated cyclin D1 expression, one of the key cell cycle-signaling proteins. Since proliferation and death processes are intimately related, the effect of leptin on cell apoptosis was investigated. Treatment with 2 μM leptin AS increased the number of apoptotic cells 60 times, as assessed by annexin V-fluorescein isothiocyanate/propidium iodide staining, and the caspase-3 activity was increased more than 2 fold. This effect was prevented by the addition of 100 ng leptin/ml. In conclusion, we provide evidence that suggests that leptin is a trophic and mitogenic factor for trophoblastic cells by virtue of its inhibiting apoptosis and promoting proliferation.

apoptosis, growth factors, leptin, mechanisms of hormone action, placenta

INTRODUCTION

Leptin hormone, the product of the *LEP* gene, formerly known as the *OB* gene, is a 16-kDa nonglycosylated polypeptide of 146 amino acids discovered in 1994 by Zhang et al. [1]. It is a cytokine-type hormone, mainly secreted by the white adipose tissue and, to a lesser extent, by placenta and gastric mucosa, although the presence of leptin has been described in other tissues, such as the skeletal muscle and mammary and salivary glands [2]. Leptin is able to exert multiple functions; the best characterized is the regulation of food intake and energy expenditure, especially under conditions of restricted energy availability. In this regard, leptin is produced by white adipose tissue and secreted in response to energy storage. Thus, plasma leptin levels correlate with total adipose mass. Circulating leptin is actively transported through the blood-brain barrier to act on the hypothalamic satiety center [3].

Recently, pleiotropic effects of leptin have been identified, involved in the modulation of several processes, such as thermogenesis, homeostasis, angiogenesis, hematopoiesis, osteogenesis, chondrogenesis, neuroendocrine and immune functions, as well as arterial pressure control [4]. Compelling evidence in recent years also implicated leptin in reproductive functions, such as the regulation of ovarian function, oocyte maturation, and embryo development and implantation [5–7]. The process of embryo implantation and trophoblast invasion is currently considered as the most limiting factor for the establishment of pregnancy. Molecular interactions at the embryo-maternal interface during the time of adhesion and subsequent invasion are crucial to the process of embryonic implantation [8]. This process takes place during the first weeks of pregnancy, when the well-differentiated primary cells of the placenta, known as trophoblast cells, grow in an invasive fashion. There is evidence suggesting that cytokines produced by the maternal endometrium and the developing embryo play an important role in this signaling process. Although numerous cytokine-receptor pairs are expressed by the maternal endometrium and the embryo during implantation, knowledge of the cytokine functions is limited [9]. Leptin sequencing indicated that it could belong to the long-chain helical cytokine family (e.g., interleukin [IL]2, IL12 proteins, and growth hormone [GH]) [10]. In fact, leptin receptors are single transmembrane glycoproteins that display sequence homology to IL6 signal transducer (also known as gp130) and members of the class I cytokine receptor family. Several cytokines and growth factors are known to influence trophoblast migration, proliferation, and invasion [11]. In particular, leptin has been proposed to play a relevant role in implantation by virtue of its stimulatory effect on matrix metalloproteinase expression in cytotrophoblast [12]. On the other hand, deregulation of leptin metabolism and/or leptin function in the placenta has been implicated in the pathogenesis of various disorders during pregnancy, such as recurrent miscarriage, gestational diabetes, intrauterine growth retardation, and preeclampsia [4, 13].

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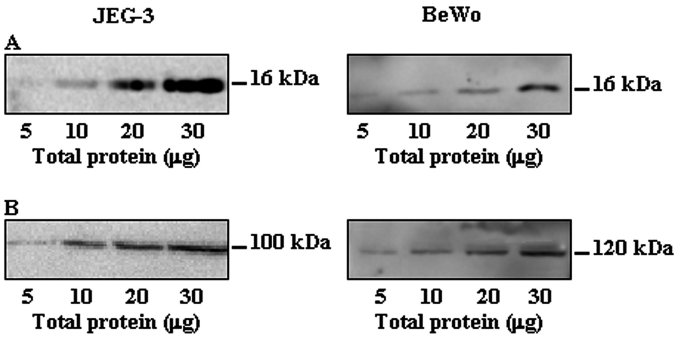

FIG. 1. Western blot analyses for leptin or leptin receptor expression in JEG-3 and BeWo cells. Cells (1×10^6 cells) were plated in complete DMEM-F12 media supplemented with 10% FCS. After 48 h of culture, cell extracts were prepared as indicated in *Materials and Methods*. Proteins (10–30 μg) were separated on SDS-PAGE gels. Immunobloting for leptin (**A**) or leptin receptor (**B**) was performed as indicated in *Materials and Methods*. Molecular weights were estimated using standard protein markers.

The human leptin receptor gene encodes four transmembrane proteins with different C-terminal lengths and sequences, and one soluble isoform lacking the transmembrane region. All transmembrane isoforms are identical in their extracellular region and transmembrane domain, and also share the first 29 amino acids of the intracellular tail [14]. Short and long transmembrane leptin receptors are expressed in the trophoblast, and indicate that leptin synthesized by the placenta can act locally through both receptor isoforms. Being also accessible to leptin from maternal origin, these transmembrane receptors may be activated to different extents in pregnancies with normal and increased leptin production [15].

