Suppression of hepatocellular carcinoma growth in mice via leptin, is associated with inhibition of tumor cell growth and natural killer cell activation

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Background/Aims: Leptin exerts potent immune modulatory properties. The aim of this study was to determine leptin’s anti-tumor effect in a murine model of hepatocellular carcinoma (HCC).

Methods: In vivo, athymic nude mice were transplanted with Hep3B cells, followed by daily leptin administration for 6 weeks.

Results: Leptin administration induced a significant reduction in tumor size, improved survival rate, and was associated with a significant increase in peripheral natural killer (NK) cell number. Splenocytes from leptin-treated mice featured decreased expression of CIS mRNA. SCID mice featured a similar leptin-associated tumor suppression. In contrast, NK-deficient SCID-beige mice developed larger tumors which were unresponsive to leptin. NK cells incubated in vitro with increasing doses of leptin demonstrated increased cytotoxicity and proliferation. Incubation of leptin with hepatoma cells induced a dose-dependent reduction in proliferation, suggesting a direct anti-tumor effect. Leptin induced increased mRNA expression of STAT2 and SOCS1 on HCC cell lines.

Conclusions: Leptin administration induces a significant suppression of human HCC. This effect is mediated by induction of natural killer cell proliferation and activation, along with direct inhibition of tumor growth. Decreased NK expression of inhibitory CIS and over-expression of the antiproliferative STAT2 and SOCS1 proteins in HCC lines may underline the anti-cancerous effects of leptin.

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Keywords: Leptin; Natural killer cell; Hepatocellular carcinoma

1. Introduction

Leptin, the 16 kDa product of the ob gene [1] is secreted almost exclusively by adipose cells, and acts centrally at the hypothalamic region in regulation of energy expenditure and appetite [2]. Leptin has been extensively studied as a regulator of energy expenditure and appetite [2]. It has also been suggested, to have potent immunomodulatory properties [3]. Structurally, leptin is similar to IL2, IL6, and IL15, making it a member of the helical cytokine superfamily [4]. Leptin receptors are structurally similar to hematopoietic cytokine receptors [5]. These receptors are found on CD4 and CD8 lymphocytes, monocytes [6], natural-killer lymphocytes [7,8], and hepatic stellate cells [9]. Leptin was shown to enhance T cell proliferation and pro-inflammatory cytokine secretion [10–12] by activation of the JAK/STAT signal transduction pathway [13]. Leptin-deficient ob/ob mice are resistant to Th1 mediated-immune disorders [14–17] but are vulnerable to LPS-induced hepatic...
damage [18]. Data on the possible leptin-induced effect on tumor development and growth is based on a small number of in vitro studies. Ablerrant leptin receptors have been found on numerous cancer cells lines [19–22]. Leptin was shown to enhance proliferation of breast [23] and prostate [24] cancer cell lines by direct activation of the JAK/STAT pathway. In contrast, leptin was shown to inhibit proliferation of pancreatic [26], colon [25], and hepatocellular carcinoma cells [26,27]. While leptin levels are low in patients with gastrointestinal [28,29] and pancreatic malignancies [30], they were normal in patients with breast cancer [31], high in patients with colorectal cancer [32], and variable in patients receiving chemotherapy for hematological malignancy [33].

Data regarding leptin’s effect on hepatocellular carcinoma cells has been sparse and contradictory, with leptin found to be inhibitory to HCC growth in one in vitro study [26], and with no antiproliferative effect in another [34]. In a recent study, involving a small group of cirrhotic male [26], and with no antiproliferative effect in another [34]. In the present study, we demonstrated that leptin exerts a significant anti-tumor activity in human hepatocellular carcinoma, via both direct tumor cell inhibition and NK cell activation.

2. Materials and methods

2.1. Chemicals

Highly purified human and mouse leptin were obtained from R&D Co. (USA). Anti-CD45 and anti-Pan-NK antibodies were obtained from eBioscience Co. (USA).

