Chapter II

Leptin is a growth factor for colonic epithelial cells

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Gastroenterology 2001; 121: 79-90
Abstract

*Background and aims:* Obesity increases the risk of colon cancer whereas physical activity reduces the risk. Plasma levels of leptin rise in proportion to the level of obesity and are reduced by physical activity. Leptin acts as a growth factor for several cell types and thus may provide a biological explanation for the observed epidemiological risk factors. We aimed to investigate whether leptin is a growth factor for colonic epithelial cells.

*Methods:* The presence of the leptin receptor in human colon cancer cell lines was assessed using RT-PCR, immunoblotting and binding studies, and its presence in human colonic tissue by immunohistochemistry. We assessed the effects of leptin *in vitro* on HT29 cells by assessing p42/44 MAP kinase phosphorylation, thymidine incorporation and cell numbers, and *in vivo* in C57BL/6 mice by colonic BrdU incorporation.

*Results:* The leptin receptor is expressed in human colon cancer cell lines and human colonic tissue. Stimulation with leptin leads to phosphorylation of p42/44 MAP kinase and increases proliferation *in vitro* and *in vivo*.

*Conclusions:* Leptin is a growth factor in colonic epithelial cells and one that may provide a biological explanation for the observed associations between obesity, physical activity and colon cancer.
**Introduction**

Investigation into the aetiology of colon cancer has been guided since the outset by epidemiological data. Research into biological explanations for the increased risk of colon cancer seen within certain families (Familial Adenomatous Polyposis, Hereditary Non-Polyposis Colon Carcinoma) and the reduced risk in non-steroidal anti-inflammatory drug users have been particularly fruitful in furthering understanding of the disease. However, for other associations the biological mechanisms involved have received less attention. Two of these are the increased risk of colon cancer with body mass and the even stronger relationship between physical activity and reduced risk of colon cancer. Here we show evidence that the hormone leptin, a hormone whose levels vary with body mass and physical activity, may provide a possible biological explanation for these associations.

Leptin was originally described as the product of the mouse *obese* (*ob*) gene. Further characterisation of the *ob* gene revealed that it encodes a hormone that is expressed predominantly in adipose tissue. This 167 amino-acid protein is secreted into blood and circulates to control energy balance. Mice with recessive *ob* mutations develop obesity and diabetes in a syndrome resembling morbid human obesity. In addition these mice show many of the abnormalities seen in starvation, such as decreased activity, decreased body temperature, infertility and decreased immune function. Leptin replacement corrects all these abnormalities.

Leptin exerts its action through the leptin receptor (Ob-R), a member of the cytokine family of receptors. It consists of a single membrane spanning protein that, through alternative mRNA splicing, exists in several isoforms. These isoforms differ only in the length of their intracellular domains and can be divided into those with a full-length intracellular domain (Ob-Rb in mice, homologous to huOb-Rb in humans), often referred to as the long isoform of the leptin receptor, and the short isoforms.

The expression of one of the short isoforms, (Ob-Ra in mice, huOb-Ra in humans), dominates over the other short isoforms. Ob-Ra expression is widespread and has been found in most tissues that have been tested. The long isoform Ob-Rb is highly expressed in hypothalamic neurones where it is responsible for the anorectic action of leptin. The consequences of disruption of the *Ob-Rb* gene are seen in the *db/db* mouse, which has the same obese phenotype as the *ob/ob* mouse. In this model only the long isoform of the leptin receptor, Ob-Rb, is disrupted but the anorectic function of leptin is altered just as severely as when production of leptin hormone itself is disrupted. From this it has been assumed that the
other isoforms of the leptin receptor play a relatively unimportant role in the anorectic function of leptin. \textit{db/db} mice do, however, differ from \textit{ob/ob} mice in their development of glomerulosclerosis.\textsuperscript{70} Glomerular endothelial cells have been found to express only the short Ob-Ra receptor and yet exhibit increased proliferation in response to leptin exposure.\textsuperscript{71} In addition the short isoform Ob-Ra has been shown to be able to activate the mitogen-activated protein kinase (MAP kinase) signal transduction pathway.\textsuperscript{72,73} These data may suggest that the effects of leptin on peripheral tissues can be mediated by the short isoform whose expression dominates in tissue other than the brain. However, the exact functions of the short isoforms remains disputed with many groups unable to show proliferative effects or signal transduction mediated by the short isoforms.