Several in vitro studies have explored the effects of leptin administration on cell growth. Lang et al. showed that leptin induced cell proliferation of hepatic stellate cells in vitro by the activation of phosphatidylinositol 3-kinase/Akt pathway [16]. Another work showed that leptin stimulated proliferation and vascular endothelial growth factor secretion of endothelial cells in vitro [17]. Leptin also stimulated proliferation and activation of human circulating T lymphocytes when they were costimulated by phytohemagglutinin or concanavalin A [18]. Moreover, leptin promoted proliferation and cell survival of human peripheral blood mononuclear cells via mitogen activated protein kinase activation [19, 20].

Cytotrophoblastic cells are undifferentiated cells able to fuse and differentiate to form syncytia [21]. In this study, we tested the hypothesis that leptin plays a role in human placental cells JEG-3 and BeWo inducing proliferation and cell survival.

MATERIALS AND METHODS

Cell Culture

The human choriocarcinoma cell lines JEG-3 and BeWo (BeWo and JEG-3 cells were generously provided by Susana Genti-Raimondi, Universidad Nacional de Córdoba, Córdoba, Argentina) were grown in 45% Dulbecco modified Eagle medium (DMEM) and 45% HAM F-12 (Invitrogen) supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine (Invitrogen), and 1 mM sodium pyruvate at 37°C in 5% CO_2.

Cell Count and [^{3}H] Thymidine Incorporation

Cells were grown in 12-well plates (5×10^5 cells/well) in complete medium with 10% fetal bovine serum. Immediately after treatment, cells were incubated with 1 μCi/ml [^{3}H]-thymidine (81 Ci/mmol) (Amersham Biosciences) for 6 h. Cells were washed three times with cold PBS, harvested, and centrifuged at

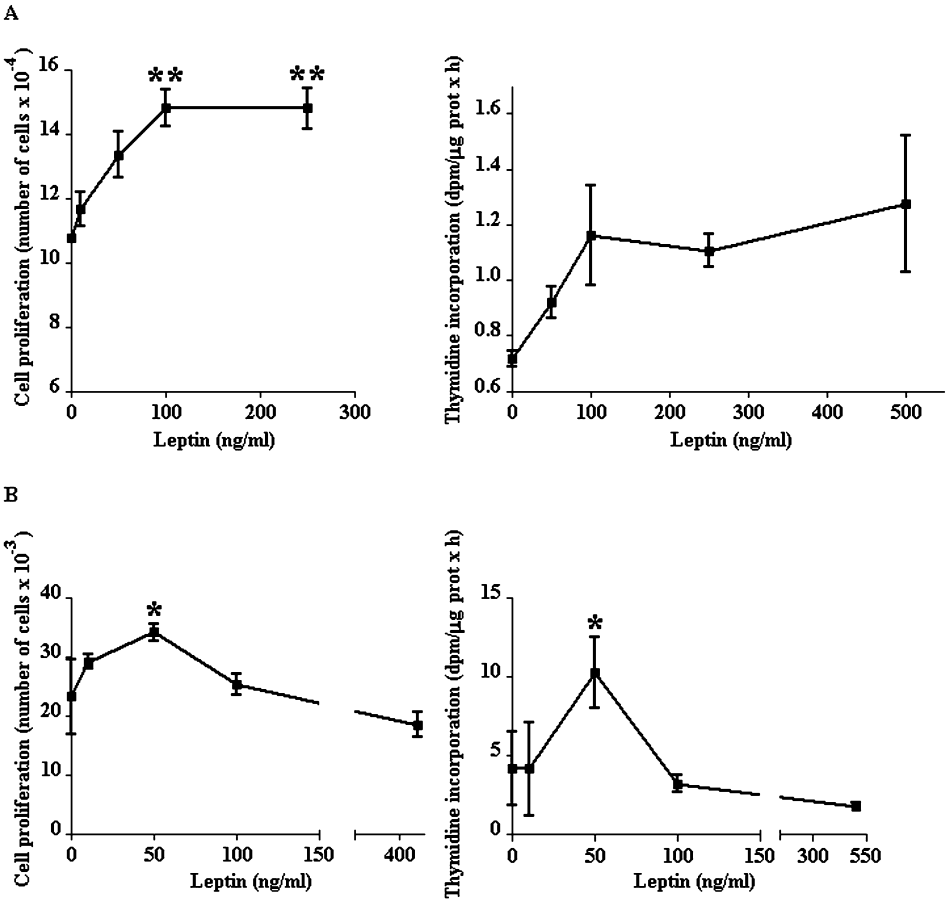

FIG. 2. Dose responses of JEG-3 and BeWo cell proliferation to exogenous leptin addition. JEG-3 (**A**) and BeWo (**B**) cells (50 000 cells/well) were plated in 24-well plates in complete DMEM-F12 media supplemented with 10% FCS. After 24 h, media were replaced by DMEM-F12 supplemented with 1% FCS, and cells were cultured for another 24 h, at which time leptin or vehicle was added for an additional 72 h. Cell count and ^{3}H-thymidine incorporation were determined as indicated in *Materials and Methods*. Data are expressed as means ± SEM from four independent experiments. ANOVA was followed by Bonferroni analysis. $^*P < 0.05$; $^{**}P < 0.01$.

