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Expression of leptin receptor in lung: leptin as a growth factor

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Abstract

Leptin receptors are expressed in various tissues in rodents but their function is not clear. The present studies were undertaken to investigate the function of the leptin receptor in mouse and human lungs. Cell proliferation, assessed with [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (MTT), was significantly less in primary cultures of tracheal epithelial cells of *db/db* mice than in those of their lean littermates. Mouse recombinant leptin significantly increased cell proliferation only in lean mice, but not in *db/db* mice. Reverse transcription–polymerase chain reaction (RT-PCR) study demonstrated the existence of a long form, OB-Rb type leptin receptor in both human lung tissue and lung squamous cell line (SQ-5). Cell proliferation, assessed with MTT, was dose-dependently increased in SQ-5 cells incubated with 10–1000 ng/ml human recombinant leptin for 6 h. The 5-bromo-2′-deoxyuridine (BrdU) uptake into SQ-5 cells was also increased by the addition of 10–100 ng/ml human recombinant leptin. Mitogen-activated protein (MAP) kinase activity was significantly increased by 10 and 100 ng/ml human recombinant leptin in SQ-5 cells. MAP kinase kinase (MEK)-1-specific inhibitor, (2-[2-amino-3-methoxyphenyl]-4*H*-1-benzopyran-4-one) (PD98059), blocked the increase in BrdU uptake into SQ-5 cells caused by human recombinant leptin. In conclusion, leptin (OB-Rb) receptors exist in human lung tissue and leptin may have stimulatory effects on the proliferation of cells of a human cell line and mouse tracheal epithelial cells through its specific leptin receptor.

Keywords: Leptin receptor; Lung; *db/db* Mouse; Cell proliferation; MAP (mitogen-activated protein) kinase

1. Introduction

The *ob* gene has been cloned in genetically obese (*ob/ob*) mice (Zhang et al., 1994) and codes for an anorexigenic peptide, leptin. A leptin receptor has been identified in a cDNA library prepared from mouse choroid plexus (Tartaglia et al., 1995). Leptin receptors are expressed in various tissues in rodents, including pancreatic islets and lungs (Tartaglia et al., 1995; Cioffi et al., 1996; Kieffer et al., 1996; Lee et al., 1996; Wang et al., 1997). Several isoforms of leptin receptors, such as OB-Ra, -Rb, Re, exist in rodents, and the expression of leptin receptor isoforms is tissue-specific (Wang et al., 1996).

Evidence is accumulating that leptin has various extrahypothalamic functions in rodents. Leptin activates signal transducers and activators of transcription (STAT) proteins in a hepatoma cell line (Wang et al., 1997), stimulates embryonic C3H10T1/2 cell proliferation (Takahashi et al., 1997), modulates insulin activity in human hepatic cells (Cohen et al., 1996), and modulates insulin secretion in pancreatic β-cells (Kieffer et al., 1997; Shimizu et al., 1997a) and β-cell proliferation (Tanabe et al., 1997). Extrahypothalamic leptin receptors have been investigated in several human peripheral tissues (Couce et al., 1997; Mikhail et al., 1997). The issue whether leptin receptors exist in human lung tissue has not been determined yet, and the details of leptin effects on the respiratory system are not known at all.

Morbidly obese patients demonstrate hypoxemia, hypercapnia, and a reduced ventilatory response to hypercapnia and hypoxemia (Hackney et al., 1959; Rochester and Enson, 1974; Lopata and Onal, 1982). The severity of sleep apnea increases with increasing obesity (Browman et al., 1984; Smith et al., 1985). However, the exact mechanism of sleep apnea still remains to be determined in obese patients. Serum leptin concentration is well correlated with body fat mass in humans (Shimizu et al., 1997b). While an increase in circulating leptin concentration may be involved in the genesis of sleep apnea in morbidly obese patients, the relationship between leptin and respiratory function is still unclear in humans.

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Leptin receptors exist in the mouse lung, although their function is not still clear (Chen et al., 1996). The present studies were undertaken to investigate the function of leptin receptors in airway tissue from mice and humans.

2. Materials and methods

2.1. Tissue preparations

Human normal lung tissue was obtained at the time of lobectomy for lung cancer. The sample was dissected out from lung tissue to isolate the peripheral bronchus and lymphoid tissues.