Leptin receptor isoforms have been reported in a wide variety of human and rodent tissues: Heart, placenta, lung, liver, muscle, kidney, pancreas, spleen, thymus, prostate, testes, ovary, small intestine and colon. Leptin receptor expression in the various tissues of the mouse gastrointestinal tract has been shown by RT-PCR with Ob-Rb seen only in the jejunum and the other isoforms found throughout.\textsuperscript{74} Human colonic tissue has never been studied in detail, and no functional relevance of leptin receptor presence on colonocytes has been demonstrated.

The wide expression of leptin receptors suggests a possible wider role for leptin than fat mass regulation. Indeed, leptin has been found to have profound effects in various human tissues including monocytes,\textsuperscript{75} respiratory epithelium,\textsuperscript{76} glomerular endothelial cells,\textsuperscript{71} vascular endothelium,\textsuperscript{77} and adrenal cells.\textsuperscript{78} It has been reported to increase proliferation in all these cell types and to have an effect on monocytes equally dramatic as stimulation with lipopolysaccharide.\textsuperscript{79}

Leptin levels in humans and animals closely reflect the percentage of body fat, thus obesity is associated with hyperleptinaemia.\textsuperscript{80-89} Research into the effects of exercise on leptin levels has shown reductions independent of levels of obesity.\textsuperscript{81-90} Leptin levels are likely to be higher in colon cancer patients who have higher levels of obesity and lower levels of physical activity than controls. Leptin is a growth factor in many cell types. We therefore propose that leptin acts as a growth factor in colon cancer and is a putative biological mechanism for the observed differences in the risk of developing colon cancer associated with changes in body mass and physical activity.

To investigate this possibility we first assessed human leptin receptor expression in human colonic epithelial cells. We used immunohistochemistry in the normal colon, colonic adenomatous polyps and colon cancer specimens, and Reverse Transcribed Polymerase Chain
Reactions (RT-PCR) and immunoblotting in colon cancer cell lines. We chose not to use human colon tissue for the RT-PCR and immunoblotting because of the likelihood of contamination of colonocytes by other cell types using this method. The expression of leptin receptors on adipocytes, monocytes, and endothelial cells are likely to lead to false positives using this material, especially when using PCR.

Secondly, we assessed the affinity and numbers of leptin receptors on colon cancer cell lines. We then demonstrated the functional significance of the receptors by stimulating colon cancer cell lines with recombinant human leptin and assessing its effect on proliferation and mitogenic signal transduction pathway activation.

Lastly, to confirm that our in vitro findings are relevant in vivo, we injected mice with recombinant mouse leptin and assessed its effect on epithelial proliferation in the colon and examined the colons of db/db mice for evidence of increased colonic epithelial proliferation.

Methods

Immunohistochemistry

The ten most recent cases of adenomatous polyp removed at colonoscopy, and the ten most recent surgically resected colonic adenocarcinomas together with normal tissue from the same patients were taken from the archives of the Pathology Department at the AMC. 4 μm sections were prepared from the formalin fixed, paraffin embedded tissue and mounted on slides coated with polylysine. Sections were dewaxed and rehydrated in graded alcohols. Endogenous peroxidase activity was quenched with 1.5% H₂O₂ in Phosphate Buffered Saline (PBS) for 30 mins and then washed in PBS. Non-specific binding sites were blocked with TENG-T (10mM Tris, 5 mM EDTA, 0.15 M NaCl, 0.25% gelatin, 0.05% (v/v) Tween 20, pH 8.0) for 30mins, and then washed (3 x 5 mins in PBS). Slides were incubated at 4°C overnight with M-18 Goat Polyclonal anti Ob receptor antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) in PBS with 0.1% Triton and 1% bovine serum albumin. These are raised against the intracellular portion of mouse Ob-Ra and reactive against all isoforms of the human leptin receptor. After washing (3 x 10 mins in PBS), slides were then incubated with biotinylated rabbit anti-goat antibodies at room temperature for one hour in PBS with 10% Human serum. Slides were washed (3 x 5 mins in PBS), incubated with streptavidin- biotin-horseradish peroxidase (Dako, Glostrup, Denmark) for 1 hour, washed again (3 x 5 mins in PBS), and peroxidase activity was detected with 3,3’-diaminobenzidine (0.5mg/ml, Sigma, St. Louis, MO) in 0.05M Tris Ph
7.6 with hydrogen peroxide (0.05%). Finally, sections were counterstained with Mayer's haematoxylin, dehydrated and mounted in 'Entellan' (Merk, Darmstadt, Germany) under cover slips.