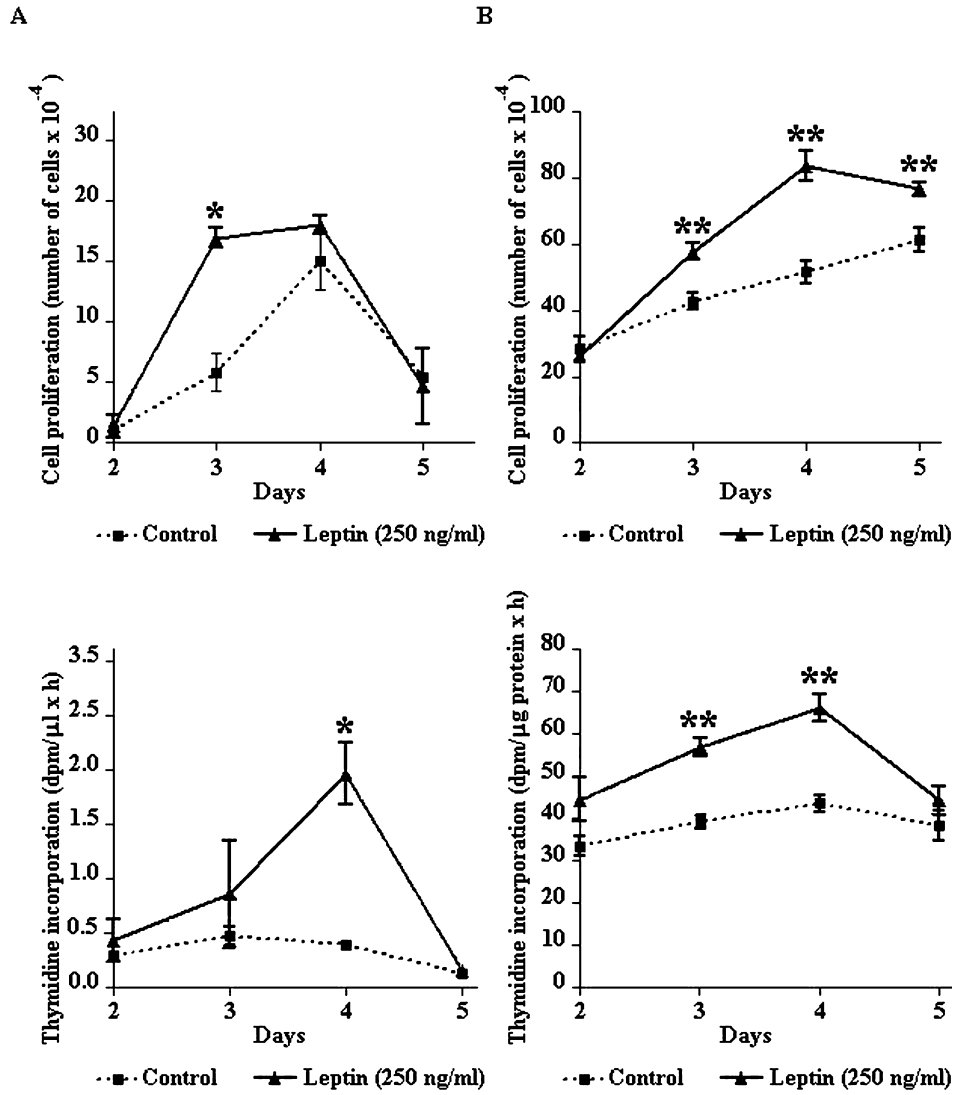

FIG. 3. Time-course response of JEG-3 cell proliferation to exogenous leptin addition. Cells (50 000 cells/well) were plated in 24-well plates in complete DMEM-F12 media supplemented with 10% FCS. After 24 h, media were replaced by DMEM-F12 supplemented with 1% FCS, containing 250 ng leptin/ml or vehicle, and remained without change for 2, 3, 4, and 5 d (**A**), or changed daily, with fresh leptin added, for 2, 3, 4, or 5 days (**B**). Cell count and ^{3}H-thyimidine incorporation were determined as indicated in *Materials and Methods*. Data are expressed as means ± SEM from four independent experiments. ANOVA was followed by Bonferroni analysis. Means that differ significantly from the control are indicated as: * $P < 0.05$; ** $P < 0.01$.

5000 × *g* for 5 min. The cellular pellet was lysed with 5% trichloroacetic acid (TCA) for 30 min, centrifuged, and washed twice with cold PBS. The pellet was resuspended in 150 µl 1 M NaOH for 1 h at room temperature. The incorporated radioactivity was quantified by scintillation counting and DNA synthesis estimated as dpm/µg protein × h. In parallel, the number of viable cells was determined by counting in a Neubauer chamber.

Treatments

Leptin treatments with different concentrations from 25 ng leptin/ml to 500 ng leptin/ml were performed in all cases in DMEM-F12 supplemented with 1% FCS. Serum present in the media of incubation was reduced from 10% to 1% to lower the basal growth rate. Once leptin maximal effect on cell proliferation was determined, in the following experiments, 250 and 100 ng leptin/ml was used for JEG-3 and BeWo cells, respectively. Time treatments were performed from 2 to 5 d. In experiments designed to diminish endogenous leptin expression, an antisense oligonucleotide (AS), complementary to the first five amino acids of leptin mRNA sequence [1] was used. The sequence is: GCACAGGGTTCCCCAATGCAT. Different concentrations of AS from 0.5 µM to 2 µM were assessed during 24-h to 96-h incubations. Treatments with the AS were performed in most experiments in DMEM-F12 media supplemented with 10% FCS unless indicated. In these experiments, a control oligonucleotide containing random sequence was used.

