SPANDIDOS PUBLICATIONS

Differential roles of leptin in regulating cell migration in thyroid cancer cells

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Abstract. Excess body weight is associated with a moderately increased risk of thyroid cancer. Adipocyte-derived hormone, leptin, has been shown to enhance cell growth and migration in many cancer types. Limited evidence suggests that leptin has direct actions on the thyroid gland, but there are no data available on the effect of leptin on thyroid cancer cells. We evaluated the action of leptin on gene expression, cell growth, cell cycle, and cell migration in anaplastic (ARO), follicular (WRO) and papillary (CGTH-W3) thyroid carcinoma cell lines. Expression of long-form leptin receptors was observed in all thyroid cancer cell lines. Leptin stimulation did not alter the expression levels of leptin, leptin receptor and sodium-iodide symporter. Cell growth and cell cycle were not changed after leptin treatment. However, leptin was able to promote cell migration of papillary thyroid cancer cells, but inhibited migration of anaplastic and follicular cancer cells. In summary, our study suggests that leptin modulates cell migration of thyroid cancer cells in a cell type-specific manner.

Introduction

The incidence of thyroid cancer has been dramatically increasing over the past decades (1). It is believed that the increase can be attributed to improved diagnostic accuracy and better detection of small papillary cancers. The prevalence of obesity has also increased rapidly, and obesity has become the most common nutritional disorder in developed countries. Growing evidence indicates that excess body weight is associated with increased risk of various types of cancer (2). In accordance, both height and body mass index are associated with a small but significant increase of thyroid cancer risk (3). The increasing risk of thyroid cancer showed similar magnitude in older and younger women (4), suggesting that factors other than steroid hormones may contribute to this association. However, thus far the relationship between obesity and thyroid cancer is not well studied.

Leptin was first discovered in 1994 as an adipocyte-derived pleiotropic hormone regulating body weight and energy balance (5). Circulating leptin reflects the total amount of adipose tissue in the body. Leptin-deficient *ob/ob* mice become morbidly obese, hyperphagic, and diabetic due to unopposed appetite (6). The activities of leptin are mediated through the transmembrane leptin receptors. Long-form leptin receptor with a full signaling potential is crucial for leptin action, and *db/db* mice lacking the long-form receptor have a strong resemblance to leptin-deficient *ob/ob* mice (7).

Leptin is mitogenic for various cell types, including hematopoietic cells, normal and transformed epithelial cells and vascular endothelial cells (8,9). The only exception is pancreatic cancer. Conversely, leptin inhibits the cell growth of Mia-PaCa and PANC-1 pancreatic cancer cells through unknown mechanisms (10). In line with other studies, we have demonstrated that leptin stimulates proliferation of breast cancer and hepatoma cells (11,12). Furthermore, among patients with breast cancer, serum leptin concentrations were higher in women with high-grade tumors (13). Overall, the current study indicates that leptin plays some roles in development and progression of different cancer types.

Leptin has a close relationship to hypothalamo-pituitary-thyroid axis (14). Leptin stimulates thyrotropin-releasing hormone (TRH) expression in the hypothalamus, and subsequently production of thyroid-stimulating hormone (TSH). Peripherally, limited studies suggest that leptin has direct actions on the thyroid gland (15). It has been demonstrated that rats receiving prolonged leptin treatment had increased thyroid gland weight (16). In leptin-administered rats, plasma TSH concentration was decreased, while free triiodothyronine and thyroxine were remarkably raised. This observation suggests that the leptin effect is through a direct mechanism involving leptin receptor of the thyroid gland. Nonetheless, little is known about the effects of leptin on thyroid cancer cell biology.

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Given that leptin is a mitogenic factor in many types of cancer and leptin may directly participate in physiological regulation of thyroid follicular cells, we postulated that leptin might play certain roles in regulating thyroid cancer phenotypes such as proliferation and migration. Since follicular epithelial cell-derived thyroid cancers are not homogeneous and different histological types have distinct clinical characteristics, different cell lines including follicular, anaplastic and papillary thyroid cancer cell lines were examined and compared.