2.2. Cell culture

SQ-5 cells are a clonal cell line derived from human lung squamous cell cancer and were obtained from the Institute of Physical and Chemical Research (RIKEN) Cell Bank (Tsukuba, Japan). The cells were routinely cultured with minimum essential medium (MEM) supplemented with 10% fetal bovine serum.

2.3. Human leptin receptor mRNA expression determination by reverse transcription–polymerase chain reaction (RT-PCR) method

To obtain total RNA from human lung tissue and human lung cancer cell lines, all samples were sonicated in 0.8 ml of Isogene (Nippon Gene, Tokyo, Japan) and centrifuged at $12{,}000 \times g$ for 10 min. Total RNA was extracted from the supernatants. Human leptin receptor mRNA was measured by RT-PCR, using the GeneAmp EZ rTth RNA PCR kit (Perkin Elmer, USA). The synthetic human leptin primer sequences used in the present study were as follows: Exon 3, sense primer 5′-CCTTTTCC-CAGGTGTACTTCTCTG-3′ (primer 1); antisense primer 5′-CACCATCCAGGTTGTCTTTAGGAG-3′ (primer 2); Exon 20, sense primer 5′-GTGGTCCTCTTCTTTTG-GAGCC-3′ (primer 3); antisense primer 5′-AGCCCTTGT-TCTTCACCAGTTTC-3′ (primer 4), according to the human leptin receptor cDNA sequence obtained by Thompson et al. from GenBank. On the basis of the results from our preliminary experiment, the reverse transcription step at 60°C for 30 min was followed by PCR was performed for 35 cycles using a 30-s denaturation step at 94°C, 30-s annealing step at 55°C and 1 min extension at 72°C. An additional 2 min extension step at 72°C was added after the 35 cycles. The PCR product was loaded on to a 6% acrylamide gel and the intensity of fluorescence of the band stained by ethidium bromide was recorded.

2.4. Primary culture of mouse tracheal epithelial cells

C57BL/KsJ (*db/db*) mice and their lean littermates were obtained from Japan Crea (Tokyo, Japan) and killed by cervical dislocation at 24 weeks of age. Tracheas were dissected out, just below the larynx to the bronchial bifurcation and washed with phosphate-buffered saline (PBS) containing 5 mM dithiothreitol, 50 U/ml penicillin G, and 50 μg/ml streptomycin. After being rinsed with PBS containing antibiotics only, the tracheas were incubated overnight at 4°C in PBS with 0.5 mg/ml protease, 50 U/ml penicillin G and 50 μg/ml streptomycin. Following the overnight incubation, an equal volume of the culture medium with 5% newborn calf serum was added to the incubation tube. The culture medium added was a mixture of 50% Ham's F12 medium and 50% Dulbecco's minimum essential medium containing 50 U/ml penicillin G and 50 μg/ml streptomycin. Then, the incubation tubes were shaken vigorously for 1 min. After the tracheas were removed from the incubation tube, the tubes were centrifuged at $400 \times g$ for 5 min and the supernatant was discarded. Then, the cell pellets were dispersed by pipetting in 5% newborn calf serum containing culture medium, and the cells were seeded into collagen-coated 96-well plates at 10^4 cells/well and were incubated in a 5% CO_2–95% O_2 incubator at 37°C. After a 24-h incubation, the incubation medium was discarded and the cells were incubated with serum-free culture medium for more than 7 days. Primary cultured tracheal epithelial cells were incubated with 100 ng/ml mouse recombinant leptin (R&D Systems, Minneapolis, MN, USA) at 37°C for 24 h. Then, cell proliferation was measured by MTT assay, described below.

2.5. Cell proliferation assay using colorimetric [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (MTT)

SQ-5 cell proliferation was assayed by the method of Mosmann (1983). The incubation medium was completely aspirated after the incubation with 10, 100, and 1000 ng/ml human recombinant leptin (R&D Systems, Minneapolis, MN, USA) for 6, 24, and 48 h. The MTT-formazon product was dissolved in a phosphate buffer solution. Following the addition of MEM containing 10% MTT, the cells were incubated at 37°C for 4 h, the medium was aspirated, and the cells were lysed by the addition of 100 μl dimethyl sulfoxide (DMSO). Then 10 μl was collected from each sample and diluted in 90 μl of fresh DMSO. After the sample was mixed with a mechanical plate mixer, the optical density of each sample was measured by the Kinetic Microplate Reader (Molecular Devices, CA, USA), using test and reference wavelengths of 550 nm and 650 nm.