Leptin receptor staining was assessed by two independent observers who were blinded to the origin of the sections. Staining of epithelial cells was scored on a scale from 0-3 for intensity where 0 represents no staining, 1 weak staining, 2 moderate staining, and 3 strong staining.

**Cell culture**

HT 29, Caco-2, DLD-1, SW 480, HCT 116 and LS 174-T colon cancer cell lines were cultured in Dulbecco's Modified Eagles Medium (DMEM) (Gibco, Paisley, Scotland) with 4.5g/L Glucose and l-Glutamine. This was supplemented with penicillin (50U/ml) and Streptomycin (50μg/ml) and, where serum was used, with 10% foetal calf Serum (Gibco). Cells were grown in monolayers in a humidified atmosphere containing 5% CO₂.

**Immunoblotting**

Confluent monolayers of cells from 6 well plates (Nalge Nunc, Denmark) were washed in ice cold PBS and scraped into 100μl of 4x sample buffer (125 mM Tris/HCl, pH 6.8; 4% SDS; 2% β-mercapto ethanol; 20% glycerol, 1mg bromphenol blue). 30μl of each sample was loaded onto SDS-PAGE and blotted onto PVDF membrane (Millipore). Equal loading was confirmed by Coomassie brilliant blue staining. The blots were blocked with 2% low fat milk powder in PBST (PBS with 1% Triton) for one hour at room temperature and washed 3x 10 mins in wash buffer (PBST with 0.2% low fat milk powder) before overnight incubation in 1/1000 primary antibody in wash buffer at 4°C. Blots were then washed 3x 10 mins in wash buffer and incubated for 1 hour at room temperature in 1/2000 Horse Radish Peroxidase (HRP) conjugated secondary antibody in wash buffer. After a final 3x 10 minute wash in wash buffer, blots were incubated for 1 minute in Lumilite plus (Boehringer-Mannheim, Mannheim, Germany) and then chemiluminescence detected using a Lumi-Imager (Boehringer-Mannheim).

For the MAP kinase studies confluent monolayers of HT29 cells in 6 well plates were serum starved overnight and then stimulated with recombinant human leptin (Sigma) for various times. Immunoblotting was then carried out as previously described using rabbit polyclonal phosphospecific p42/44 MAP kinase antibodies and rabbit polyclonal total p42/44
MAP kinase (New England Biolabs, Beverly, MA) as primary antibodies and goat anti-rabbit HRP (DAKO) as secondary antibodies.

For the Ob-receptor blots M-18 Goat Polyclonal anti Ob receptor antibodies (Santa Cruz) raised against the intracellular portion of mouse Ob-Ra and reactive against all isoforms of the human leptin receptor were used as primary antibodies. After primary antibody incubation, an extra step was added, using biotin labelled rabbit anti-goat antibodies (DAKO) 1/2000 in wash buffer for one hour, followed by a 3 x 5 minute wash and incubation with streptavidin-HRP (DAKO) 1/100 for 60 minutes before detection of HRP using the method described. The experiment was repeated with anti-human leptin receptor goat polyclonal antibodies, AF389, (R&D, Minneapolis) raised against an epitope from the extracellular portion of the human leptin receptor. Equal protein loading was further confirmed using β-actin (Santa Cruz) antibodies on the same blots.

**Thymidine incorporation assay**

Confluent monolayers of HT 29 cells were trypsinised and taken up in serum free medium supplemented with 20μg/ml of human transferrin. 10⁴ cells were seeded in flat-bottomed tissue culture treated 96 well plates (Falcon) and allowed to adhere for 6 hours. Cells were then stimulated with recombinant human leptin, vehicle or 100 ng/ml Epidermal Growth Factor (Sigma) for 50 hours. Leptin concentrations were taken that included those found in non-obese (1-3ng/ml) and obese (up to 100ng/ml) humans. Tritiated thymidine was then added for 16 hours. 10μl of 0.1 M EDTA was added to each well for 15 minutes, after which cells were lysed with distilled water and aspirated through a filter. Filters were dried at 50°C for 30 minutes, covered with 40μl of scintillation fluid per well and ³H activity measured in a top counter.