Materials and methods

Cell culture and reagents. The human anaplastic thyroid cancer cell line ARO and follicular thyroid cancer cell line WRO were kindly provided by Dr Guy Juillard (UCLA, Los Angeles, CA). The human papillary thyroid cancer cell line CGTH-W3 (17) was a generous gift from Dr Jen-Der Lin (Chang Gung Memorial Hospital, Taoyuan, Taiwan). The cells were grown in complete media containing Dulbecco's modified Eagle's medium (DMEM; Sigma, St. Louis, MO), 10% fetal bovine serum (FBS; Gibco, Carlsbad, CA), 1% (v/v) L-glutamine (Gibco), 1% (v/v) non-essential amino acids (Gibco), and 100 IU/ml of penicillin and streptomycin (Sigma) in a 37˚C humidified incubator with 5% CO_2. Starvation media for ARO and WRO cells did not contain FBS, whereas starvation medium for CGTH-W3 cells contained 1% FBS. Recombinant human leptin for treatment was purchased from Sigma (catalog no. L4146).

Cell growth. Cell growth was determined using the 3-(4,5-dimethylthiazolyl-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. In brief, after leptin (1-125 ng/ml) treatment of cells in 96-well plates for 24-48 h, cells were collected and incubated in medium containing 0.5 mg/ml MTT reagent (Sigma) at 37˚C for 4 h. The formazan crystals converted from tetrazolium salts by viable cells were dissolved in dimethyl sulfoxide (150 μl/well) and the absorbance at 570 nm was measured by a microplate spectrophotometer to reflect cell growth. In addition, trypan blue exclusion test was also performed to measure viability and cell growth. After leptin (1-125 ng/ml) treatment of cells in 96-well plates for 24-48 h, cells were harvested and stained with 4% trypan blue solution (Sigma). Viable cells were counted with hemocytometer under a light microscope.

Cell cycle analysis. The effect of leptin on cell cycle progression was analyzed by flow cytometry. The two peaks of DNA content corresponding to G0/G1- and G2/M-phase cells, respectively, and the intermediate amount of DNA corresponding to S-phase cells were counted. Briefly, cells were seeded in 6-well plates at approximately $4x10^5$/well and were allowed to grow in complete culture media for 24 h. Thereafter, the media of cells was replaced with starvation media for a further 24 h to synchronize cell cycles. Cells were then treated for 24 h with either starvation or complete culture media combined with leptin (125 ng/ml) or vehicle control. After treatment, cells were fixed in 70% cold ethanol at 4˚C overnight.

The fixed cells ($1x10^6$) were centrifuged, washed with phosphate-buffered saline, pre-treated with Triton X-100 (0.1%) and RNase (0.2 mg/ml) at room temperature for 30 min, and then reacted with propidium iodine (20 μg/ml). Subsequently, the cells were analyzed on a FASCalibur flow cytometer (BD Biosciences, San Jose, CA) using an argon-ion laser (15 mW) with an incident beam at 488 nm. The red fluorescence was collected through a 585-nm filter. Data were analyzed by CellQuest (BD Biosciences) and ModFit (Verity Software House, Topsham, ME) software.

RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR) analysis. Total cellular RNA was extracted with TRIzol reagent (Gibco/Invitrogen) according to the manufacturer's recommendations. RNA integrity was verified by electrophoresis in 2% agarose gel and amplification of a housekeeping gene, ß-actin. The first strand cDNA was synthesized using a random primer $pd(N)_6$ and RTG You-Prime RXN beads (Amersham Pharmacia Biotech, Piscataway, NJ) according to the manufacturer's instructions. The RT-synthesized cDNAs were further PCR-amplified by specific primers summarized in Table I. Expression analysis was done under the following PCR amplification condition: 5 min of initial denaturation at 94˚C, followed by indicated cycles of 30 sec at 94˚C, 30 sec at indicated annealing temperature, and an extension at 72˚C and a 2-min final extension at 72˚C. The amplified products were visualized under UV in ethidium bromide-stained 3% agarose gel after electrophoresis.

Western blot analysis. Whole cell lysate was prepared by resuspending cells in M-PER protein extraction reagent (Pierce, Rockford, IL) supplemented with protease inhibitors cocktail (Pierce) and incubating the cells on ice for 30 min. Cell lysates were centrifuged at 12,000 x g for 15 min and supernatant collected. Protein concentration was measured using Bradford assay (Bio-Rad Laboratories, Hercules, CA). An aliquot of protein lysate (30 μg) from each sample was mixed with 10X Laemmli sample buffer (Bio-Rad), and protein lysate was separated in 7.5% SDS-polyacrylamide gels for 1.5 h. After transfer sample to nitrocellulose membrane, the membrane was blocked with 5% milk in 1X T-TBS buffer (25 mM Tris, 150 mM NaCl, 1% Tween-20, pH 7.2) for 1 h at room temperature. The proteins were probed with antibodies against leptin receptor (long-form specific, C-20; Santa Cruz Biotechnology, Santa Cruz, CA; 1:200) and ß-actin (Sigma) at 4˚C overnight and followed by incubation with horseradish peroxidase-conjugated secondary antibodies (Sigma). Protein visualization was performed using the enhanced chemiluminescence kit (Pierce) according to the manufacturer's protocol. Equal loading of total protein was normalized with ß-actin signal.