2.2. Animals

Eight-week old male nude mice, SCID mice, and SCID-beige mice were purchased from Jackson laboratories (Bar Harbor, ME). Animals were housed in laminar flow hoods in sterilized cages, and kept on regular 12 h light-dark cycles. All animal experiments were carried out in accordance with the guidelines of the Hebrew University-Hadassah Institutional Committee for care and use of laboratory animals.

2.3. Cell cultures

The human Hepa 3B HCC cell line was obtained from American type culture collection (ATCC), VA. Cells were grown as monolayers in cultures containing supplemented DMEM medium.

2.3.1. In vivo studies

All experiments were repeated twice.

2.4. Experimental groups

Athymic nude mice were divided into four groups (Table 1): Groups A and C mice were subcutaneously implanted with $10^6$ Hepa-3B human HCC cells; Groups B and D were subcutaneously administered 100 µl saline solution. Following tumor implantation, groups A and B mice were administered two daily intraperitoneal doses of 0.5 mcg/g highly purified mouse leptin for a period of 6 weeks, while groups C and D were administered two daily intraperitoneal doses of 0.5 mg/g saline. An identical experimental protocol was employed using SCID mice and SCID-beige mice. Mice were followed at 2-weekly intervals for 6 weeks for body weight and tumor volume using calipers. Tumor growth was monitored using caliper measurement of tumor length (L) and width (W), and the equation $V= LW^2/2$.

2.5. Splenic and hepatic natural killer cell isolation

Splenocytes were isolated and red blood cells removed as previously described [36]. Livers were placed in a 10-ml dish in cold sterile PBS. Livers and spleens were crushed through a stainless mesh (size 60). Cells were washed twice in 45 ml PBS (1250 rpm at room temperature). For liver and spleen lymphocyte isolation, 20 ml of histopage 1077 (Sigma Diagnostics, St Louis, MO) was slowly placed underneath the cells suspended in 7 ml of PBS, in a 50-ml tube. Cells at the interface were collected, diluted in a 50-ml tube, and washed twice with ice-cold PBS (1250 rpm for 10 min).

2.6. Flow cytometry analysis for determination of NK cell population

Following NK cell isolation, triplicates of $2-5 \times 10^5$ cells/500 µl PBS were put into Falcon 2052 tubes incubated with 4 ml of 1% BSA for 10 min, and centrifuged at 1400 rpm for 5 min. Cells were resuspended in 10 µl FCS with 1:20 CY5-conjugated CD45 antibody and PE-conjugated anti-Pan NK antibody (eBioscience, USA), and mixed every 10 min for 30 min. Cells were washed twice in 1% BSA, and kept at 4 °C until reading. For the control group, only 5 µl of 1% BSA was added. Analytical cell sorting was performed on $1 \times 10^5$ cells from each group with a fluorescence-activated cell sorter (FACSTAR plus, Becton Dickinson). Data was analyzed with Consort 30 two-color contour plot program (Becton Dickinson, Oxnard, CA), using the CELLQuest program.

2.7. Lymphocyte RNA isolation and semi-quantitative RT-PCR

Total RNA from $10^6$ splenocytes of each mouse experimental group was isolated and transcribed into complementary DNA using Promega Reverse Transcriptase Kit (USA). PCR products were obtained after 35 cycles of amplification with an annealing temperature of 56–62 °C, and visualized by ethidium bromide staining after agarose electrophoresis. RT-PCR products were semiquantified by visual analysis after normalization against the actin internal control. Primer sequences used for mouse target gene detection are depicted in Table 2.

2.8. Cytokine measurement

Serum cytokine levels, including leptin, IL10, IL12, IL4, TNF-α, and TGF-β were measured in all mice by a ‘sandwich’ ELISA method, using Genzyme Diagnostic kits (Genzyme Diagnostics, MA, USA) in accordance with the manufacturer’s instructions.

