Leptin stimulates the proliferation of human colon cancer cells in vitro but does not promote the growth of colon cancer xenografts in nude mice nor intestinal tumourigenesis in ApcMin/+ mice

T Aparicio, L Kotelevets, A Tsocas, J-P Laigneau, I Sobhani, E Chastre and T Lehy
INSERM, U 683, IFR 02, Faculté de Médecine Xavier Bichat, 16 rue Henri Huchard, 75018 Paris, France

Short title
Leptin and intestinal tumourigenesis

Correspondence to
Dr. T Lehy, INSERM U 683, Faculté de Médecine Xavier Bichat, 16 rue Henri Huchard, BP 416, Paris, Cedex 18, F-75870, France.
e-mail: tlehy@bichat.inserm.fr

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ABSTRACT

Background/Aims. Leptin, the product of ob gene, has been suggested to increase the colon cancer risk. However, we have shown that although leptin stimulates epithelial cell proliferation it reduces the development of carcinogen-induced preneoplastic lesions in the rat colon. Here, we explored the leptin effect in vitro on the proliferation of human colon cancer cells, and in vivo on the growth of HT-29 xenografts in nude mice and the development of intestinal tumours in Apc\textsuperscript{Min+} mice.

Methods. Proliferation of HT-29, LoVo, Caco2 and SW 480 cells was assessed in the absence or presence of leptin (20-500 ng/ml) by \textsuperscript{3}H-thymidine incorporation and cell count. Leptin (800 µg/kg/d) or its vehicle was delivered for 4 weeks to nude mice, inoculated with HT-29 cells on day 0, and 6 weeks to Apc\textsuperscript{Min+} mice.

Results. Leptin dose-dependently stimulated cell DNA synthesis and growth in all cell lines. In nude mice, leptin caused a 4.3-fold increase in plasma leptin levels as compared with pair-fed controls. This hyperleptinemia, despite leptin receptor expression in tumours, did not induce significant variation in tumour volume or weight. Tumour Ki-67 index was even inhibited. In leptin-treated Apc\textsuperscript{Min+} mice, a 2.4 fold increase in plasma leptin levels did not modify the number, size and distribution of intestinal adenomas as compared with pair-fed controls.

Conclusions. Leptin acts as a growth factor on colon cancer cells in vitro but does not promote tumour growth in vivo in the two models tested. These findings do not support a pivotal role for hyperleptinemia in intestinal carcinogenesis.

Key words
Hormone, carcinogenesis, animal models, cell lines
INTRODUCTION

Leptin, the product of the mouse ob gene (1), is a 167 amino-acid peptide hormone involved in energy balance and regulation of food intake. It was initially found to be produced in adipocytes. Later, other sources of leptin have been described, notably the stomach (2;3). In human, serum leptin levels are high in obese subjects and decrease in case of weight loss (4). Epidemiological studies have revealed that overweight raises the risk for colon adenomas (5) and colorectal cancers (6;7). Leptin effects are mediated through the binding of specific cell surface receptors (Ob-R) coupled to the activation of the PI3 Kinase and the Jak/Stat signalling. Both pathways exert a critical role in the control of many cellular functions, including survival, proliferation, and differentiation.

In this context, several lines of evidence suggest that leptin may be involved in carcinogenesis. Indeed, in vitro, leptin is able i) to stimulate the proliferation of different types of cancer cell lines, ii) to induce angiogenesis through interaction with Ob-R expressed on the surface of endothelial cells (8;9), angiogenesis being essential for tumour growth, invasion and metastasis and, iii) to increase the secretion of metalloproteinases, key enzymes for tumorous invasion (10). With regards to the digestive tract, the long isoform of leptin receptor (Ob-Rb), which is the functional form, is expressed all along this tractus (3;11-14). This Ob-Rb expression is preserved in human colonic adenomas and carcinomas as well as in human colon cancer cell lines (11;13). The hypothesis that leptin is related to the development of digestive cancers is supported by the fact that leptin promotes the proliferation of several cell lines derived from human adenocarcinomas such as Barrett’s and squamous esophageal cancer cell lines (15;16), the AGS gastric cancer cell line (17) and the HT-29 colon cancer cell line (13;18). Leptin is also able to promote the invasiveness of human colon cancer cells in collagen gel (11) and to counteract sodium butyrate-induced apoptosis in HT-29 cells (19).

Nevertheless, in vivo, data concerning the action of leptin on the intestinal epithelial cell growth are contradictory. Thus, in human, although in some reports there was no evidence of elevated leptin levels in patients with colorectal cancers (20-22), a recent study showed that the risk for colonic cancer, but not for rectal cancer, increases with high serum leptin concentration (23). In mice, leptin injection either stimulated (13), or was without effect or even inhibited the proliferation of the colonic epithelial cells (24). Recently, in rats, we confirmed the promoting effect of leptin on the cell proliferation of the right, but not of the left colonic mucosa. More interestingly, in the same work, we showed that leptin significantly reduced the development in the colonic epithelium of aberrant crypt foci induced by azoxymethane, a colon carcinogen, aberrant crypts being considered as preneoplastic lesions (25). This was intriguing and indicated that leptin exerts a more complex action on the gut than suspected at first evidence.