Transwell migration assay. Cells were trypsinized, resuspended in culture media containing 1% FBS; then $1x10^5$ cells were added to the top chambers of 24-well transwell plates. Transwell migration chambers (8-μm pore size) were purchased from Corning Inc. (Corning, NY). Complete medium containing 10% FBS as the chemoattractant was added to the bottom chambers. After 8 h (WRO and CGTH-W3 cells) or 24 h (ARO cells) incubation at 37˚C, top chamber (non-migrated) cells were removed by PBS rinsed cotton-swab, bottom chamber (migrated) cells were fixed by cold

rimers, product sizes and conditions in RT-PCR.

Gene	Primer	Sequence	Product size (bp)	Annealing temperature (˚C)	Extension time (sec)	No. of cycles
Leptin	Forward	GGA CTT CAT TCC TGG GCT CCA CC	237	63	20	35
	Reverse	CCT GAA GCT TCC AGG ACA CC				
OBRl	Forward	CAG AAG CCA GAA ACG TTT GAG	344	55	40	35
	Reverse	AGC CCT TGT TCT TCA CCA GT				
NIS	Forward	CCC CAG CTC AGG AAT GGA	74	55	15	35
	Reverse	CGT AAT AGA GAT AGG AGA TGG CAT AGA A				
ß-actin	Forward	TGG CAT TGC CGA CAG GAT	100	60	15	28
	Reverse	GCT CAG GAG GAG CAA TGA TCT				

OBRl, long isoform of leptin receptor; NIS, sodium-iodide symporter.

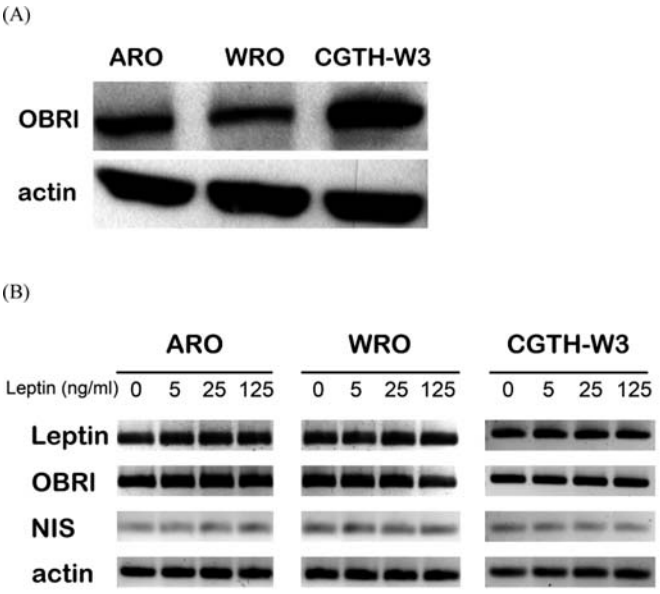

Figure 1. Expression of leptin receptor and effects of leptin on gene expression of thyroid cancer cells. (A) Total protein was isolated from ARO, WRO and CGTH-W3 thyroid cancer cells, 30 μg of proteins were subjected to immunoblotting using specific antibody for long isoform of leptin receptor (OBRl). (B) Total RNA was extracted from ARO, WRO and CGTH-W3 thyroid cancer cells treated with or without leptin, and analyzed by RT-PCR using specific primers for leptin, OBRl, and sodium-iodide symporter (NIS). A primer set for ß-actin was used as an internal control. Note that mRNA expression of leptin, OBRl, and NIS are not changed in response to various doses of leptin treatment for 24 h. The presented images are representative of three separate experiments.

100% methanol for 20 min and stained with Giemsa solution (Sigma). The numbers of migrated cells were counted using an inverted Zeiss microscope at x100 magnification in ten randomly selected fields.