**Cell counting assay**

Confluent monolayers of HT 29 cells were trypsinised and taken up in medium with 1% FCS. 5x10⁴ cells were seeded in flat-bottomed tissue culture treated 12 well plates (Falcon) and then stimulated with recombinant human leptin, vehicle or 100 ng/ml epidermal growth factor (Sigma) for 66 hours. Cells in each well were then trypsinised and taken up in 1ml of medium. Cells were then counted manually in a standard cell counter (Bürker).
RT PCR

Total RNA was isolated from HT 29, CACO-2, DLD-1, SW 480, HCT 116 and LS 174-T cells as well as fresh human placenta. A one-step RT-PCR kit (Qiagen, Hilden, Germany) was used and 1 μg of RNA added. Primers were designed with the aid of primer designing software using the human leptin receptor (huOb-Rb) cDNA sequence and the huOb-Ra sequence obtained from Entrez. These were designed either to detect the presence of any of the leptin receptor isoforms (common) or to be specific to the long isoform (huOb-Rb) or the short isoform (huOb-Ra). Care was taken to design primers that flanked several introns, and the long isoform sense primer hybridises across the junction of two exons. False positives arising from chromosomal DNA contamination of the RNA are thus eliminated or will result in significantly larger products than those obtained from the cDNA. The expected product lengths were 489 base pairs for the common receptor primers, 481 base pairs for the long receptor primers, and 200 base pairs for the short isoform, and primer sequences were as follows:

<table>
<thead>
<tr>
<th>Receptor Type</th>
<th>Sense Primer</th>
<th>Antisense Primer</th>
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<tbody>
<tr>
<td>Common</td>
<td>5'-ctttcactgttgctttcgg-3'</td>
<td>5'-tcttgtgttccatatgccaaacc-3'</td>
</tr>
<tr>
<td>Long</td>
<td>5'-ccatgatcattttatccca-3'</td>
<td>5'-gtcactaatccaaacagaccaacc-3'</td>
</tr>
<tr>
<td>Short</td>
<td>5'-ttgtgccagtaattatttccttt-3'</td>
<td>5'-agttggcacattggttcat-3'</td>
</tr>
</tbody>
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PCR was performed for 40 cycles using a 60-s denaturation step at 94°C, 60-s annealing step at 55° and a 90-s extension step at 72°C. 10 microlitres of the product was loaded onto agarose gel and the fluorescence of the Ethidium Bromide stained band recorded.

BrdU incorporation in leptin treated mice

In a protocol approved by the relevant animal ethics review board, eight seven week old female C57BL/6 mice were treated with a single intraperitoneal injection of mouse recombinant leptin (R&D) in NaCl at a dose of 10mg/kg as used by Pelleymounter et al. Eight mice received intraperitoneal NaCl as a control. After 15 hours all mice received a single intraperitoneal injection of BrdU at a dose of 150mg/kg and were sacrificed one hour later.
Colonic proliferation in \(db/db\) mice

Six 10 week old \(db/db\) mice of the C57BL/6J background and six wild type littermates were sacrificed having received no intervention.

Scoring of proliferation

Colons were removed, rolled up and fixed in 4% paraformaldehyde and embedded in paraffin. Sections were prepared and stained as for other immunohistochemistry except for BrdU stainings where the addition of a one-hour incubation in 2M HCl at 37°C followed by washing in Boric acid pH 8.5 before the blocking step was necessary. Mouse monoclonal antibodies against BrdU, 5-bromo-2’-deoxyuridine, (Roche, Almere, The Netherlands) or PCNA, proliferating cell nuclear antigen (DAKO), were used as primary antibodies. Nuclei staining positively for BrdU or PCNA were scored by taking five pictures of each section at 200x magnification from the proximal colon. In each picture positive nuclei in five well-orientated crypts were counted, blind to treatment, with the help of image analysis software (EFM Software, Rotterdam, The Netherlands).

Results

Colon cancer cell lines express the short and long isoforms of the leptin receptor.