Western Blot Analysis

Total cell lysates were prepared in lysis buffer (1× PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and 10 mg/ml PMSF). The lysates were centrifuged at 10 000 × *g* for 10 min to remove cellular debris. The protein concentration of the supernatant was determined by the brilliant blue Coomasie G staining method [22] with BSA as standard. Lysates were mixed with Laemmli sample buffer containing 2% SDS and 30 mM β-mercaptoethanol, boiled for 5 min, resolved by SDS-PAGE on a 12% gel, and thereafter electrophoretically transferred to a nitrocellulose membrane (Hybond; Amersham Pharmacia). Membranes were equilibrated in 1× PBS, and nonspecific binding sites were blocked by 5% nonfat milk in PBS at room temperature for 1 h. The membranes were then immunoblotted with polyclonal rabbit anti-human leptin Y20 (1:1000) or anti-human leptin receptor H300 (1:1000), or mouse monoclonal anti-cyclin D1 (1:500) (Santa Cruz Biotechnology). The antibodies were detected using horse radish peroxidase-linked goat anti-rabbit/anti-mouse immunoglobulin G (1:1000) (Santa Cruz Biotechnology), and visualized by the Amersham Pharmacia enhanced chemiluminescence signaling system and a bio-imaging analyzer (Fujifilm LAS-1000). Control for equal gel loading was carried out by Coomasie Blue staining.

Cell Cycle Analysis

Adherent cells were washed and harvested in 1 ml PBS containing 10% FCS. Cellular pellet was resuspended in 100 µl PBS and fixed by drop-wise addition of 1 ml 70% ethanol and rested overnight at 4°C. The fixed cells were then centrifuged, washed twice with cold PBS, and resuspended in 200 µl of ribonuclease A (250 µg/ml) and incubated for 30 min at 37°C. Cells were centrifuged and resuspended in 200 µl of 50 µg/ml propidium iodide (PI). Cells were then analyzed for DNA content by flow cytometry on a FACScalibur (BD Biosciences). Histograms show cell distribution among the different stages in cell cycle due to the fluorescence of PI staining (see Figure 4). Data were analyzed using the computer program WinMDI version 2.8 (Scripps Research Institute).

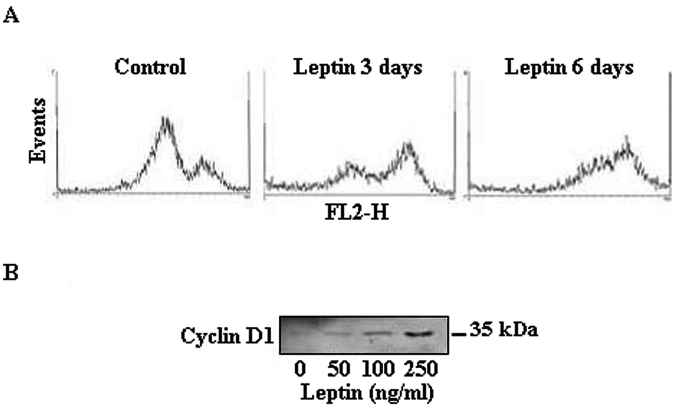

FIG. 4. Cell cycle progression analysis in response to the addition of exogenous leptin. **A**) JEG-3 cells (500 000 cells/well) were plated in 6-well plates in complete DMEM-F12 media supplemented with 10 % FCS. After 24 h, media were replaced by DMEM-F12 supplemented with 1% FCS and cultured for another 24 h, at which time 250 ng leptin/ml or vehicle was added for an additional 3 or 6 d. In the 6-d incubation experiments, media were replaced at Day 3 and fresh leptin was added. Cells were processed and analyzed by flow cytometry, as indicated in *Materials and Methods*. FL2-H, Intensity of PI fluorescence. **B**) JEG-3 cells (1×10^6 cells) were plated in complete DMEM-F12 media supplemented with 10% FCS. After 24 h, media were replaced by DMEM-F12 supplemented with 1% FCS and cultured for another 24 h. Next, different leptin concentrations or vehicle were added, and cells were incubated for an additional 3 d. Cyclin D1 was detected by Western blot analysis, as indicated in *Materials and Methods*, and its molecular weight was estimated using standard protein markers.