Wound healing assay. Cells were seeded onto a culture insert (0.22 cm^2 growth area with a cell-free gap of 500 μm; ibidi GmbH, Munich, Germany) in complete culture media. After 24 h, the culture insert was removed allowing cells to grow across the gap. Cells were photographed after incubation with or without various concentrations of leptin at the indicated time points.

Statistical analysis. Statistical differences were calculated by the two-tailed Student's t-test. Bar graphs with error bars represent mean ± standard error of the mean. $P<0.05$ was considered as statistically significant.

Results

Expression of leptin receptors and leptin in thyroid cancer cell lines. Leptin elicits its biological functions through binding to its receptors (7). We examined the expression of leptin receptors in anaplastic (ARO), follicular (WRO), and papillary (CGTH-W3) thyroid cancer cells. The expression of leptin receptor mRNA and protein was examined using RT-PCR and Western blot analysis, respectively. A predicted PCR product of long isoform of leptin receptor (OBRl) was obtained by specific primers in all thyroid cancer cell lines examined. Immunoprecipitation using a specific antibody (recognizing only long form of the leptin receptor) confirmed the presence of OBRl in thyroid cancer cells (Fig. 1A). COLO 320 DM cell lysate was used as positive control.

We next examined the mRNA expression of leptin in thyroid cancer cells. Interestingly, leptin mRNA could be detected in all thyroid cancer cells, suggesting the possibility of its autocrine/paracrine role. After leptin treatment for 24 h, the mRNA levels of leptin and OBRl remained unchanged (Fig. 1B). In addition, leptin treatment did not change the expression of sodium-iodide symporter (NIS). These results were confirmed using real-time quantitative PCR (data not shown).

Effects of leptin on cell proliferation. Cell growth of thyroid cancer cells in response to various doses of leptin was tested for 48 h in starvation media. The results from MTT assay showed that leptin treatment did not induce significant changes in cell growth of all thyroid cancer cell lines examined (Fig. 2). Similar experiments performed in complete media containing