2.8.1. In vitro studies

All experiments were repeated twice in quadruplets.
2.9. Natural killer cell cytotoxicity determination

NK cells were incubated for 4 h with several concentrations of target YAC cells (NK:YAC ratio of 1.5:1.0) in the presence of 0, 0.01, 0.1, 1 mcg/ml mouse leptin. Cytotoxicity was determined by measuring lactate dehydrogenase (LDH) activity released in the media following 4 h of incubation, using the CytoTox96 non-radioactive assay (Promega) and normalized to a maximal release of LDH upon lysis of YAC cells (100%). Data were quantitated by measuring wavelength absorbance at 490 nm. Data were corrected for spontaneous baseline LDH release from YAC cells, normalized to a maximal release of LDH upon lysis of YAC cells (100%), and were corrected for spontaneous baseline LDH release from YAC cells, NK cells and the buffer.

2.10. Proliferation assays

Splenocytes were collected and prepared as described above. NK cells were isolated from splenocytes using double passage through magnetic beads conjugated to monoclonal hamster anti-mouse pan-NK antibody (Miltenyi Biotec, Bergisch Gladbach, Germany). Hepa 3B cells and NK cells were seeded in RPMI medium in quadruplicates. Each quadruplicate was added with 0, 0.01, 0.1, or 1 mcg/ml mouse highly purified leptin. After 5 days of incubation, methyl-H3thymidine was added to all wells (1 Ci/ml, Amersham Pharmacia Biotech, Little Chalfont, UK). Cell cultures were harvested following 16 h.

2.11. HCC Hepatoma cell mRNA determination

HEPA 3B cells were cultured in quadruplicates for 1, 2, and 6 h in RPMI medium containing 10% fetal calf serum, at a density of 1 × 10^6 cells/16-mm well of a 24-well plate, in the presence of 0.5, 5, 50 mcg/ml human purified leptin. Total RNA was isolated and transcribed into complementary DNA, using Promega Reverse Transcriptase Kit (USA). PCR products were obtained after 35 cycles of amplification with an annealing temperature of 56–62°C and visualized by ethidium bromide staining after agarose electrophoresis. RT-PCR products were semi-quantified by visual analysis after normalization against the actin internal control. Primer sequences used for mouse target gene detection are depicted in Table 3.

2.12. Statistical analysis

Data are expressed as means ±SEM of at least six mice. Statistical analysis was performed using student t-test and 2-way Anova, where appropriate.

### Table 3

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### Table 2

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3. Results

#### 3.1. In vivo effect of leptin administration on hepatocellular carcinoma in athymic mice

Leptin administration resulted in significant inhibition of HCC growth and improved survival rate in athymic mice. Differences in tumor size between leptin-administered and saline-administered mice were noted within 2 weeks of tumor implantation. Following 6 weeks, mean tumor volume in leptin-administered mice (1.04 ± 0.12 cm^3^) was significantly lower than in saline-administered mice (1.9 ± 0.5 cm^3^, P < 0.001, Fig. 1(A)). Mean tumor weight was lower (0.31 ± 0.05 vs. 0.63 ± 0.37 g, P = 0.002). Mortality was significantly lower (0 vs. 20%, P < 0.001). Macroscopically, leptin-administered mice featured a small mass and a large necrotic center, whereas saline-administered mice were manifested by a larger solid mass and a smaller interphase area, composed of a mixture of lymphocytes and neutrophils (Fig. 3), that was not notable in saline-administered mice. Mice in control groups B and D did not feature tumor growth or mortality.

#### 3.2. In vivo effect of leptin administration on NK subsets

Peripheral NK cell population was expanded in leptin-administered, HCC-implanted mice (6.16 ± 2.19%) in comparison with saline-administered mice (3.25 ± 0.67%, P = 0.03). A similar increase in NK cell population was
noted in leptin-administered control group B, as compared with saline-administered group D (1.27 ± 2.73 vs. 0.58 ± 0.37%, in groups B and D, respectively, \( P = 0.007 \), Fig. 4). No significant difference in the hepatic/splenic NK cell ratio was noted between the groups (1.02 ± 1.41, 0.45 ± 0.64, 1.05 ± 1.05, 0.25 ± 0.24, for groups A–D, respectively, \( P = \text{NS} \)).