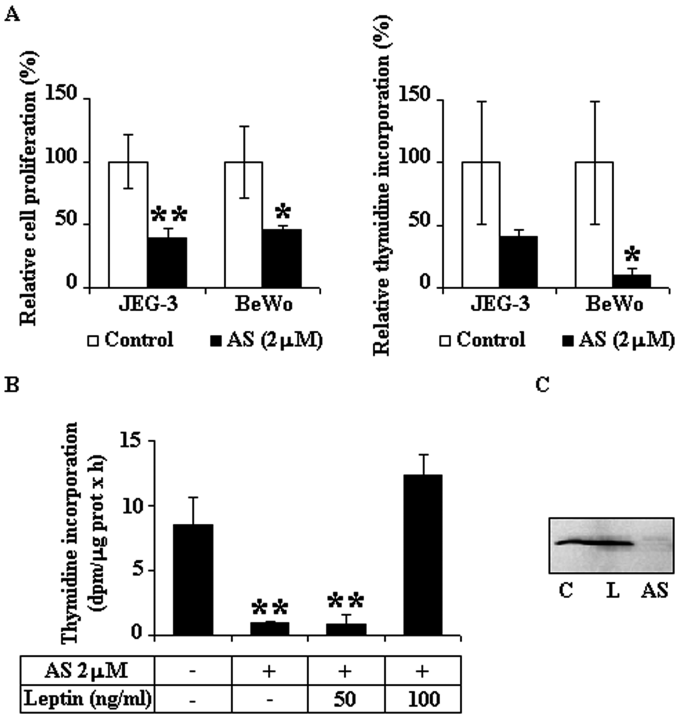

FIG. 5. Effect of diminished endogenous leptin expression on cell proliferation. **A**) As indicated, JEG-3 or BeWo cells (50 000 cells/well) were plated in 24-well plates in complete DMEM-F12 media supplemented with 10% FCS. After 24 h, 2 μM AS or 2 μM control oligonucleotide was added, and cells were incubated for 3 d. Cell count and ^{3}H-thymidine incorporation was determined as indicated in *Materials and Methods*. Data are expressed as means ± SEM from four independent experiments. ANOVA was followed by Student two-tailed analysis. * $P <$ 0.05; ** $P < 0.01$ compared with control group. **B**) JEG-3 cells (50 000 cells/well) were plated in 24-well plates in complete DMEM-F12 media supplemented with 10% FCS. After 24 h, 2 μM AS or 2 μM control oligonucleotide was added, and cells were incubated for 3 d. Subsequently, media were replaced with DMEM-F12 supplemented with 1% FCS in the presence or absence of 2 μM AS or 2 μM AS plus 50 or 100 ng leptin/ml, and cells were cultured for an additional 3 d. ^{3}H-thymidine incorporation was determined as indicated in *Materials and Methods*. Data are expressed as means ± SD from four independent experiments. ANOVA was followed by Bonferroni analysis. ** $P < 0.01$ compared with control group. **C**) JEG-3 cells (1×10^6 cells/well) were plated in 10-cm-diameter plates in complete DMEM-F12 media supplemented with 10% FCS. After 24 h, 250 ng leptin/ml (L), 2 μM AS (AS), or vehicle (C) was added, and cells were incubated for 3 d. Cellular leptin was detected by Western blot analysis, as indicated in *Materials and Methods*.

Caspase-3 Activity

Cells were cultured at 3×10^6 cells/well, harvested with lysis buffer (50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 10 mM EGTA, 10 μM digitonine, 0.5 mM PMSF, 10 μg/ml bisbenzamide, 10 μg/ml pepstatin, and 10 μg/ml aprotinin), incubated for 30 min at 37°C, and centrifuged at $12\,000 \times g$ for 20 min. The activity of caspase-3 in 150-μl cell lysates was determined using 100 μM of the synthetic caspase-3 substrate Ac-DEVD-pNA (Sigma) in reaction buffer (100 mM Hepes, pH 7.5, 0.5 mM EDTA, 5 mM dithiothreitol, and 20% glycerol) in a final volume of 300 μl, and incubated at 37°C for 4 h. Color development was measured at 405 nm. Caspase-3 activity was estimated as A_{405}/μg protein h, where A_{405} indicates absorbance at 405 nm.

Fluorescein Isothiocyanate-Annexin V/Propidium Iodide Double Staining and Analysis. Cells were washed with PBS and resuspended in binding buffer (10 mM Hepes, pH 7.4, 140 mM NaCl, and 2.5 mM $CaCl_2$). Fluorescein isothiocyanate (FITC)-annexin V and PI were added to a final concentration of 1 μg/ml [23]. The mixture was incubated for 10 min and then analyzed by flow cytometry. A total of 20 000 cells were routinely acquired in a FACScalibur flow cytometer. Data were analyzed using CELLQuest software (BDIS). Trophoblastic cell population was gated in side-forward scattering to analyze FL-1 (FITC-Annexin) and FL-2 (PI). This test discriminates intact cells (annexin V^-/PI^-), early-apoptotic cells (annexin V^+/PI^-), and late-apoptotic necrotic cells (annexin V^+/PI^+). Data were also analyzed using the computer program WinMDI version 2.8.

Data Analysis

Experiments were repeated separately at least three times to assure reproducible results. Results are expressed as the means ± SD. The statistical significance was assessed by ANOVA, followed by different tests indicated in each figure legend, and was calculated using the GraphPad Instat computer program. A P value < 0.05 was considered statistically significant.

RESULTS

Both Trophoblastic Cell Lines JEG-3 and BeWo Express Leptin and the Large Isoform of Its Receptor

The choriocarcinoma cell lines JEG-3 and BeWo were used as a model for trophoblastic cells, as previously reported [24, 25]. Leptin and leptin receptor expression were characterized in these cell cultures by Western blot analysis. Results shown in Figure 1 demonstrate that both proteins are expressed in these systems, suggesting that leptin is probably exerting an autocrine/paracrine effect.

Treatment with Recombinant Leptin Promotes Trophoblastic Proliferation in a Dose-Dependent Manner

Leptin effect on cell proliferation was investigated in JEG-3 and BeWo cells by ^{3}H-thymidine incorporation and cell count. Cells were seeded at 50%–60% confluence in complete DMEM-F12 medium (10% FCS) and, 24 h before leptin treatment, cells were starved in media supplemented with 1% FCS. Leptin treatment was performed in the same media during 3 d. As seen in Figure 2A, leptin enhanced cell proliferation in JEG-3 cells. This effect of leptin on JEG-3 cell proliferation was dose-dependent, reaching a 1.6-fold increase that turned