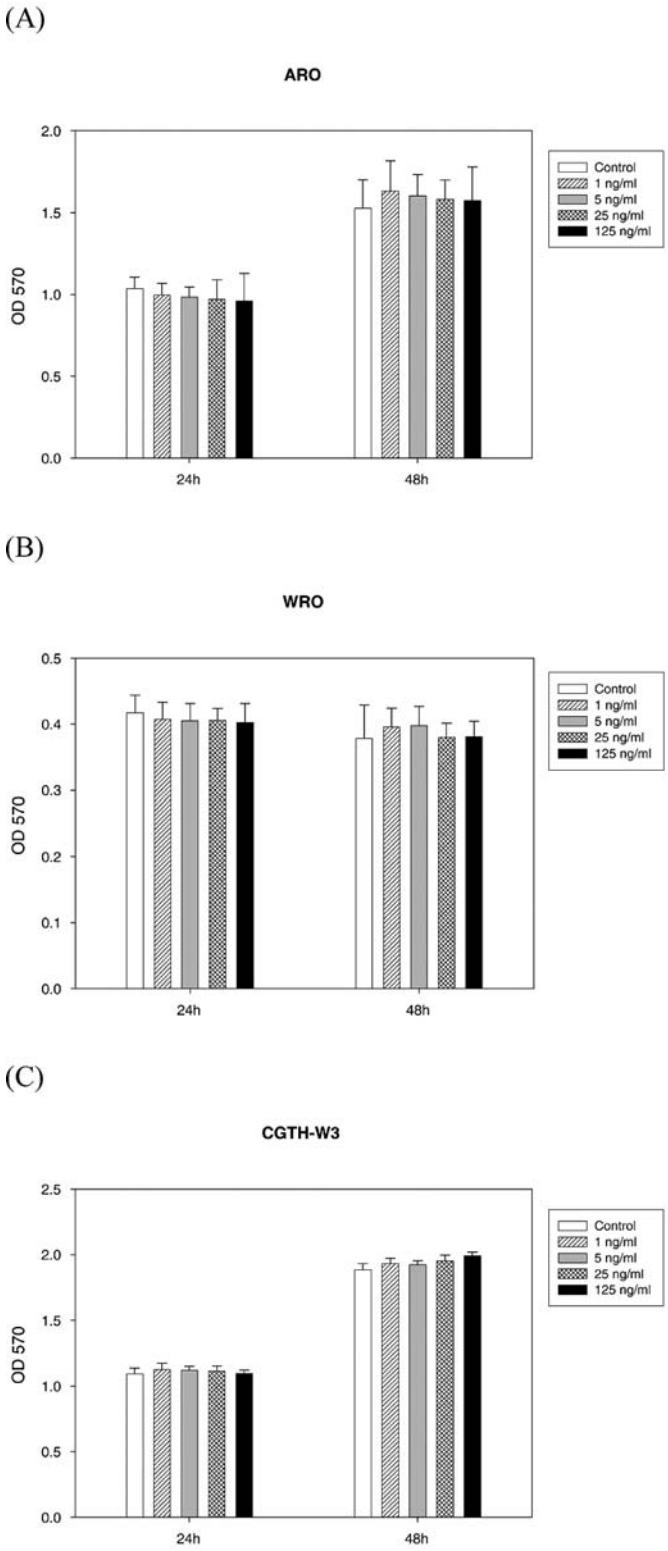

Figure 2. Effect of leptin on thyroid cancer cell growth. After ARO (A), WRO (B) and CGTH-W3 (C) thyroid cancer cells were seeded in 96-well plates, and serum starved for 24 h after semiconfluence, cells were then incubated with indicated serial doses of leptin in starvation media for an additional 24 to 48 h. Cell growth was measured using MTT assay. Data (mean ± SEM) represent four independent experiments.

10% FBS did not show significant changes in cell growth either (data not shown). These observations were confirmed with trypan blue exclusion test.

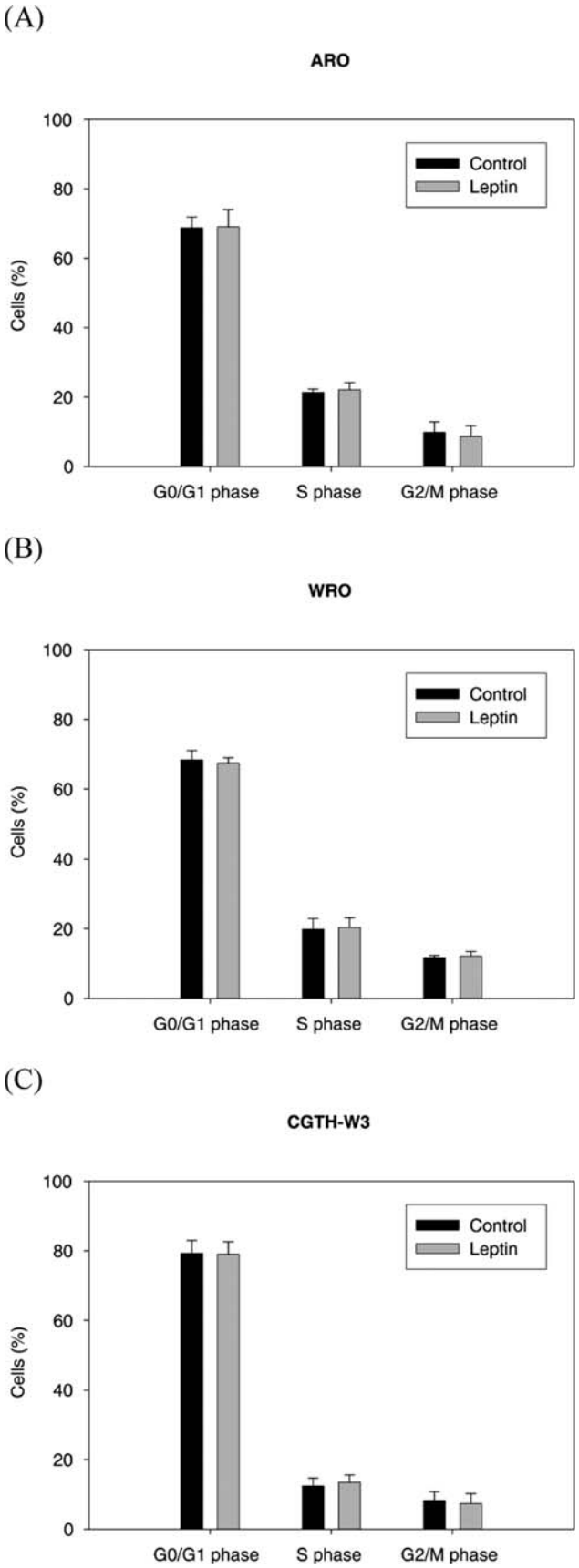

Figure 3. Effect of leptin on cell cycle of thyroid cancer cells. ARO (A), WRO (B) and CGTH-W3 (C) thyroid cancer cells were synchronized by serum starvation for 24 h. Serum-starved cells were exposed to leptin (125 ng/ml) or control starvation media for an additional 24 h. The distribution of thyroid cancer cells in various phases of cell cycle was determined by flow cytometry using propidium iodide-stained nuclei. Error bars indicate the standard error of the mean from three independent experiments.