3.3. In vivo effect of leptin administration on NK cell mRNA expression:

Semiquantitative RT-PCR analysis of peripheral lymphocyte mRNA expression revealed significantly decreased expression of CIS protein in leptin-administered, as compared with saline administered mice (Fig. 5). Lymphocyte mRNA expression of STAT1-6 and SOCS1-3 did not differ between groups.

Fig. 1. (A) Leptin-administered athymic mice developed tumors (top row) that were significantly smaller in volume and weight than tumors from saline-administered mice (bottom row). (B) Natural killer cell-deficient SCID-beige mice developed tumors that were significantly larger than those of nude mice. Leptin-administered mice developed tumors (top row) that were not different in size from tumors in saline-administered mice (bottom row).

3.4. In vivo effect of leptin administration on cytokine profile:

Serum leptin levels were significantly higher in the leptin-treated groups A and B mice (7.05 and 6.29 ng/ml, respectively) than the saline-treated groups C and D mice (3.24 and 1.57 ng/ml, respectively, \( P < 0.01 \) between leptin and saline-treated groups). No significant difference between groups was noted in any of the other serum cytokine levels (\( P = \text{NS} \)).

3.5. In vivo effect of leptin administration on HCC in SCID and in SCID-beige mice

In vivo experiments were repeated using T and B cell deficient SCID mice and T, B and NK deficient SCID-beige mice. The administration of leptin to SCID mice resulted in tumor inhibition (tumor volume and weight of 2.4 ± 0.8 cm\(^3\) and 0.72 g in leptin-administered mice, vs. 1.2 ± 0.6 cm\(^3\) and 0.48 g in saline-administered mice, respectively, \( P < 0.01 \)). Microscopic evaluation of tumors in leptin-administered SCID mice revealed an intense lymphocytic inflammatory reaction in interphase areas and within the tumors and large necrotic areas.

Fig. 2. A demonstrative photo of a nude mouse from leptin-administered group A featuring a small tumor and a large central area of necrosis, as compared to a nude mouse from saline-administered group B, featuring a much larger tumor mass.

Fig. 3. In nude mice, leptin administration was associated with the development of a dense mixed lymphocytic and neutrophilic infiltrate around and within tumors (A), as compared to no infiltrate in tumors of saline-administered mice (B).

Fig. 4. In HCC harboring mice, leptin administration was associated with a significant natural killer cell expansion. Even in the two control groups, leptin administration resulted in relative expansion of natural killer population.
In NK cell-deficient SCID beige mice, tumor volume and weight was significantly greater ($5.05 \pm 2.65$ cm$^3$ and $2.73 \pm 1.61$ g in SCID-beige saline-administered mice vs. $1.9 \pm 0.9$ cm$^3$ and $0.63 \pm 0.37$ g in saline-administered nude mice, $P < 0.001$ for both). Leptin and saline-administered SCID beige mice suffered from 30 to 40% mortality rate, respectively. In contrast to nude and SCID mice, leptin-administration to SCID-beige mice resulted in no tumor inhibitory effect (mean tumor volume of $4.62 \pm 2.29$ cm$^3$ and weight of $2.12 \pm 0.8$ g in leptin-administered mice, $P = NS$ for both parameters, Fig. 1(B)). Tumors in SCID beige mice, and leptin and saline-administered mice featured no areas of necrosis, and no lymphocytic inflammatory reaction was noted in SCID-beige mice.