Colon cancer cells were investigated for the presence of three sequences using RT-PCR on RNA isolated from six different colon cancer cell lines with human placental tissue as a positive control. The sequences were firstly, a common sequence from the extracellular domain of the human leptin receptor, secondly, a sequence exclusive to the long isoform of the leptin receptor (huOb-Rb), and lastly a sequence exclusive to the short isoform, huOb-Ra. As shown in Figure 1, a product of the expected length was obtained in all cell lines with all three primer sets, whereas no RNA controls gave no products.
To further confirm the expression of this receptor, immunoblotting was performed for the leptin receptor. Figure 2 shows that all of the colon cancer cell lines tested show a band at approximately 100 kDa on the immunoblot consistent with the presence of the short (huOb-Ra) isoform of the leptin receptor. In addition several showed a band at approximately 130 kDa consistent with the long (huOb-Rb) isoform of the receptor. The same two bands can be seen in human placenta, which is known to express both short and long isoforms of the leptin receptor and was thus used as a positive control. A repeat of this experiment using an antibody raised against an epitope from the human extracellular region common to all isoforms yielded the same two bands of the same sizes. (Not shown). Simultaneous loading controls using β-actin antibodies are shown.
Leptin and colon cancer

Figure 2 Immunoblot for the human leptin receptor using an antibody raised against an epitope of mouse Ob-Ra, which is reactive against all human isoforms with an intracellular portion. Two bands are seen, one dominant band running at a size of 100 kDa which corresponds to the huOb-Ra protein and a second weaker band running at 130 kDa corresponding to the huOb-Rb isoform. Both of these bands are also seen in human placental tissue which is known to express both long and short isoforms and is used here as a positive control. Simultaneous loading controls using β-actin antibodies are shown.

We conclude from these results that both the short (huOb-Ra) and long (huOb-Rb) isoforms of the leptin receptor are expressed on colon cancer cells.

Expression of the leptin receptor in human colon tissue in vivo.

To investigate whether the expression of the leptin receptor in human colon cells in vivo concurred with our findings in colon cell lines in vitro, we took human colonic adenomatous polyp tissue, normal colonic tissue and tissue from colonic adenocarcinomas and stained for the presence of leptin receptors (all isoforms). As evident from figure 3, clear immunoreactivity was observed in the normal colonic epithelium and adenomatous polyp epithelium. Staining appeared to be largely on cell membranes and in the cytoplasm consistent with results from transfection experiments showing that 85% of leptin receptors are usually in the Golgi apparatus and 15% on the cell surface at any given time. Receptor expression was similar in normal and polyp epithelial cells. Staining was also seen on
adipocytes and the infiltrating mononuclear cells, which are both known to express leptin receptors. Control sections with no primary antibody showed no staining (not shown).

Figure 3 Immunohistochemistry, staining for leptin receptors in normal human colonic tissue (a+b) and in a whole adenomatous polyp (marked P) arising from surrounding normal colonic epithelium (c-f). A cross section of normal colon (a) and an enlargement of the area marked with a rectangle (b) shows strong staining (brown) of the epithelium. Images (c-f) show that staining for the leptin receptor is predominantly in the epithelial cells and is seen in close-up in both the surrounding normal (d) and polyp (e and f) tissue. Staining appears to be largely localised to the cytoplasm and cell membrane. Magnifications: A x50, B x 600, C x50, D+E x200, F x800. D, E and F are enlargements of the areas within C marked with dashed, dotted and solid lines respectively.
Two independent observers scored epithelial staining intensity for all specimens and the results are displayed in Figure 4.

Figure 4  Scoring of Ob-R staining intensity in normal colon and adenomatous polyps. Staining was scored on a scale from 0-3 where 0 represents no staining, 1 weak staining, 2 moderate staining, and 3 strong staining.

We conclude that expression of the leptin receptor is a consistent feature of colonic epithelial cells in vivo and in vitro.

The leptin receptor is functional and linked to proliferative signalling in colon cancer cells

To investigate whether leptin receptors are functional and linked to proliferation in colon cancer cells we measured the influence of leptin on p42/44 MAP kinase phosphorylation. Phosphorylation at Thr\textsuperscript{202} and Tyr\textsuperscript{204} leads to activation of p42/44 MAP kinase and this phosphorylation was assessed using phospho-specific antibodies. Figure 5a shows that leptin stimulation causes biphasic phosphorylation of MAP kinase. MAP kinase activation marks the onset of proliferation. This suggests that leptin may lead to proliferation. In figure 5b we show the effect of a range of different concentrations of leptin on MAP kinase phosphorylation.
Figure 5a Immunoblot for the phosphorylated form of p42/44 MAP kinase in HT29 cells treated with recombinant human leptin (2μg/ml) for various times (shown in minutes). The same blot was stripped and reprobed for total MAP kinase demonstrating presence of equal amounts of the protein in each lane.