In the present study, in an effort to analyse further the relationship between leptin and intestinal cancer, we investigated the potential effect of leptin i) in vitro, on the proliferation of HT-29 cells and three other colon cancer cell lines known to express the leptin receptor Ob-Rb (11;13), and ii), in vivo, on the growth of HT-29 cell xenografts in nude mice, and on the progression of spontaneous intestinal tumourigenesis in the model of ApcMin/ mice.

MATERIAL and METHODS

Cell lines and culture
The human colon cancer cell lines SW 480, LoVo, Caco-2, and HT-29, the latter used as controls, were routinely grown in 25 cm² plastic flasks (Costar, Cambridge, MA, USA). Cells were maintained at 37°C in a humidified atmosphere of 5% CO2/95% air in DMEM (GIBCO,
Grand Island, NY) containing 10% decomplemented FCS (GIBCO) for HT-29 and SW 480 cells, and 20% decomplemented FCS and 1% non-essential amino-acids for Caco-2 cells. LoVo cells were grown in the same condition in Ham’s F12 medium (GIBCO) containing 10% decomplemented FCS.

**In vitro tritiated-thymidine incorporation assay**

Colon cancer cells (10^5 cells/well) were seeded in 12-well clusters (Costar) in medium supplemented with FCS and cultured for 24 h to allow cell adhesion. After washing, the cells were cultured for another 24 h in serum-free medium. Human recombinant leptin (R&D Systems Europe Ltd, Abingdon, UK) was then added in the same medium at a concentration of 20 to 500 ng/ml and cells cultured for further 48 h. At the end of that time, 0.1 µCi of ^3H-thymidine (specific activity 5 Ci/mmol, Amersham Biosciences, Saclay, France) was added in each well for 2 h. Incorporation radioactivity was determined in cell pellets using a β scintillation counter. Results are expressed in d.p.m per well. For each leptin concentration, the experiment was performed in 3 to 6 wells. Two to three experiments were carried out for each cell type.

**Assessment of cell growth in vitro**

Cells (1.10^4 cells/well) were seeded in 96-well clusters and cultured for 24 h in medium supplemented with 10% FCS for HT-29, SW 480 and LoVo cells or 20% FCS for Caco-2. Then, attached cells were washed and grown in their respective serum-free culture medium in the absence or presence of leptin (20-500 ng/ml for 8 days, the medium being renewed every 48 h. Then, cells were harvested then those which excluded Trypan blue were counted with a hemacytometer. For each leptin concentration, the experiment was performed in 3 to 6 wells. For each cell type, 2 to 3 experiments were carried out.

**Leptin receptor immunoprecipitation and phosphorylation assay**

Sixty per cent confluent HT-29 cells were starved for 24 h in serum-free DMEM. Then, the medium was renewed and the cells were incubated in the absence (control) or presence of 100 ng/ml of leptin for 30 seconds, 2 or 5 min. Then, cells were harvested at 4°C. The pellet was resuspended and incubated in ice-cold RIPA buffer containing a cocktail of protease inhibitors (Sigma) and 1mM of sodium orthovanadate and sodium fluoride. Ob-Rs were immunoprecipitated from cell lysates (200 µg) overnight at 4°C using M18 polyclonal anti-Ob-R antibody -cross-reacting with human and murine leptin receptors (Santa Cruz Biotechnology, CA, USA)- and protein G sepharose beads (Amersham). Immunoprecipitates were resolved on 7.5% SDS PAGE gel and transferred to nitrocellulose sheets. Phosphorylated Ob-Rs were detected by Western blot with an anti-phospho-tyrosine monoclonal antibody (4G10) (Upstate Biotechnologies, Lake Placid, NY). Signals were visualized with the peroxidase-labelled secondary antibody using enhanced chemiluminescence (ECL, Amersham).

**Animals**

Six-week-old male BALB/c nu/nu athymic mice, referred to as nude mice, were obtained from Iffa Credo (L’Arbresle France) and housed in our animal quarters in individual cages, kept in temperature controlled room with a 12-hr light-dark period. They were given standard mice food pellets (SAFE A 03, Scientific Animal Food, Villemoisson sur Orge, France) and water ad libitum until the beginning of experiment, one week later.