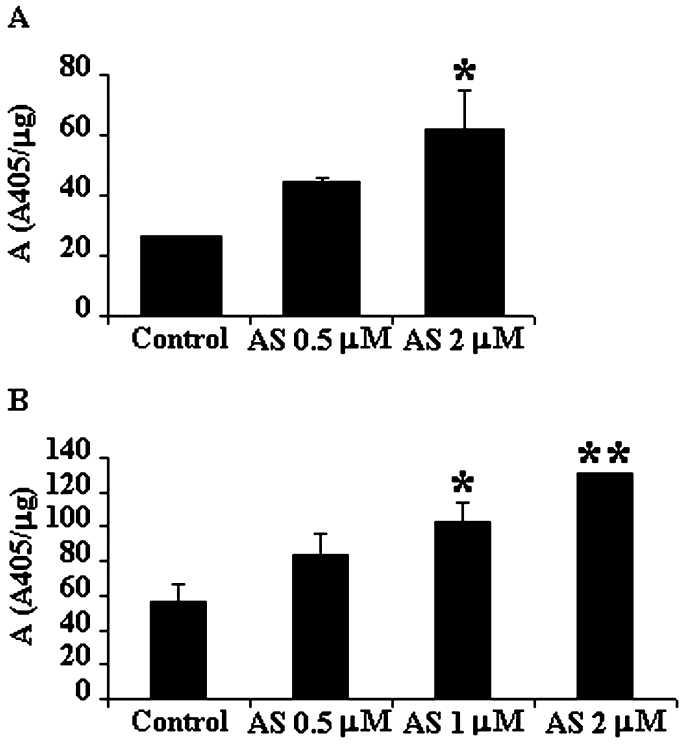

FIG. 6. Effect of leptin AS on caspase-3 activity. JEG-3 (**A**) or BeWo (**B**) cells (500 000 cells/well) were plated in 10-cm-diameter plates in complete DMEM-F12 media supplemented with 10% FCS. After 24 h, media were replaced by DMEM-F12 supplemented with 1% FCS and cultured for another 24 h, at which time control or leptin AS (in the concentration indicated) was added for an additional 3 d. Caspase-3 activity was determined as indicated in *Materials and Methods*. Data are expressed as means ± SD from four independent experiments. ANOVA was followed by Bonferroni analysis. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with control group.

out to be statistically significant. Maximal effect was achieved at 250 ng leptin/ml. As shown in Figure 2B, BeWo cells seem to be more sensitive and effective to leptin treatment. Thus, 50 ng leptin/ml concentration achieved maximal effect, which represented a 2.4-fold increase in ^{3}H-timidine incorporation.

Time-Course Effect of Leptin on Trophoblast Cell Proliferation

To further characterize leptin effect on cell proliferation in trophoblastic cells, time-course experiments were carried out in JEG-3 cells stimulated with 250 ng leptin/ml, which is the maximal effective leptin concentration. Leptin treatment was performed in DMEM-F12 media supplemented with 1% FCS. In steady-state conditions, without any change of media throughout the experiment (Fig. 3A), control cells showed an increase in number and ^{3}H-thymidine incorporation according to its basal growing rate, with a maximum at 4 d. The presence of the hormone in the culture media produced a significant increase in cell proliferation rate measured by cell counting and ^{3}H-thymidine incorporation. Maximal effect was obtained between Days 3 and 4 of treatment, reaching a three-fold increase above control cells. Subsequently, the number of cells decreased, probably as a result of aging culture. In order to verify that the effect observed was not due to the fact that medium was not changed during the study; we next performed the time-course experiments with daily medium change. As can be seen in Figure 3B, similar results were obtained. All these results reinforce the notion that leptin has a role in enhancing cell proliferation.

Leptin Increases Cell Cycle Progression

To address the molecular mechanism of leptin action on cell proliferation, we next studied cell distribution during the different stages of cell cycle by fluorescence-activated cell sorting analysis. JEG-3 cells were starved during 24 h in DMEM-F12 supplemented with 1% FCS, and subsequently treated with leptin for 3 or 6 d. In samples incubated for 6 d, the culture media were replaced on Day 3 by fresh media with the addition of leptin. As seen in the histograms in Figure 4A, cells that had no treatment (control) displayed a distribution in which the left-hand peak corresponded to cells in G1 stage, and the right-hand peak to G2/M stage. The addition of 250 ng leptin/ml for 3 d changed the cell profile along the cell cycle. Compared with control sample, the hormone produced increased cell numbers in the G2/M peak. This redistribution was even more evident in the sample incubated for 6 d in the presence of leptin. Taken together, these results might indicate a quicker progression along the cell cycle towards the G2/M phase. None of the profiles obtained by this analysis displayed a sharp shape, which could be a result of the heterogeneous DNA polyploidy of this cell line [26].

The evidence that leptin treatment displaced cells toward the G2/M phase prompted us to analyze which components of the cell cycle machinery might be involved in that effect. Thus, we analyzed if a key cell cycle-signaling protein was involved in the leptin effect. Cyclin D1 expression was determined by immunoblot analysis, the results of which are shown in Figure 4B. Cell treatment with 250 ng leptin/ml promoted an increase in cyclin D1 levels. Taken together, these data demonstrate that leptin is a potent mitogen.

Role of Endogenous Leptin on Cell Proliferation

Since both JEG-3 and BeWo cell lines express leptin, we speculated that perhaps the proliferative effect exerted by exogenous leptin could be partially masked by endogenous production. To address this problem, an experimental approach was used to diminish leptin expression in these cellular models. Cells were treated with an AS complementary to the mRNA sequence of the first five amino acids in DMEM-F12 supplemented with 10% FCS. As demonstrated in Figure 5C, treatment with 2 µM AS for 3 d abolished leptin expression, as analyzed by immunobloting. Thymidine incorporation and cell count analysis showed a significant reduction in cell proliferation both in JEG-3 and BeWo cells (Fig. 5A). Experiments with different doses of AS and time-course studies were performed. Maximal effect of AS on cell proliferation in both JEG-3 and BeWo was achieved at 2 µM and 3 d of incubation (data not shown). In order to confirm the specificity of the leptin effect on cell proliferation, we next tried to reverse the effect of the AS by the addition of exogenous leptin. Two different leptin doses were employed over 3 d of culture. As shown in Figure 5B, 50 ng leptin/ml was not sufficient to compensate for the lack of endogenous leptin. However, 100 ng leptin/ml fully restored the cell proliferation rate, measured as thymidine incorporation. Taken together, these results confirm that leptin may act as a potent proliferation enhancer.