2.6. 5-Bromo-2′-deoxyuridine (BrdU) uptake into SQ-5 cells

BrdU uptake into SQ-5 cells and its incorporation into DNA (Porstmann et al., 1985) was determined by using the Cell Proliferation ELISA system (Amersham Interna-

tional, England). The incubation medium was completely aspirated after the incubation with 1, 10, and 100 ng/ml human recombinant leptin for 12 h. Following the addition of 10 μM-BrdU containing MEM, the cells were incubated at 37°C for 2 h. The medium was removed, the cells were incubated for 30 min at room temperature in fixative solution and then in the blocking reagent included in the assay kit. Peroxidase-labeled anti-BrdU monoclonal antibody (from mouse cells) solution was added and incubated for an additional 90 min at room temperature. After the cells were washed three times with PBS for 5 min, substrate solution was added and the cells were incubated for 30 min at room temperature. The reaction was stopped by the addition of 1 M sulphuric acid and the optical density of each sample was measured by Kinetic Microplate Reader at 450 nm. In addition, changes in cell proliferation in response to a 12-h incubation with 10 and 100 ng/ml human recombinant leptin were also determined in the presence of the mitogen-activated protein kinase kinase (MEK)-1-specific inhibitor, (2-[2-amino-3-methoxyphenyl]-4*H*-1-benzopyran-4-one) (PD98059) (New England Biolabs, Beverly, MA, USA) (Alessi et al., 1995; Dudley et al., 1995; Lazar et al., 1995; Pang et al., 1995).

2.7. Mitogen-activated protein (MAP) kinase activity in SQ-5 cells

MAP kinase activity was measured with the p42/p44 MAP Kinase Enzyme Assay System (Amersham International, England) in SQ-5 cells. SQ-5 cells were seeded into 6-well plates and used for the experiment when the cells had reached about 90% confluence. The cells were incubated at 37°C for 30 min in the presence of 1, 10, and 100 ng/ml human recombinant leptin. The cells were then lysed with 1000 μl/well lysing buffer in 10 mM Tris, 150 mM NaCl, 2 mM EGTA, 2 mM dithiothreitol, 1 mM orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, pH 7.4 measured at 4°C, and homogenized. Cellular debris were precipitated at 13,500 rpm for 20 min at 4°C. Following the addition of 15 μl of the supernatant, 10 μl of the substrate buffer and 5 μl of magnesium [^{32}P] ATP buffer (1.0 μCi/tube), each sample was incubated for 30 min at 30°C. The reaction was terminated by the addition of 10 μl of stop reagent and 30 μl of the sample was put on the center of the binding paper disc. The discs were twice washed with 1% acetic acid for 2 min and with distilled water for 2 min. Then, each disc were cut out and placed in 10 ml liquid scintillation cocktail (Ultima Gold™F, Packard Instrument, Meriden, CT, USA) and counted in β-scintillation counter for phosphorus-32.

2.8. DNA fragmentation assay

As previously reported (Ezawa et al., 1996), the Cellular DNA Fragmentation ELISA Kit (Boehringer Mannheim, Germany) was used for the determination of DNA fragmentation induced by the addition of human leptin. SQ-5 cells were incubated with 10 mM BrdU overnight at 37°C, centrifuged at $250 \times g$ for 10 min, adjusted to 1×10^5 cells/ml in MEM and plated out in 96 multi-well plates. The cells were incubated with 1, 10, and 100 ng/ml human recombinant leptin at 37°C for 12 h and the supernatant was completely removed from each well. The cells were lysed by the addition of the incubation buffer enclosed in the kit for 30 min at room temperature. The multiplate was centrifuged at $250 \times g$ for 10 min and the supernatant was transferred directly to the well of a multiplate precoated with anti-DNA antibody. Then, samples were incubated for 90 min at room temperature. After being washed, the samples were denatured and fixed by microwave (500 W) for 5 min and frozen at −20°C for 10 min. Peroxidase-conjugated anti-BrdU solution was added and incubated for an additional 90 min at room temperature. Then, the substrate solution was added and the mixture was incubated at room temperature in the dark on a plate shaker at 250 rpm for 10 min. The reaction was stopped by the addition of 0.5 M sulphuric acid to each well and incubation of the plate for 1 min on the shaker at 250 rpm. The absorbance was measured at 450 nm (reference wavelength: 690 nm) against substrate solution as a blank.