The C57BL/6J-Apc<sup>Min/+</sup> mouse progenitors were purchased from The Jackson laboratory (Bar Harbor, ME, USA). Our breeding colony was established by crossing heterozygous male C57BL/6J-Apc<sup>Min/+</sup> mice with wild-type female C57BL/6J mice. Genomic DNA was prepared
from tail biopsies, using Qiagen DNAeasy kit (Qiagen S.A., Courtaboeuf, France) and offsprings characterized for the Min genotype by multiplex PCR, according to the recommendations of The Jackson Laboratory. We used the following sense: 5'-GCCATCCCTTCACGTTAG-3' (wild-type) and 5'-TTCTGAGAAAGACAGAAGTTA-3' (final adenosine residue corresponds to the T to A transversion at nucleotide 2549 in C57BL/6J-Apc\textsuperscript{Min/+} mice), and the antisense 5'-TTCCACTTTGGCATAAGGC-3' oligonucleotides. The expected size of the PCR products was 600 bp for wild-type animals. For the C57BL/6J-Apc\textsuperscript{Min/+} mice, two additional bands of 340 bp and 1000 bp were obtained corresponding to the amplification of the mutant allele and the heteroduplex (wild-type/mutant allele), respectively.

Animals were treated in accordance with European Community guidelines concerning the care and use of laboratory animals.

**Tumorigenicity assay in nude mice**

On day 0, exponentially growing HT-29 cells were harvested and resuspended at a concentration of 1.10\textsuperscript{8}/ml. Nude mice were anaesthetized by i.p. injection of a mixture of Rompum and ketamine in NaCl. 1.10\textsuperscript{7} cells were inoculated in the right flank of mice. A miniosmotic pump (Alzet pump, model 2002, Alza Mountain View, CA) was implanted s.c. in the left flank of mice. Pumps delivered recombinant human leptin diluted in saline + 0.3% bovine serum albumin (BSA) or leptin vehicle (saline+BSA) alone (control). The dose of leptin, based on the initial concentration and specified delivery rate of pumps, was 800 µg/kg/d for two weeks. Pumps were renewed after 14 days. Food intake of leptin-treated mice were monitored daily. Vehicle-treated mice were pair-fed, receiving the same amount of food as had been eaten by leptin-treated mice the day before. Body weight was monitored twice a week in the two groups. Tumour development was followed daily by caliper measurement along two orthogonal axes, length (L) and width (W). The volume (V) of the tumour was calculated by the equation for ellipsoid (V= L x W\textsuperscript{2} x \pi/6). At the time of killing (day 28), blood samples were collected for radioimmunoassays, and tumours were dissected out from neighbouring connective tissues and weighed. Then, they were divided into three parts, either frozen in liquid nitrogen and stored at −80°C or fixed partly in Bouin’s fluid or in 4% paraformaldehyde and paraffin-embedded.

**Estimation of Ki-67 proliferative index in nude mice tumours**

Paraffin blocks were cut into 4 µm-thick sections. Cell proliferative activity was examined after immunohistochemical detection of the Ki-67, a nuclear protein present in all phases of the cell cycle, except G\textsubscript{0}. We used a mouse monoclonal antibody (clone MIB-1, Dakopatt, Glostrup, Denmark) diluted 1:100. In each tumour, the total number of epithelial cell nuclei and that of Ki-67 immunostained nuclei were counted in five different representative areas, using a calibrated ocular grid at x400 magnification. The proliferative index was estimated from a total of at least 1000 nuclei counted per tumour.

**In situ labelling of apoptotic cells in nude mice tumours**

Apoptotic cells were identified by a terminal deoxynucleotidyl transferase (TdT) mediated dUTP nick-end labelling (TUNEL) method. After dewaxing, tissue sections were treated with proteinase K (20 µg/ml) for 10 min at room temperature. The TUNEL reaction was performed according to procedures provided with the ApopTag Plus\textsuperscript{®} Peroxidase In Situ Apoptosis Detection Kit (Serologicals Corporation, Biotech). Peroxidase activity was revealed with diaminobenzidine substrate. A negative control was run, omitting TdT from the reaction mixture. As a positive control, sections of normal human lymph node tissues were used. Apoptotic cells were identified by their brown positively labelled nuclei (including apoptotic bodies) and counted outside the necrosis areas. The apoptotic index was estimated, using the ocular grid, from a total of at least 1000 nuclei counted per tumour.
Experimental design in Apc\textsuperscript{Min/+} mice
The study was performed on 5 successive couples of animals, 8-9 week-old. For each individual experiment, the control and leptin treated-C57BL/6J-Apc\textsuperscript{Min/+} mice originated from the same littermate and were matched for weight and sex. Mice were equipped on day 0 with a miniosmotic pump model 2002 implanted s.c. and delivering murine leptin (R&D Systems) (800 µg/kg) or its vehicle (control). These pumps were renewed twice and mice killed after 6 weeks of treatment. Animals of the two groups were pair-fed and their body weight monitored once a week. At the time of killing (day 42), blood samples and the entire small and large intestines were taken.