3.6. In vitro effect of leptin administration on NK cytotoxicity:

Leptin manifested a dose-dependent increase in NK cytotoxicity in vitro (Fig. 6). A 4 h incubation of mouse-derived NK cells with YAC cells, in the presence of increasing doses of mouse leptin (0.01–1 mcg/ml) resulted in a significantly increased NK-mediated lysis of YAC cells (manifested as increased LDH release). The dose-response enhancement in cytotoxicity was notable in each of the tested NK-YAC ratios (5:1–0.04:1 NK-YAC ratios). Cytotoxicity was amplified with increasing doses of leptin. Administration of 1 mcg/ml leptin using NK-YAC ratio of 5:1, resulted in a 100% 4-h cytotoxicity.

3.7. In vitro effect of leptin administration on NK cell proliferation:

High dose leptin (1 mcg/ml) but not lower doses (0.1 and 0.01 mcg/ml) induced in vitro proliferation of NK cells. Incubation of $1 \times 10^4$ NK with 0, 0.01, and 0.1 mcg/ml mouse leptin resulted in thymidine uptake of $1361 \pm 143$, $1302 \pm 173$, and $1359 \pm 229$ CPM, respectively, $P = NS$. Incubation with 1 mcg/ml leptin, resulted in a significantly elevated thymidine uptake of $2733 \pm 52$ CPM, $P < 0.05$.

3.8. In vitro effect of leptin administration on HCC proliferation:

In vitro administration of leptin to HEPA 3B hepatocellular carcinoma cell culture resulted in a dose-dependent inhibition of tumor cell growth (Fig. 7). A 5-day incubation of $10^3$ HCC cells in the presence of increasing leptin doses resulted in a significant reduction in thymidine incorporation, from $7678 \pm 2603$ CPM in the absence of leptin, to $3371 \pm 1178$ CPM in the presence of 0.1 mcg/ml leptin, and $832 \pm 289$ CPM in the presence of 1 mcg/ml leptin, $P < 0.001$. Maximal inhibition was noted at 0.1 mcg/ml, with no added inhibition noted with the addition of 1 mcg/ml leptin ($1287 \pm 412$) as compared with 0.1 mcg/ml leptin ($P = NS$).

3.9. In vitro effect of leptin administration on HCC proliferation in the presence of NK cells:

The NK cell-mediated effect of leptin on HCC cell proliferation was assessed by incubation of $10^3$ HCC cells for 5 days in the presence of $10^4$ irradiated NK cells in the presence of increasing leptin doses. Incubation of HCC cells in the presence of NK cells resulted in further inhibition of
tumor cell growth, from $885 \pm 152$ CPM without leptin, to $815 \pm 117$ CPM, $724 \pm 44$, and $613 \pm 95$ CPM in the presence of 0.01, 0.1, and 1 mcg/ml leptin, respectively, ($P<0.05$ for 0.1 and 0.01 mcg/ml leptin).

3.10. In vitro effect of leptin administration on HCC cell mRNA expression:

HEPA 3B cells expressed leptin receptor mRNA. Hep3B cells were incubated in the presence of increasing leptin doses (0.5, 5, 50 mcg/ml) for 45 min, 2 and 6 h. Semiquantitative RT-PCR demonstrated that leptin administration, even at the lowest doses, resulted in increased mRNA expression of STAT2 and SOCS1 in HCC cells. Increased mRNA expression was notable after 2 h for STAT2 and SOCS1. No effect of leptin administration on mRNA expression was noted for STAT 1 and 3–6, SOCS 2–4, and CIS. [This figure appears in colour on the web.]

4. Discussion

Leptin administration to HCC-harboring athymic nude mice resulted in significant inhibition of tumor growth and improved survival rates. This effect was mediated via activation of NK cells and direct inhibition of tumor cell growth. The anti-tumor effect was associated with development of a mixed peri-tumor lymphocytic and neutrophilic infiltration, increase in peripheral NK population, and reduced lymphocyte CIS mRNA expression. This effect was eliminated in NK cell-deficient mice. In vitro, leptin was demonstrated to induce dose-related enhancement of NK cell cytotoxicity, in addition to a dose-related direct inhibition of HCC tumor growth.