2.9. Statistical analysis

All data represent means + S.E.M. The statistical analysis of the means was performed by analysis of variance (ANOVA), followed by Duncan's multiple range test for the individual comparisons of the means.

3. Results

3.1. Mouse tracheal epithelial cell proliferation

Fig. 1 demonstrates the changes in the proliferation of mouse tracheal epithelial cells, assessed by MTT assay. Tracheal epithelial cell proliferation was significantly lower in *db / db* mice than in their lean littermates. In lean mice, the addition of 100 ng/ml mouse recombinant leptin significantly increased tracheal epithelial cell proliferation. In contrast, mouse recombinant leptin did not cause a significant increase in tracheal epithelial cell proliferation in *db / db* mice.

3.2. RT-PCR

Fig. 2 shows the results of the RT-PCR study with human lung tissue and human lung cancer cell line. The primers were designed to detect exons 3 and 20, which code for the extracellular and intracellular domain of leptin

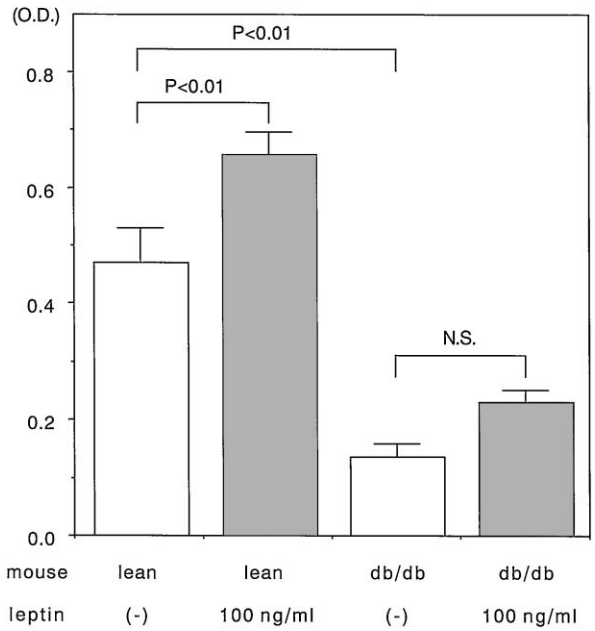

Fig. 1. Effect of 100 ng/ml mouse recombinant leptin on the proliferation of primary cultured, tracheal epithelial cells. Tracheal epithelial cell proliferation was assessed by colorimetric [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (MTT) assay. $N = 6$ (*db / db* mouse), 11 (lean littermates). O.D.: optical density. N.S.: statistically not significant.

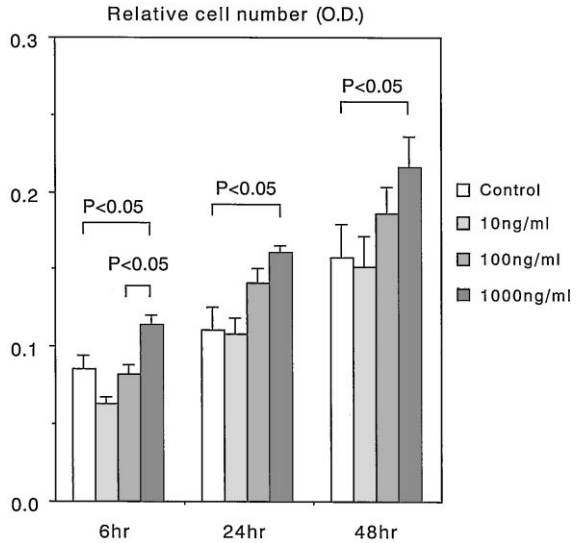

Fig. 3. Chronological changes in cell proliferation induced by 10–1000 ng/ml human recombinant leptin. Cell proliferation was assessed by colorimetric [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (MTT) assay in SQ-5 cells. The effect of 1000 ng/ml leptin was significant at 6, 24, and 48 h. But, the effect of 10 and 100 ng/ml leptin was not statistically significant during the whole observation period. $N = 6$ in each group. O.D.: optical density.

receptor, respectively. RT-PCR products were obtained with both PCR sets at 285 bp (primer 1 and 2, exon 3) and 289 bp (primer 3 and 4; exon 20), respectively. The existence of both exons 3 and 20 identifies the leptin receptor as the long-form, OB-Rb type. The RT-PCR study demonstrated the existence of OB-Rb receptor in normal human lung tissue and SQ-5 cells.