Examination of intestinal mucosa and adenoma scoring in Apc\textsuperscript{Min/+} mice
Small intestine was measured and immediately cut into 6 equal segments, numbered from the proximal duodenum to the distal ileum. The large intestine was cut into 3 segments from the caecum to the anal ring. The lumen of segments was filled with 10% neutral-buffered formol solution. After several minutes, these segments were opened, carefully pinned flat on a paraffin wax block to examine the entire mucosa with the minimum of artifact, then fixed in formalin for two days. After fixation, they were measured in length and examined under a light microscope at 40x magnification. The number of adenomas per segment was quantified and their highest diameter measured using a calibrated ocular grid by two independent observers (TA and TL). Adenomas were classified in function of their size in 6 classes 500 µm away from one another: class 1, diameter less than or equal to 500 µm, class 2, higher than 500 µm up to 1 mm, and so on until class 6, diameter higher than 2.5 mm.

Immunohistochemistry and immunoblotting of the leptin receptor Ob-R
For Ob-R immunohistochemistry, we used goat polyclonal antibodies (Santa Cruz Biotechnology) diluted 1:30 to 1:50 -C-20 for nude mice tumour xenografts, or M-18 for Apc\textsuperscript{Min/+} mice small intestinal and colonic adenomas,- and the corresponding Vectastin ABC kit (Vector Labs, Burlingame, Ca). C-20, raised against human leptin receptor, recognizes the functional Ob-Rb isoform and M18, Ob-Rb and Ob-Ra isoforms. No reaction was seen when the primary antiserum was omitted.

For Western immunoblotting, frozen tissues were disrupted in ice-cold RIPA buffer. Equal amount of protein tumour lysates (75 µg) were separated on SDS-7.5% PAGE, and transferred to nitrocellulose membranes. They were probed with M18 antibody diluted 1:50, and with horseradish peroxidase-conjugated anti-goat IgGs (Santa Cruz Biotechnology), using ECL. The specificity of the signal was evaluated by peptide neutralization of M18 antibody. The relative loading of protein tumour samples was assessed after reprobing the membrane with a monoclonal antibody directed towards \( \alpha \)-tubulin (Sigma-Aldrich, France).

Leptin and insulin radioimmunoassays
After blood centrifugation, plasma was collected and kept at -80\(^\circ\)C for analyses. Leptin and insulin plasma concentrations were measured using radioimmunoassay kits for multi-species leptin and rat insulin, respectively, (Linco Research Inc., St charles, Mo), the insulin assay recognizing mice insulin.

Statistical analysis
All results were expressed as means ± SEM. Differences in proliferation of cultured cells between control values and the different doses of leptin tested were evaluated by one-way ANOVA or the non-parametric Kruskall-Wallis test for multiple comparisons, followed by the Student’s \( t \)-test or the Mann-Whitney \( U \)-test, respectively, if significant results were obtained. For the other variables studied, statistical comparisons between control and leptin-treated groups were made with the \( t \)-test or \( U \)-test (unequal variances) when relevant. Relationships between variables were examined with the non parametric Spearman’s rank correlation. The
RESULTS

Leptin stimulates the DNA synthesis and growth of human colon cancer cells
First of all, we checked the functional activity of the leptin receptor Ob-Rb isoform known to be present in HT-29 cells by the ability of leptin to induce its phosphorylation. The Ob-Rb phosphorylation was maximal after 2 min incubation of HT-29 cells in the presence of 100 ng/ml of leptin (Fig. 1A). Secondly, leptin added in serum-free culture medium, significantly stimulated the DNA synthesis and proliferation in these cells with a maximal effect at 100 ng/ml (+46% and +58% as compared to control, respectively) (Fig. 1B). A stimulating effect was also observed on the other three human cancer cell lines tested. Figure 1B shows representative experiments for each of these cell types. The leptin effect was maximal at 50 ng/ml for DNA synthesis and growth in LoVo cells (+145% and +210%, respectively), and at 500 ng/ml in Caco-2 cells (+220% and +87%, respectively). Despite Ob-Rb expression in SW 480 cells, as evidenced by RT-PCR and Western blot (data not shown), the leptin effect was less clear-cut in these cells and not quite significant (100% increase in cell number as compared to control value at 50 ng/ml leptin, p<0.07).