NK cells were demonstrated to play a role in control of tumor growth and metastasis, via release of cytokines and chemokines. In addition, they play a role in the modulation of dendritic cells, and granulocyte growth and differentiation [37]. Activation of NK cells has been demonstrated to successfully suppress HCC growth, while suppression of NK cells contributes to HCC dissemination [38]. The profound antitumor effect of leptin in a murine HCC model was associated with NK cell activation. In vivo, T, B and NK cell-deficient SCID beige mice, but not T and B cell-deficient SCID mice, featured increased tumor size and abolished the effect of leptin administration. In vitro, leptin administration resulted in a dose-response increase in NK cell cytotoxicity. Furthermore, the addition of NK cells to HCC cell culture resulted in augmented leptin-mediated inhibition of tumor growth.

Leptin induced a dose-response inhibition of HCC cell growth in vitro, but was not associated with an anti-tumor effect in NK cell-deficient SCID-beige mice. As the in vivo mice experiments were conducted using highly purified mouse leptin which features an 85% homology to human leptin, leptin in these experiments may have had a more prominent effect on mouse NK cells than on the human-derived Hep3B cells. Human leptin was used to assess leptin’s direct effect on HCC cell line, while mouse leptin was used to assess mouse NK proliferation and cytotoxicity. Administration of mouse leptin resulted in augmented tumor cell inhibition as compared to leptin’s effect on cultured HCC cells. These results suggest that both direct and immune-mediated effects of leptin play a role in tumor suppression.

Leptin exposure resulted in decreased expression of cytokine inducible SH2-containing protein 1 (CIS1) mRNA in lymphocytes. This protein has been demonstrated to be a potent T cell and NK cell regulator [39]. In CIS transgenic mice, high levels of CIS expression were correlated with potent inhibition of T cell and NK cell-mediated pro-inflammatory response, with a reduction noted in NK cell population [40]. Leptin-induced inhibition of CIS expression may have contributed to NK expansion and enhanced the pro-inflammatory effect. The antitumor effect of leptin was associated with an increased mRNA expression of STAT2, which plays a role in IFN-α-induced STAT1 activation. STAT2 phosphorylation by JAK following IFN-α binding to its receptor enables STAT1 phosphorylation and subsequent formation of STAT1/STAT2 heterodimer. This heterodimer, binds with the p48 nuclear factor to form the IFN-stimulated gene factor that activates target genes through recognition of IFN-stimulated response elements [41]. STAT1 is a potent tumor suppressor, via activation of apoptosis by induction of caspase and p21/waf1 expression [42] and reduction of c-myc gene expression in response to IFN-αstimulation [43]. Leptin-induced STAT2 overexpression in HCC cells may enable STAT1 to exert its anti-tumor, pro-apoptotic effect. In addition, overexpression of HCC cell suppressor of cytokine signaling 1 (SOCS1) mRNA was noted. SOCS1, an inhibitor of STAT activation by binding of JAK kinases, has been demonstrated to be muted by hypermethylation in several tumor models, including hepatocellular carcinoma [44], multiple myeloma [45], and leukemia [46]. Leptin-
induced over-activation of SOCS1 in HCC cells may result in inhibition of STAT pathways, leading to suppression of HCC growth.

The use of the athymic mouse model used in the present study does not rule out additional leptin-mediated effects on regulatory T cell populations, or possible leptin-associated effect on effector cells. This model does not enable immediate quantification of leptin’s direct and indirect, immune-mediated effect on tumor growth.

In summary, leptin has a profound antitumor effect in a murine HCC model that was associated with activation of NK lymphocytes and a direct antitumor effect. NK cell modulatory effect and a direct anti-proliferative effect. STAT-related pathways. Leptin may have both an immune and cellular effect on effector cells. This model does not enable regulatory T cell populations, or possible leptin-associated study does not rule out additional leptin-mediated effects on HCC growth.

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Acknowledgements

References