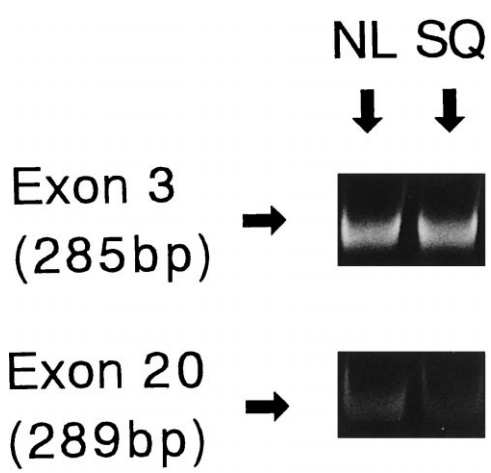

Fig. 2. Expression of OB-R mRNA in human lung tissue and SQ-5 cells. The RNAs extracted from normal human lung (NL) and squamous cancer (SQ-5 cell: SQ) were analyzed by the RT-PCR method. The upper panel shows the PCR product obtained by RT-PCR with primers 1 and 2 (Exon 3), and the lower panel shows the PCR product obtained by RT-PCR with primers 3 and 4 (Exon 20).

3.3. Cell proliferation

To determine the effect of human recombinant leptin on lung cell function, SQ-5 cells were used in this study. Figs. 3 and 4 show human recombinant leptin-induced changes in cell proliferation, as measured by colorimetric MTT assay and BrdU uptake into SQ-5 cells. Cell proliferation, assessed by colorimetric MTT assay, was dose-dependently increased in SQ-5 cells incubated with human re-

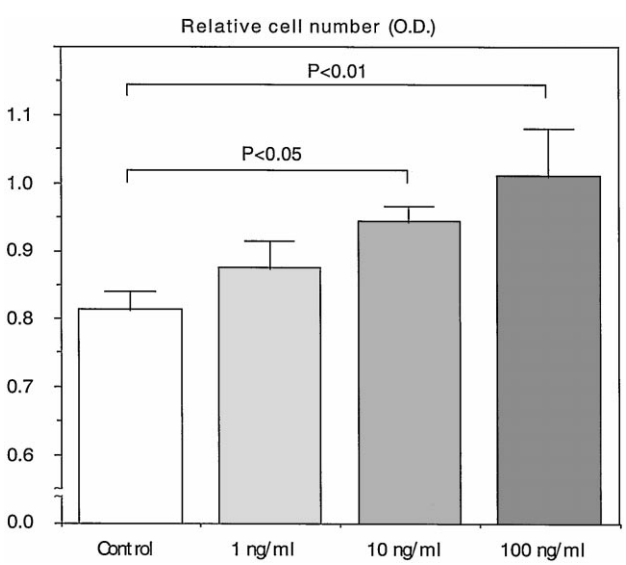

Fig. 4. Changes in BrdU uptake into SQ-5 cells elicited by 1–100 ng/ml human recombinant leptin. $N = 10$ in each group. O.D.: optical density.

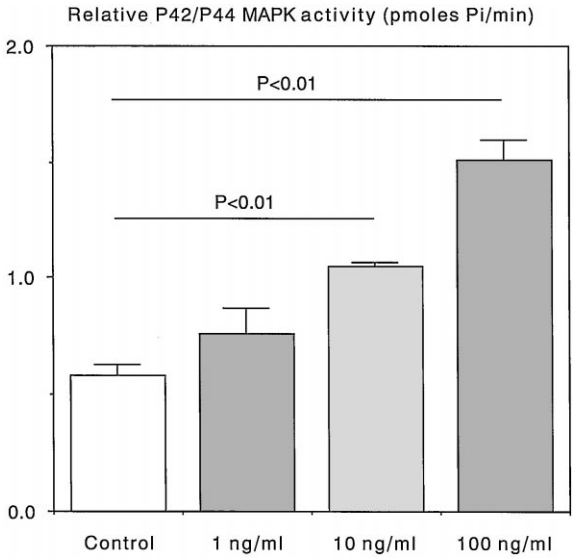

Fig. 5. Changes in MAP kinase activity elicited by 1–100 ng/ml human recombinant leptin in SQ-5 cells. $N = 3$ in each group.