Leptin does not promote the growth of HT-29 cancer cell xenografts in nude mice
The promoting effect of leptin on the in vitro growth of HT-29 cells leads us to investigate whether this effect occurs in vivo in nude mice. Mice treatment with leptin or its vehicle (10 mice/group) began the day of tumor cells inoculation (day 0). At the beginning of treatment, the mean weights of mice were identical in the two groups : 21.5±0.6 g and 21.7±0.7 g. During the experiment (28 days), leptin- and vehicle–treated mice were pair-fed and had similar body weight curves, showing a decrease at the end of the experiment with a mean weight of 17.9±0.8 g for leptin-treated mice and 18.6±0.7 g for vehicle-treated mice. Two mice died in the course of the experiment, one control on day 15 and one leptin-treated mouse on day 24. Inoculation of HT-29 cells in nude mice resulted in the development of tumours, detectable at day 6. At that time, their volumes were significantly larger in leptin-treated mice than in control mice, P= 0.02. Apart from this day, they were no longer significantly different from those of controls until the time of sacrifice, although tumour volumes had tendency to grow more quickly under leptin treatment (Fig. 2A). Confirming the lack of obvious effect of leptin on tumour volumes, no variation was found in the weight of tumours at the end of the experiment, 689±99 mg for leptin-treated mice and 663±149 mg for vehicle-treated mice (Fig. 2B). Autopsy of mice and histological examination of livers after fixation did not reveal any metastatic site of tumour development in both groups. Histologically, HT-29 tumour xenografts were moderately differentiated adenocarcinomas which displayed very large areas of necrosis. We verified by Western immunoblots that leptin receptors remained expressed in HT-29 tumour cells (Fig. 3A). Immunohistochemistry confirmed the presence of these receptors in tumours of the two groups of mice (Fig. 3B). Then, we examined the state of proliferation of epithelial tumour cells. The mean Ki-67 proliferative index was significantly lower in leptin-treated mice, 35.5±5.5, than in controls, 55.1±3.4 (P<0.008) (Fig. 2C and Fig. 3C) whereas the mean apoptotic index was unchanged, 1.90±0.16 in leptin-treated mice vs 1.77±0.15 in controls (Fig. 2D). Plasma leptin levels measured at the end of the experiment were however 4.3-fold higher in leptin-treated than in vehicle-treated mice (9.1±2.4 ng/ml vs 2.1±0.4 ng/ml respectively, P<0.0003) (Fig. 4A). We established that this hyperleptinemia was biologically functional because plasma insulin levels were 4.1-fold decreased in hyperleptinemic mice as compared to controls (0.18±0.04 ng/ml vs 0.74±0.20 ng/ml, respectively, P=0.008), (Fig. 4B).

We concluded that continuous s.c. leptin delivery for 4 weeks in nude mice induced a strong and sustained hyperleptinemia which exerts a clear-cut effect on insulinemia but has no
noticeable effect on the growth of HT-29 colon cancer cell xenografts.

**Leptin does not promote intestinal tumourigenesis in \( Apc^{Min/+} \) mice**

We also investigated the possible influence of leptin in another tumour model, \( Apc^{Min/+} \) mice which have a mutation in codon 850 of the \( Apc \) tumour suppressor gene, leading to the spontaneous development of intestinal adenomas and carcinomas (26). Three successive couples of male and two couples of female \( Apc^{Min/+} \) mice were divided in two groups with an identical weight at the beginning of experiment, 21.7±0.35 g and 21.4±0.34 g. After six weeks of continuous infusion of leptin or its vehicle, leptin-treated mice weighed 22.1±1.82 g and pair-fed controls 22.1±1.15 g. At that time, leptin levels were 2.4-fold higher in leptin-treated than in vehicle-treated \( Apc^{Min/+} \) mice (7.4±2.3 ng/ml vs 3.1±0.4 ng/ml, \( P<0.03 \)), (Fig. 4C). To evaluate whether \( Apc \) mutation influenced leptinemia, the latter was also determined in wild-type C57BL/6J counterpart mice restrained in feeding for a week and found roughly of the same order as in vehicle-treated \( Apc^{Min/+} \) mice (Fig. 4C). Insulin levels were not significantly modified in leptin-treated \( Apc^{Min/+} \) mice as compared with vehicle–treated \( Apc^{Min/+} \) mice. The latter had values similar to those of control wild C57BL/6J mice (Fig. 4D). The small intestine exhibited numerous adenomas, most often solitary but sometimes developing in narrow proximity and forming clusters (Fig. 5A and B). The largest adenomas often displayed areas of high dysplasia (Fig. 5C and D). Immunolabelling with Ob-R antibody (Fig. 5E-H) indicated that epithelial cells of adenomas expressed leptin receptors but heterogeneously.

For the whole of experiments, the mean numbers of tumours in the small intestine of leptinand vehicle-treated \( Apc^{Min/+} \) mice were similar : 114.9±14.3 and 123.0±23.1, respectively (Fig 6A). No significant difference was found between control and leptin-treated males or between control and leptin-treated females. The density of adenomas per cm of intestine was also unchanged, whether small intestinal length was measured before fixation : 3.19±0.55 vs 3.07±0.59 per cm for controls and leptin-treated mice, respectively, or after fixation, 3.07±0.42 vs 3.25±0.67 per cm (Fig 6A). Moreover, the distribution profiles of tumour size were similar for the two groups of \( Apc^{Min/+} \) mice (Fig 6B). Tumours most frequently encountered measured between 500 µm and 1.5 mm in the largest diameter. The distribution of tumours along the small intestine also did not differ between the two groups (Table 1).