Figure 5b Immunoblot for the phosphorylated form of p42/44 MAP kinase in HT29 cells treated for 5 minutes with recombinant human leptin at various concentrations (shown in ng/ml)

These results suggest that leptin might act a growth factor for colon cancer cells. To confirm this we measured the effect of leptin on colon cancer cell proliferation by assessing the effects of leptin on thymidine incorporation and cell numbers in HT29 colon cancer cells. Figure 6 demonstrates the changes induced by leptin in the incorporation of tritiated thymidine into the colon carcinoma cell line, HT29.
Leptin leads to a highly statistically significant and dose dependent increase in thymidine incorporation. These effects are seen at levels found in obesity in human subjects (up to 100ng/ml). Surprisingly, leptin was nearly as potent in its stimulation of thymidine incorporation as epidermal growth factor (EGF). Maximal stimulation of thymidine incorporation with EGF was seen at 100ng/ml concurring with the findings of Ryder et al.\textsuperscript{95}

Figure 7 shows the same experiment repeated but this time showing the effects of leptin on cell numbers. It should be noted that in the thymidine incorporation and cell numbers experiments degradation of the recombinant leptin will occur and that \textit{in vivo} where leptin levels remain constant, greater effects for comparable leptin concentrations might be expected.
Figure 7 Cell numbers, counted in a Bürker cell counting chamber, of HT 29 cells stimulated for 66 hours with various concentrations of leptin. The negative control was leptin heated to 95°C for 10 mins, labelled vehicle, and the positive control was 100 ng/ml of EGF. Values were compared to the vehicle control using a one-way ANOVA with the Tukey post hoc test. Significance was taken as a P value of <0.05 marked *. P values of <0.01 are marked **. Error bars show the standard error of the mean. Probability of linear trend P<0.0001.

These results demonstrate that the leptin receptor is expressed, functional and linked to cell growth in colon cancer cells.

**Leptin acts to promote colonic epithelial cell proliferation in vivo**

To confirm the relevance of our results in vivo we injected mice with recombinant leptin and assessed the colon for changes in epithelial cell proliferation. We found that leptin caused a significant increase in epithelial cell proliferation as judged by nuclear BrdU incorporation (See figures 8 and 9).
Figure 8 Numbers of nuclei per crypt staining positive for BrdU in leptin treated mice versus controls. Statistical analysis was performed using the Students t-test.

Figure 9 Representative sections from leptin treated (a) and untreated (b) sections of mouse colon stained for BrdU.

Further confirmation of the relevance of our in vitro findings was obtained by investigating a mouse model of hyperleptinaemia, the db/db mouse. We assessed colonic epithelial cell proliferation in db/db mice compared with their wild type littermates. In figure 10 we show a statistically significant increase in proliferation in the hyperleptinaemic mice as judged by proliferating cell nuclear antigen staining.
Discussion

In this study we show that leptin is a growth factor for colonic epithelial cells, which may provide a biological explanation for the observed associations between obesity, physical activity and colon cancer. The evidence that body mass and levels of both leisure time and occupational activity are associated with the development of cancer of the colon has mounted steadily and now receives widespread acceptance. A full review of the epidemiology is beyond the scope of this manuscript but a recent review\(^6\) supplemented with more recent studies gives the following evidence.

For body mass, four cohort studies and eight case-control studies have found that men who are in the highest quintile for body size have as much as a two to threefold increased risk of colon cancer. Two more recent large cohort studies\(^5\) confirm this as well as adding weight to the evidence that the same holds true for women.

The relationship between physical activity and a reduced risk of colon cancer is among the most consistent findings in the epidemiological literature. Seven cohort studies and ten case controlled studies have found an association, with two and one respectively finding no association.