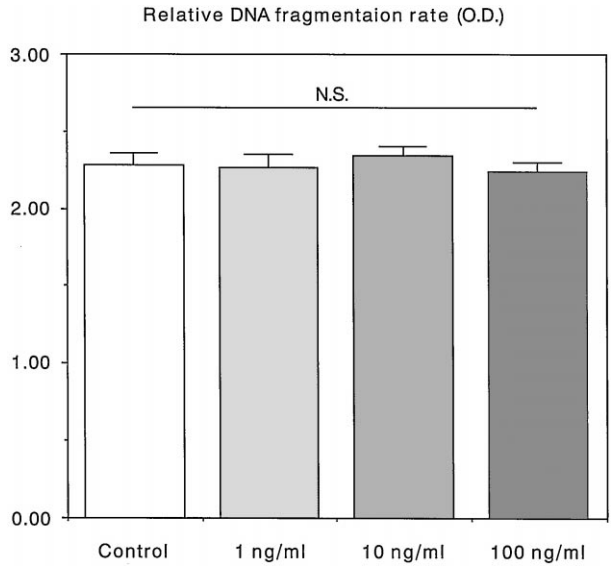

Fig. 7. Effect of 1–100 ng/ml human recombinant leptin-induced DNA fragmentation in SQ-5 cells. $N = 10$ in each group. O.D.: optical density.

combinant leptin for 48 h (Fig. 3). As shown in Fig. 3, 1000 ng/ml human recombinant leptin significantly increased cell proliferation from 6 h after the start of incubation. The effect of 10 and 100 ng/ml leptin was not statistically significant during the whole observation period. In addition, BrdU uptake into SQ-5 cells was also increased by human recombinant leptin in a dose-dependent manner and the effect of 10 and 100 ng/ml human recombinant leptin was significant (Fig. 4). These data clearly indicate that human recombinant leptin stimulates SQ-5 cell proliferation.

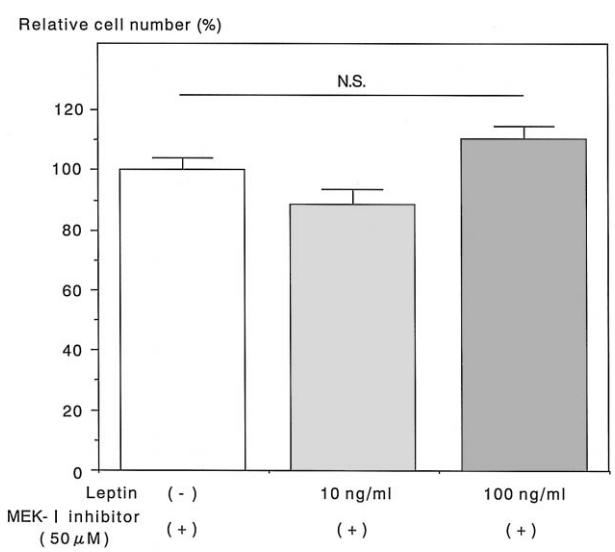

Fig. 6. Changes in BrdU uptake elicited by 1–100 ng/ml human recombinant leptin in the presence of 50 μM MEK-1-specific inhibitor, PD98059. $N = 10$ in each group. N.S.: statistically not significant.

3.4. MAP kinase activity

We measured MAP kinase activity to determine the mechanism of SQ-5 cell proliferation produced by human recombinant leptin. MAP kinase activity was significantly increased by the addition of 10 and 100 ng/ml human recombinant leptin to SQ-5 cells (Fig. 5). In addition, we used the MEK-1-specific inhibitor, PD98059, to determine if MAP kinase activation is responsible for the human recombinant leptin-induced proliferation of SQ-5 cells. The addition of 50 μM PD98059 blocked the increase in BrdU uptake into SQ-5 cells caused by 10 and 100 ng/ml human recombinant leptin (Fig. 6).

3.5. DNA fragmentation

Leptin affects cell proliferation, and because there is a possibility that leptin may have a direct effect on DNA, we investigated the effect of human recombinant leptin on DNA fragmentation in SQ-5 cells. Fig. 7 demonstrates the effect of human recombinant leptin on DNA fragmentation in SQ-5 cells. The addition of 1–100 ng/ml recombinant human leptin did not affect DNA fragmentation in SQ-5 cells at all.