**Table 1 : Distribution of adenomas in function of the small intestinal segment localisation**

<table>
<thead>
<tr>
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<th>Segment 1</th>
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<tr>
<td>Total number</td>
<td>8.4 ± 1.7</td>
<td>6.4 ± 0.5</td>
<td>22 ± 6.6</td>
<td>37.4 ± 9.3</td>
<td>31.3 ± 4.2</td>
<td>9.5 ± 5.1</td>
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<tr>
<td>%</td>
<td>7.9 ± 2.7</td>
<td>5.9 ± 0.8</td>
<td>18.2 ± 4.6</td>
<td>31.0 ± 4.6</td>
<td>28.2 ± 3.2</td>
<td>8.8 ± 4.8</td>
</tr>
<tr>
<td><strong>Controls</strong></td>
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</tr>
<tr>
<td>Total number</td>
<td>9.5 ± 3.0</td>
<td>7.2 ± 1.8</td>
<td>25.1 ± 7.5</td>
<td>39.3 ± 11.3</td>
<td>33.1 ± 5.6</td>
<td>8.8 ± 2.5</td>
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<tr>
<td>%</td>
<td>7.7 ± 1.5</td>
<td>5.9 ± 1</td>
<td>19.0 ± 3.8</td>
<td>29.5 ± 5.7</td>
<td>28.6 ± 3.9</td>
<td>8.8 ± 3</td>
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Adenomas developed preferentially in the distal jejunum and proximal ileum, their number decreasing abruptly in the distal ileum. Adenomatous clusters had tendency to be larger in vehicle-treated than in leptin-treated mice. Since no difference was seen between leptin- and vehicle-treated mice, we analysed the number of tumours of small intestine in function of
gender. Females had noticeably more tumours than males: 150.9±14.7 vs 97.7±12.8, P<0.03 (Fig 6A). Finally, when examining the relationship between total numbers of small intestinal adenomas and plasma leptin levels in mice, we observed that the two variables tended to be inversely correlated (r = -0.34, Spearman’s rank correlation) but this was not significant (P=0.3). Adenomas were rare in the caecum, colon and rectum and their numbers did not differ any more: 1.3±0.4 in control Apc<sup>Min/+</sup> mice vs 0.9±0.5 in leptin-treated Apc<sup>Min/+</sup> mice.

We concluded that hyperleptinemia in Apc<sup>Min/+</sup> mice did not enhance the development of adenomas in the small as in the large intestine nor the size and distribution of adenomas in the small intestine. Besides, in those mice, hyperleptinemia was not associated with a change in plasma insulin levels.

**DISCUSSION**

The present study provides original data about the *in vitro* growth action of leptin on three human colon cancer cell lines characterized for the expression of functional leptin receptors and explores for the first time the potential role of hyperleptinemia on intestinal tumourigenesis in two animal models, HT-29 xenografts in nude mice and Apc<sup>Min/+</sup> mice. The results stressed the lack of obvious effect of leptin on tumour growth in the two models.

Herein, we confirmed that leptin *in vitro* stimulates the DNA synthesis and proliferation of HT-29 cells and thus is a growth factor for these cells as previously reported (13;18;19). We also extended leptin effects to three other colon cancer cell lines, namely LoVo, Caco-2 and SW 480. A variability in leptin response was noted in these cell lines which was apparently not related to p53 mutation or microsatellite instability. A differential effect of leptin on the *in vitro* proliferation of other cancer cell lines has also been reported (16). The doses tested here were in the range of those used by others (13;18). However, if we refer to the mean serum leptin concentration reported in obese human subjects, around 30 ng/ml, -40-100 ng/ml in some individuals (27), the maximal effects on DNA synthesis and cell growth were obtained with either doses corresponding to the highest levels measured in humans or with supraphysiological doses.