dy the possible effect of leptin on regulating cell yroid cancer cells were synchronized with serum starvation for 24 h and then exposed to leptin (125 ng/ml) or control starvation media for additional 24 h. Flow cytometric analysis was carried out after propidium iodide staining. Treatment with leptin for 24 h did not significantly change the cells in various phases as compared with the control group (Fig. 3). Similarly, there was no difference in cell cycle progression in complete media between leptin treatment and vehicle control (data not shown).

Effects of leptin on thyroid cancer cell migration. Previous studies demonstrated that leptin promotes cell migration and invasion in various types of cancer cells (18-21). To investigate the effect of leptin on cell motility, using transwell migration assay we exposed the cells to different concentrations of leptin. Unexpectedly, ARO cells exhibited dose-dependent migration inhibition in response to leptin treatment (Fig. 4A). A similar response was seen in WRO cells, although to a lesser extent (Fig. 4B). Of interest, migration of CGTH-W3 cells was enhanced with increasing concentrations of leptin (Fig. 4C).

To verify our observations, we analyzed the effect of leptin on migration potential using a wound healing assay. As shown in Fig. 5, both ARO and WRO cells cultured in the presence of different concentrations of leptin had reduced migration in a dose-dependent manner compared with the untreated controls. In contrast, the ability of CGTH-W3 cells to migrate was remarkably increased when they were treated with increasing concentrations of leptin.

Discussion

The present study revealed that leptin modulates migration of thyroid cancer cells without influencing cell proliferation. To our knowledge, no experimental study has investigated the effects of leptin on thyroid cancer cells. This is the first study showing that thyroid cancer cells exhibit differential responses to leptin stimulation *in vitro*.

Direct peripheral effects of leptin on the thyroid gland have been suggested by a couple of studies. In rat thyroid FRTL-5 cells, leptin induced an increase in *c-fos* expression and TSH-stimulated cAMP production (15). However, leptin inhibited iodide uptake induced by TSH as well as TSH- and cAMP-induced NIS and thyroglobulin expression. The authors postulated that leptin inhibits TSH-induced NIS expression at steps distal to cAMP production in murine thyroid follicular cells. An unpublished work also indicates that leptin inhibits the TSH-stimulated cAMP and thyroglobulin release from primary cultures of normal human thyrocytes (14).

A subsequent study showed that thyroid ^{125}I uptake was not changed in rats receiving a single dose of leptin whereas rats receiving chronic leptin administration had a higher uptake (22). In human thyroid cancer, we have observed that leptin and leptin receptors are overexpressed in some surgical specimens of thyroid tumors (Cheng *et al*, unpublished data). In this study, we found that leptin and its receptor present in thyroid cancer cells but have no effect on NIS expression. It is plausible that leptin signaling in transformed epithelial cells is different from that in normal thyroid follicular cells.