Leptin Prevents Apoptosis in Trophoblastic Cells

Since proliferation and death processes are intimately related, the effect of leptin on apoptosis of BeWo and JEG-3 cells was

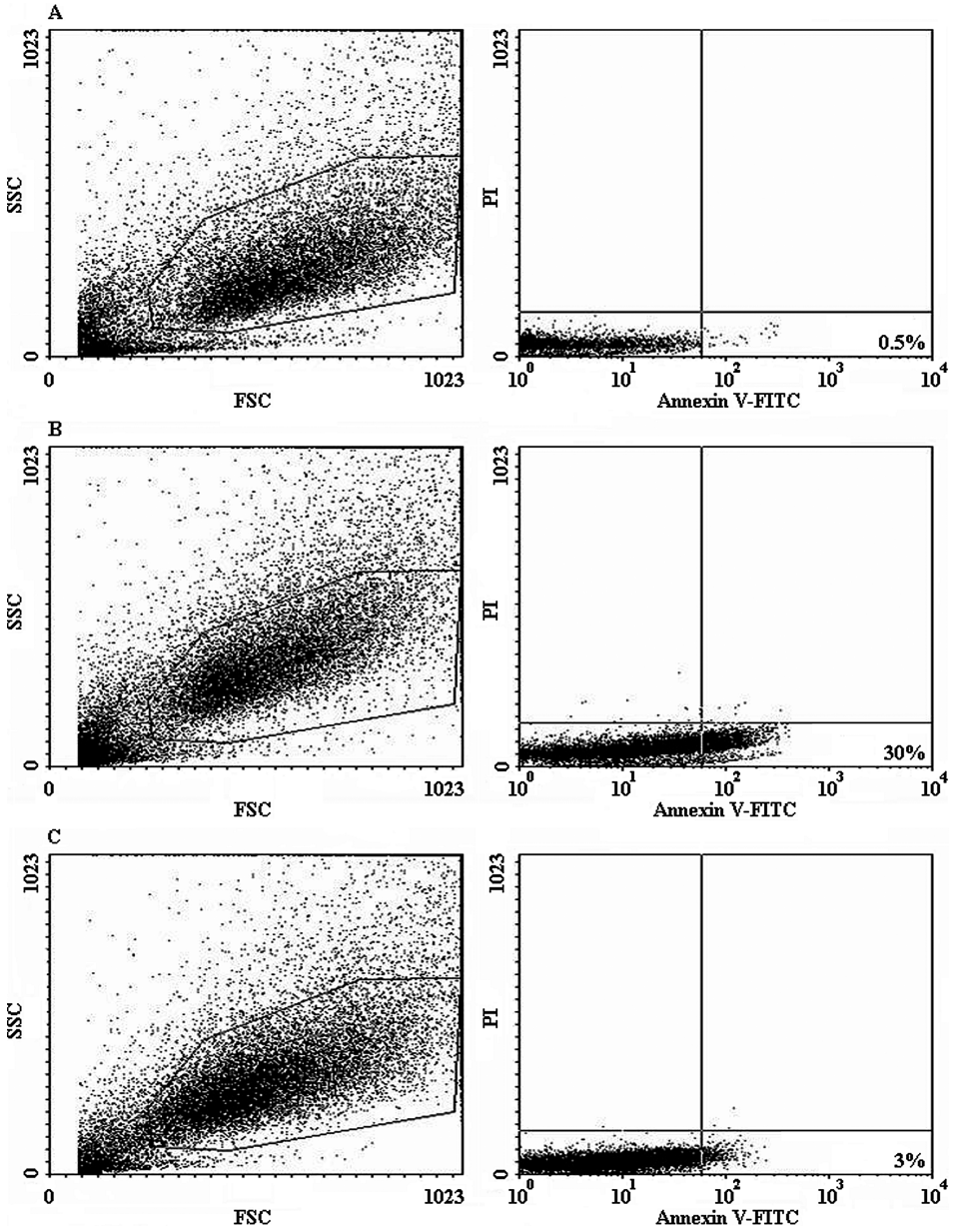

FIG. 7. Leptin prevents apoptosis in JEG-3 cells. JEG-3 cells (1×10^6 cells) were plated in 10-cm-diameter plates in complete DMEM-F12 media supplemented with 10% FCS. After 24 h, media were replaced by DMEM-F12 supplemented with 1% FCS in the presence of control oligonucleotide (**A**), 2 μM leptin AS (**B**), or 2 μM leptin AS plus 250 ng leptin/ml (**C**), and cells were cultured for 3 d. Cells were processed and analyzed by flow cytometry, as indicated in *Materials and Methods*. Data shown on the left side are dot-plot profiles of cell size and cell complexity according to forward scatter (FSC) and side scatter (SSC) in linear scale. In each of these graphs, cell gated events chosen for analysis are marked. Data shown on the right side are dot-plot diagrams of FITC-annexin V/PI flow cytometry. The lower left quadrants show the viable cells, which exclude PI and are negative for FITC-annexin V binding. The upper right quadrants contain the nonviable, necrotic, and late-apoptotic cells, positive for FITC-annexin V binding and for PI uptake. The lower right quadrants represent the apoptotic cells, positive for FITC-annexin V and negative for PI. One representative experiment of four independent experiments is shown.

assessed by two different approaches: caspase-3 activity and annexin V binding. Different apoptotic pathways converge in the late activation of the key apoptotic effector, caspase-3. Thus, we measured the effect of the lack of leptin on caspase-3 activity in both cell types. Figure 6 shows that cells treated with the AS dose-dependently increased caspase-3 activity compared with control cells. Maximal effect was observed at 2 μM AS, which increased caspase-3 activity more than two-fold.