In nude mice, we showed here that the sustained delivery of exogenous leptin led to a 4.3-fold increase in plasma leptin levels as compared with controls. This value is in the range of the 4.2-fold increase in plasma leptin levels reported between obese and normal-weight humans (27). In these mice, the hyperleptinemia induced a decrease of the same order in plasma insulin levels. We and others had already observed such action of leptin on insulin levels in Fisher 344 rats (25;28). This is in line with the majority of studies in rodents indicating that leptin inhibits insulin secretion (29). Although tumours seemed to develop somewhat more quickly in leptin-treated than in vehicle-treated mice, the difference did not reach significance except on day 6, day on which tumours began to be measurable. It should be reminded that measurement of tumour volume through the skin entails some degree of imprecision inherent to the technique. At the end of the experiment, mean weight of tumours - which is a more exact variable - was in the same range in the two groups since there was only a 4% increase in leptin-treated mice as compared to controls. Thus, leptin did not exert a noticeable action on the growth of xenografted HT-29 tumours. These results could not be imputed to the absence of leptin receptors because they remained expressed in tumour cells. The estimation of the Ki-67 proliferative index in the epithelial tumour cells was another way to assess the influence of leptin on HT-29 xenografts. Surprisingly, this index was significantly lower whereas the apoptotic index was slightly higher in leptin-treated mice than in controls. At first insight, the proliferative effect was the opposite of the *in vitro* one observed on HT-29 cells herein and in previous works (13;18;19). In the same way, by contrast to the present *in vivo* data, we have previously shown an anti-apoptotic effect of leptin on HT-29 cells *in vitro* (19). These data raised two hypotheses: i) this decrease in tumour cell proliferation is directly due to leptin. However, although some authors have
shown that leptin inhibits the mitotic activity of epithelial cells in the mouse large intestine in vivo (24), it is unlikely that leptin exerted such a direct effect on HT-29 cell proliferation since tumours in the leptin-treated group grew slightly faster than in controls; ii) More plausibly one may evoke that consequently to their accelerated growth, tumours of leptin-treated mice had begun at the time of sacrifice an involution process which was accompanied by reduced cell replication. Consistent with this hypothesis, histologic examination of tumours revealed that necrotic areas were somewhat larger in tumours from leptin-treated mice than from controls. This might result from an altered balance in tumour growth and angiogenesis. Nevertheless, freezing large sample from each tumour prevented us from doing a global evaluation of necrosis.

The lack of leptin effect on the growth of HT-29 cells in nude mice might be further explained by the decrease in insulinemia. Accordingly, insulin has been reported to stimulate in vitro the proliferation of colon cancer cell lines, among them HT-29 cells (30-33), to promote colon carcinogenesis in the rat (34;35) and to be related to a higher risk of colonic neoplasia (31;36;37). Consequently, insulin decrease might counteract a potential growth effect of leptin. Alternatively, leptin might be not involved at all in cancer growth.

In this connection, we did not evidence any effect of leptin on the promotion of intestinal tumours in ApcMin/+ mice, as evaluated by the size, number and distribution of adenomas. The hyperleptinemia obtained in leptin-treated ApcMin/+ mice was significant but only moderate (2.4 fold-increase), although a similar dose of leptin as for nude mice was used. Moreover, the lack of leptin effect on tumourigenesis in ApcMin/+ mice could not be explained by a decrease in plasma insulin levels since the latter did not significantly change in leptin-treated mice and were in the range of those in wild-type counterpart control mice. No significant effect of leptin on insulin release has already been observed only in a few rodent studies (29). Our data get closer to those recently reported in ApcMin/+ mice submitted to physical exercise (38). Authors observed a significant decrease in plasma leptin levels in those animals as compared to controls but no change in the total number and size of intestinal polyps. Besides, they noted that leptin levels and total polyp numbers were significantly and inversely correlated (38). In our ApcMin/+ mice, we found also a trend toward an inverse correlation between plasma leptin levels and polyp numbers in the small intestine.

So, the present data as well as our previous findings in the rat showing that leptin significantly reduced the development of carcinogen-induced aberrant crypt foci in the colonic mucosa (25) do not support a promoting role for leptin on intestinal tumourigenesis. Several reports seem to go in the same direction. Thus, in bearing-tumor animals i.e., rats inoculated with hepatoma cells and mice inoculated with Lewis lung carcinoma cells, the circulating leptin levels, measured in the absence of anorexia, were lower than in pair-fed control animals (39). In some studies on human there is no evidence of increased leptin levels in patients with colorectal cancers (20-22). However, from a recent report, the risk for colonic cancer, but not for rectal cancer, increases with increasing serum leptin concentration (23). Nevertheless, in that study, it must be noticed that the risk is significant only in individuals in the highest quartile of leptin levels and, when colonic segments are studied separately, the risk exists only for the left colon. All these facts suggest that leptin is not involved in intestinal tumourigenesis. Nevertheless, if leptin is implicated in this process, it is likely that not mild but very high hyperleptinemia may play a role.

In conclusion, our results show that leptin exerts differential effects. In vitro, it does act as a growth factor for colon cancer cell lines. However, in vivo, in the rodent models tested and under our conditions, leptin exerts no influence on HT-29 colon cancer growth in nude mice nor on the promotion of intestinal tumourigenesis in ApcMin/+ mice. These latter findings do not support a pivotal role for hyperleptinemia on intestinal carcinogenesis.
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FOOTNOTE

Competing interests: none.