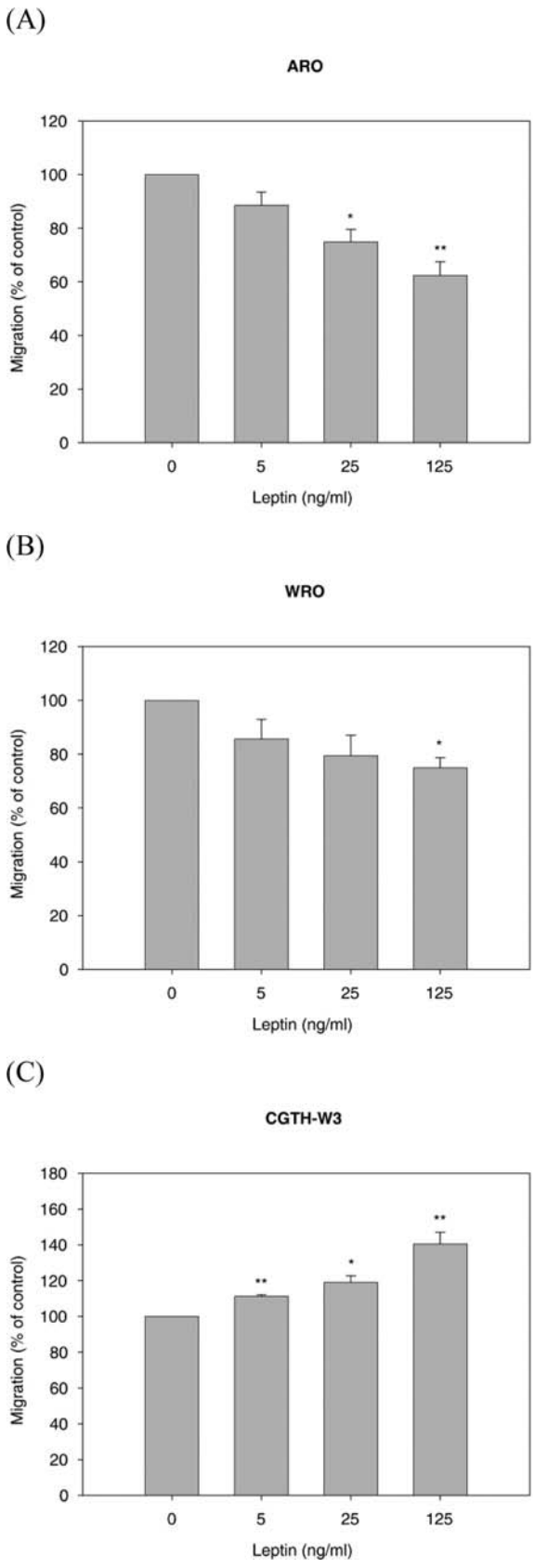

Figure 4. Effect of leptin on thyroid cancer cell migration. Migration of ARO (A), WRO (B), and CGTH-W3 cells (C) was evaluated in transwell chambers as described in Materials and methods. Cells were incubated in the presence of different concentrations of leptin. After incubation at the indicated time points, cells that had migrated to the bottom surface of the filter were fixed and stained with Giemsa solution, and counted under a light microscope. Data (mean ± SEM) are representative of five independent experiments. The statistically significant differences compared with the control are noted as $^{*}P<0.01$ and $^{**}P<0.005$.