4. Discussion

The data for *db/db* mice clearly indicated that the action of leptin on tracheal epithelial cell proliferation was mediated by the specific leptin receptor. In addition, leptin may affect tracheal epithelial cell proliferation in vivo, because there was an obvious difference in tracheal epithelial cell proliferation between *db/db* mice and their lean

littermates. The present study also demonstrates that human leptin receptor mRNA is expressed in human normal lung tissue and SQ-5 cells. These results are compatible with the recent finding that a 97-kDa fragment is detected in human lung tissue in the cytoplasmic fraction (Miller and Bell, 1996). Our finding indicate that the leptin receptor expressed in human lung is a long-form, OB-Rb type, which has boxes 1, 2 and 3 in its intracellular domain (Tartaglia et al., 1995).

Human recombinant leptin had a stimulatory action on SQ-5 cell proliferation, accompanied by an increase in MAP kinase activity. In contrast, the addition of human recombinant leptin did not affect DNA fragmentation in SQ-5 cells. The dose-related effect of leptin on cell proliferation measured by two different methods (Figs. 3 and 4) did not correlate in the present study. The results obtained for BrdU uptake showed that DNA synthesis was altered in SQ-5 cells. In contrast, the results obtained with the MTT assay show the number of living cells and thus the results may be affected by changes in the cell death rate. Therefore, the results obtained for BrdU uptake may more directly reflect cell proliferative activity and this may explain the difference in the dose-related effect of leptin on SQ-5 cell proliferation.

The leptin receptor is similar to other cytokine receptors such as interleukin-6 (Tartaglia et al., 1995). It is proposed that gp-130 mediates transduction of the leptin signal in the intracellular space (Baumann et al., 1996). Leptin appears to modulate cell function through the activation of the Janus kinase (JAK)-STAT system. The activation of STAT 1 or 3 by leptin is supposed to involve cell proliferation in the liver (Takahashi et al., 1997; Wang et al., 1997). Leptin increases insulin secretion from pancreatic islets (Shimizu et al., 1997a; Tanizawa et al., 1997) and activation of STAT 3 may involve β-cell proliferation (Tanabe et al., 1997). Leptin also induces the proliferation, differentiation, and functional activation of hematopoietic cells (Gainsford et al., 1996). The activation of MAP kinase by leptin has been also reported in C3H10T1/2 cells (Takahashi et al., 1997). These previous findings indicate the possible involvement of leptin in cell proliferation through the JAK-STAT system and/or MAP kinase activation.

The present experiments demonstrated that the addition of human recombinant leptin stimulated MAP kinase activity in a dose-dependent manner, accompanied by an increase in the proliferation of SQ-5 cells, lung squamous cancer cells, as assessed by MTT assay and BrdU uptake studies. PD98059 acts as a highly selective inhibitor of the MEK-1 and MAP kinase cascade (Alessi et al., 1995; Dudley et al., 1995; Lazar et al., 1995; Pang et al., 1995). The fact that the addition of PD98059 prevented the human recombinant leptin-induced increase in cell proliferation indicates the involvement of MAP kinase in SQ-5 cell proliferation. These findings are compatible with previous observations in mesenchymal cells such as hepatic cells (Takahashi et al., 1997) and hematopoietic cells (Gainsford et al., 1996). Recently, a link between the JAKs and the Raf/MAP kinase signaling pathways has been reported to be involved in the effect of β-interferon and oncostatin M in Hela and U4A cells (Stancato et al., 1997). It is possible that the observed SQ-5 cell proliferation induced by leptin may be mediated by MAP kinase activation through the JAK-dependent pathway.

The severity of sleep apnea increases with increasing obesity (Browman et al., 1984; Smith et al., 1985). An increase in total body fat mass is accompanied by an increase in circulating leptin concentrations in humans (Shimizu et al., 1997b). The present finding that OB-Rb type human leptin receptors exist in normal lung tissue raises the possibility that leptin may be involved in the peripheral regulation of respiratory function in humans. However, the exact cellular localization of the leptin receptor in human lung tissues was not identified in the present study and is now being investigated in our laboratory by using immunohistochemical techniques with specific antibodies against the human recombinant leptin receptor in human lung tissues.

In conclusion, the human leptin (OB-Rb) receptor exists in human lung tissue and in SQ-5 cells. Human recombinant leptin may stimulate SQ-5 cell proliferation by increasing the activity of cell proliferation-related enzymes such as MAP kinase.

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