FIGURE LEGENDS

Figure 1. Biological effects of leptin in the human colonic HT-29, LoVo, Caco-2 and SW 480 cell lines

(A), Tyrosine phosphorylation assay of Ob-R leptin receptor in HT-29 cells in the absence or presence of 100 ng/ml of leptin. Ob-Rb was immunoprecipitated with the M18 anti-Ob-R antibody, and its phosphorylation was detected by Western blot with the 4G10 anti-phospho-tyrosine antibody (P-tyr). The functional isoform Ob-Rb was identified as a protein band around 120 kilodaltons. Its phosphorylation was maximal after two minutes incubation with leptin. (B), DNA synthesis and cell growth of HT-29, LoVo, Caco-2 and SW 480 human colonic cancer cells cultured in serum-free medium in the absence (control) or presence of 20 to 500 ng/ml of leptin. Each histogram is representative of two to three experiments except for HT-29 cells which served as controls and were studied only once. *, P<0.05 ; **, P<0.03 to P<0.02 ; ***, P<0.005 to P<0.002 vs controls.

Figure 2. Effect of a 4-week continuous s.c. infusion of leptin on the growth of HT-29 cells xenografted in nude mice.

(A), tumour volume during the course of treatment ; (B), tumour weight on day 28, at the time of sacrifice ; (C), proliferative index and (D), apoptotic index in epithelial tumour cells at day 28. *, P=0.02 and **, P<0.008 vs vehicle-treated nude mice.

Figure 3. Effect of leptin treatment on the expression of Ob-Rb and on the mitotic activity of HT-29 tumour xenografts.

(A), Western immunoblots of Ob-Rb from homogenates of HT-29 tumour xenografts in leptin- and vehicle-treated nude mice using M18 Ob-R antibody. A band around 120 kilodaltons corresponding to the Ob-Rb functional isoform of leptin receptor was identified in all tumors. This band disappears after immunoneutralization of Ob-R antibody with the corresponding blocking peptide (not shown). Membranes were reprobed with an antibody against α-tubulin (diluted 1:1000) to assess the relative equal loading of protein samples. (B), Ob-R immunohistochemistry. Insets illustrate detail of labelling on membranes and in cytoplasm of tumour epithelial cells ; (C), Ki-67 immunohistochemistry in tumours (MIB antibody) showing a higher proliferative index in the tumour of a vehicle-treated mouse than in that of a leptin-treated mouse. Bar = 20 µm.

Figure 4. Effects of s.c. leptin treatment on the leptinemia and insulinemia in nude mice and ApcMin/+ mice.

(A), Plasma leptin and (B), plasma insulin after 28 days of s.c. continuous infusion of leptin or its vehicle in nude mice. *, P=0.008 and **, P<0.0003 vs vehicle-treated mice. (C), Plasma leptin and (D), plasma insulin after 42 days of s.c. continuous infusion of leptin or its vehicle in ApcMin/+ mice. These variables were also measured in the C57BL/6J wild mice studied as controls for Apc mutation. *, P<0.03 vs vehicle-treated mice (U test).
Figure 5. Aspect and Ob-R expression in intestinal adenomas in \textit{Apc}^{Min/+} mice.
Macroscopic aspect of polyps, single (A) or in cluster (B). (C), histologic pattern of adenoma. Here, a sessile duodenal polyp. The duodenal mucosa under the polyp appears atrophic (arrow). (D), detail of the polyp showing areas of moderate to high dysplasia. Asterisks indicate glandular tubes only slightly modified. (E-H), immunohistochemical Ob-R signal. (E), part of the duodenal polyp. The labelling is seen in the epithelium surrounding the adenoma (arrow head) and in the duodenal villi (arrow). (F), colonic adenoma. The labelling is seen in the surrounding epithelium (arrow head) and in some dysplastic glandular tubes (arrows). (G-H), detail of the reaction in the duodenal polyp showing heterogeneous Ob-R expression in the surrounding epithelium (G) and in epithelial cells of dysplastic glands in another area (H). Bar= 40 µm.

Figure 6. Numbers and size distribution of adenomas in the small intestine of leptin- and vehicle-treated \textit{Apc}^{Min/+} mice.
(A), No difference was observed in the total number or the number per cm of intestine (here, considering the length before fixation) between the two groups. By averaging values in all females and these in all males, the total number of adenomas was found to be significantly higher in the small intestine of female than in males, \(P<0.03\). (B), Histogram showing the distribution profile of adenomas in the small intestine in function of their size. Polyps were classified into six classes. There was no difference between leptin- and vehicle treated \textit{Apc}^{Min/+} mice for any class. In this study, the size of most polyps ranged between 500 µm and 1.5 mm.
REFERENCES


Figure 1
Figure 2
Figure 3
Figure 4
Figure 6