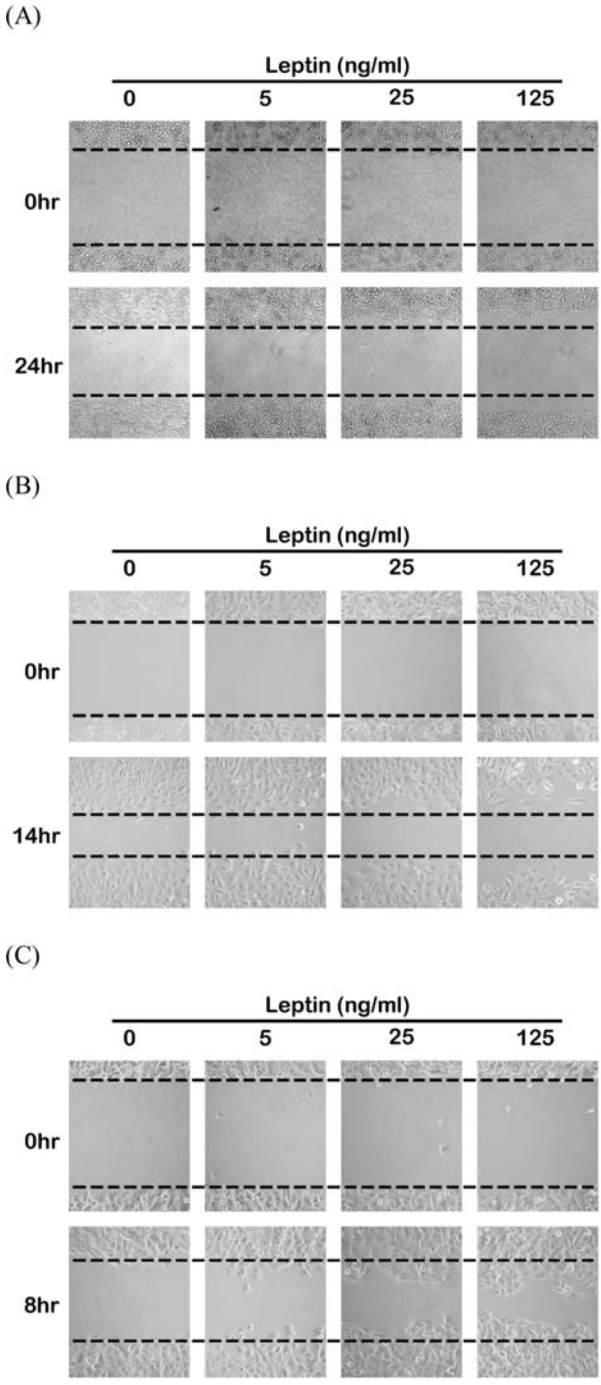

Figure 5. Effect of leptin on *in vitro* wound-healing motility assay of thyroid cancer cells. Cells were seeded onto a culture insert, which was removed 24 h later allowing cells to grow across the gap. 'Wound healing' is moderately inhibited in ARO (A) and WRO (B) cells with leptin treatment, whereas CGTH-W3 (C) cells treated with leptin have a more motile and spindle-shaped fibroblast-like appearance and migrate faster than controls. Representative photomicrographs are shown from one of four independent experiments.

Although the *in vivo* role of leptin in carcinogenesis has not been well characterized, leptin promotes *in vitro* cell proliferation in many, but not all, types of cancer (8-12). In pancreatic cancer cells, leptin inhibits cell growth in a dose-dependent manner (10). As observed in the present study, leptin had no significant effect on cell growth and cell cycle progression of thyroid cancer cells in either complete or starvation media. One possibility is that insulin resistance and inflammation, rather than adipocyte-derived factors, are probably more responsible for the link between obesity and the risk of developing cancers (23). The other possibility is that leptin and other adipokines may play a more important role in the initiation stage of carcinogenesis and the role of other adipokines in thyroid cancer needs further investigation.

In addition to tumor growth, leptin also participates in tumor migration/invasion. Cancer cells possess a broad spectrum of migration and invasion mechanisms to invade adjacent tissues and to travel to distant sites (24). There are several studies showing leptin promotes migration/invasion in different cancer types (18-21). In this respect, it is of interest that leptin has differential effects in different cell types of thyroid carcinoma; namely, leptin promoted cell migration in papillary thyroid cancer cells but inhibited migration in follicular and anaplastic thyroid cancer cells. The opposing effects were an unexpected but reproducible finding. It is well known that the various types of thyroid cancer have distinct clinical presentations and disease course. For example, papillary thyroid cancer, but not follicular cancer, tends to develop lymphatic metastasis. It remains unclear whether these intrinsic differences determine the leptin susceptibility and effects.

It is worth pointing out that altered motile phenotypes are often associated with the cancer development and progression, and eventually metastases (25). Most obese individuals have higher serum leptin concentrations, which may act in an endocrine fashion on tumors with overexpression of leptin receptors. A poor prognosis has been observed in breast cancer patients with high intratumoral expression levels of leptin and leptin receptor mRNA, combined with elevated serum levels of leptin (26). However, not all cancer types with overexpression of leptin or leptin receptor are associated with worse prognosis (27,28). Our observations, therefore, may not be readily translated into clinically relevant context. As for thyroid cancer, increased body weight or body mass index has not been proved to have any effect on disease recurrence or mortality. Analysis of our thyroid cancer database did not demonstrate evidence of any correlation between body mass index and tumor characteristics (29). Further study on clinical implications of thyroid cancers overexpressing leptin receptors and/or leptin is warranted.

The mechanism of action accounting for the differential effects of leptin on thyroid cancer cells observed in our study remains to be elucidated. There are at least four tyrosine phosphorylation signaling pathways that can derive from leptin receptors (7-9). Mitogenic effects of leptin are mainly induced via activation of the ERK1 (extracellular signal-regulated kinase 1) and ERK2 pathway, the JAK2-STAT3 (Janus kinase 2-signal transducer and activator of transcription 3) pathway, and PI3K-Akt (phosphatidylinositol 3-kinase-protein kinase B) survival pathway. It will be interesting to see whether differential effects in different cancer subtypes are mediated via different signaling pathways. Despite these limitations, this study shows a first step towards appreciating the intricate interplay between adipokines and thyroid cancer.

To summarize, we have clearly shown that leptin increases the migration of CGTH-W3 papillary thyroid cancers *in vitro*,

its the migration of ARO and WRO thyroid cancer iout affecting cell proliferation of these cells. The complexity of pleiotropic effects of leptin is highlighted by its cell type-specific modulation of cell locomotion. Further studies are expected to explore the mechanistic and functional bases of leptin-mediated alterations in thyroid cancer motility.

Acknowledgements

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