Early apoptotic events were next evaluated by measuring the exposure of phosphatidylserine on the cell membrane, which binds annexin V. Double staining using annexin V-FITC and PI was performed to discriminate viable, apoptotic, and late apoptotic/necrotic cells. Cells were incubated for 3 d with the addition of control oligonucleotide or 2 μM AS, or 2 μM AS plus 100 ng leptin/ml in DMEM-F12 (1% FCS). As shown in Figure 7, treatment with 2 μM AS increased the number of apoptotic cells 60 times, as represented by cells that were annexin V^+/ PI^- (value of 0.5% compared with 30% of the lower right quadrants). Moreover, when cells were incubated in the presence of AS and leptin, apoptotic cells were reduced by 90%; thus, only 3% of cells were annexin V^+/PI^-. These results are consistent with findings obtained from caspase-3 activity measurement, and further demonstrate that endogenous leptin is important for trophoblastic cell viability. Moreover, leptin added to cells can compensate for the lack of endogenous leptin, preventing trophoblastic death by apoptosis.

DISCUSSION

Previously published results have suggested that leptin might play a role in reproduction, particularly in the fetoplacental physiology. Circulating leptin levels are elevated during pregnancy, reaching a peak during the second trimester and at the end of pregnancy; maternal plasma leptin levels decline to normal values 24 h after delivery [27]. Consistent with this, leptin is produced by the human placenta [24]. In humans, serum leptin concentration in normal pregnancy was determined to be between 7.4 and 19 ng/ml [13, 28]. But, as trophoblastic cells produce leptin locally, the effective concentration of this hormone is probably greater in the

placenta. This might be the reason we observed leptin effects at higher concentrations (50–250 ng leptin/ml). Little is known about the physiological role of leptin during human pregnancy, and many observations suggest that this small polypeptide could be a key player in the regulation of the embryo implantation as well as its maintenance. In this study, leptin regulation of cell growth and survival in JEG-3 and BeWo human choriocarcinoma cells was investigated. These cells maintain many characteristics of human trophoblast cells, and have been widely used to study placental cellular signaling [29–31]. Moreover, these cells express both leptin and its receptor. In the present study, we provide evidence for a stimulatory effect of leptin on placental cell proliferation. Both leptin and its receptor share structural and functional similarities with the IL6 family of cytokines [10, 32]. The leptin receptor also has signaling capabilities comparable with IL6-type cytokine receptors [33]. In fact, leptin has been shown to induce proliferative activity in monocytes, lymphocytes, hematopoietic progenitors, and osteoblasts, among others [18, 19, 34, 35]. The molecular mechanisms underlying the effect of leptin on cell proliferation in our system remain unknown, but our results indicate that leptin stimulates cell cycle progression to the G2/S phase. It is well known that progression through the G1 phase is accomplished by the expression of cyclin D, among other proteins [36]. Moreover, it was recently reported that leptin increased cyclin D1 expression in hepatic stellate cells [37]. Therefore, we investigated whether this key cell cycle-signaling protein was involved in the effect of leptin. We found an enhancement of cyclin D1 expression in response to leptin. This observation is consistent with recently published results showing that leptin upregulates AP-1 gene expression, which promotes cyclin D1 expression, resulting in osteoblast proliferation [38].

In addition, our findings provide evidence for an inhibitory effect of leptin on the cell apoptosis program, suggesting a trophic role of leptin in the physiology of trophoblast cells. This antiapoptotic role of leptin has been previously found in other systems, such as blood monocytes, eosinophils, vascular endothelial cells, osteoblasts, neuroblastoma cells, and follicular cells [20, 39–43]. Moreover, it has been published that leptin increased the proportion of oocytes that develop into blastocysts [44]. In addition, these blastocysts exhibit increased numbers of cells, while the proportion of apoptotic cells is reduced [44]. Since we have found that the inhibition of leptin expression in trophoblast cells results in the induction of apoptosis, and this effect can be fully reverted by the addition of exogenous leptin, the autocrine action of leptin may be important for trophoblast cell survival and, therefore, for the maintenance of the placenta. This may also be relevant for pathophysiological conditions, since mRNA leptin expression is known to be increased in pre-eclamptic placenta in which the fetus would be under chronic stress [45]. On the other hand, previous data have shown that leptin can not counteract apoptosis of trophoblastic cells promoted by pathophysiological conditions, such as hypoxia [46], even though it is a well-known stimulatory factor for leptin expression [47].

To our knowledge, this is the first time that this antiapoptotic role of leptin in human trophoblastic cells has been suggested. Therefore, we have provided some evidence for the possible role of the leptin produced by trophoblastic cells in the physiology of the placenta. However, further studies are needed to explain the molecular mechanisms underlying these effects. Besides, different spliced isoforms of leptin receptor have distinct signaling capabilities. It remains to be clarified which isoforms and which specific signaling pathways are mediating these effects.

In summary, our results further support the importance of leptin in the biology of reproduction. More precisely, we have demonstrated the autocrine antiapoptotic and proliferative effect of leptin in trophoblastic cells.

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