The suggested biological explanations for these associations include stimulation of peristalsis and reduced transit time, favourable effects on the immune system,\(^6\) and lower insulin and glucose levels in thinner and more active individuals.\(^7\)\(^8\) However, transit time despite much attention, is not a recognised risk factor for colonic neoplasia. Insulin is a growth factor for colon cancer cell lines but no association has been found between HbA1c

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**Figure 10** Numbers of nuclei per crypt staining positive for PCNA in Leptin receptor mutant db/db mice (db/db) and wild type mice. Statistical analysis was performed using the Students t-test.
levels and colon cancer \(^9\) and while leptin levels rise with small weight gain within the same individual the same is not found consistently with insulin levels.\(^{10}\) Having said this, there is extensive evidence that leptin and insulin levels are closely linked \(^{101}\) and both may act in concert.

Body mass and physical activity are intrinsically linked and it is difficult to analyse their impact independently. Several papers have instead referred to metabolic state or energy balance as being associated with colon cancer.\(^{102-104}\) The startling changes in incidence within the same population exposed to a change in environment have been difficult to correlate with any particular dietary constituent and may instead be associated with energy imbalance. In other words the increased colon cancer risk seen in ‘Western’ diets may not be due to what we eat but how much, especially when combined with low levels of exercise or energy expenditure. This is supported by the finding that caloric restriction reduces colonic epithelial proliferation in humans.\(^5\) The relevance of this is that leptin is associated with other factors that regulate energy homeostasis.\(^{105}\) High levels of leptin are seen in individuals whose balance of calorie intake and expenditure has resulted in obesity.

Leptin is the product of the \(Ob\) gene and was initially reported as being secreted by adipocytes and controlling appetite, activity, metabolic rate and fertility through receptors in the hypothalamus. The leptin receptor has been identified as a single membrane spanning protein with multiple isoforms that arise from alternative RNA splicing. Of these, the long isoform, \(Ob-Rb\), is proposed as the most important signalling isoform as it contains a full length cytosolic domain of 302 amino acids that includes binding motifs required for activation of the Janus kinase/STAT signalling pathway. This is the isoform found in the hypothalamus and is also the isoform which when disrupted in mice leads to the same obesity syndrome as seen in \(Ob\) mice, while the other isoforms remain unaltered. Leptin has also been shown to activate the MAP kinase signal transduction pathway both through the \(Ob-Rb\) receptor isoform and by some groups, also via the \(Ob-Ra\) short isoform. The function of other isoforms is unknown but may include leptin transport or repression of signalling activity by competing in the homodimerisation of receptors needed for effective signal transduction.

In humans, levels of leptin in blood are closely correlated with body mass and may rise from levels of 1-3 ng/ml in non-obese subject to as high as 100ng/ml in obese individuals. There is also considerable evidence that leptin levels depend, albeit to a lesser extent, on physical activity. Increased physical activity appears to reduce leptin levels by around 20% independent of obesity.
Recently, it has been shown that leptin also has profound effects in various extraneurial tissues including monocytes, respiratory epithelium, glomerular endothelial cells, vascular endothelium and adrenal cells. It has been reported to increase proliferation and differentiation in all these cell types and to have an effect on monocytes equally dramatic as stimulation with LPS.

In this paper we hypothesise that high levels of leptin in obese and inactive humans may lead to growth promotional effects in colon cancer cells, thus providing a biological explanation for the associations between obesity, exercise and the incidence of colon cancer in humans.

To confirm our hypothesis we felt that the following steps were necessary. Firstly, confirmation of the presence of the leptin receptor on colonic cells using different methods, in both colon cancer cell lines and patient material. Secondly, demonstration of the functional relevance of these receptors by their ability to activate signal transduction pathways. Thirdly, confirmation of leptin as a growth factor for colon cancer cells and finally confirmation of the importance of leptin as a growth factor in vivo.

The first step was accomplished using RT-PCR, immunoblotting, immunohistochemistry, and binding studies. We show that all colon cancer cell lines tested expressed a common sequence, as well as sequences exclusive to the short and long isoforms of the human leptin receptor. We then show that human adenomatous polyps and normal colonic tissue also express the leptin receptor. We investigated adenomatous polyps, as they are a likely point in the stepwise progression to invasive cancer to be affected by growth factors. After the development of invasiveness or metastatic potential, the effects of growth factors such as leptin are unlikely to have a significant impact on the outcome. The incidence of colonic polyps is also related to body mass and exercise in the same way as the incidence of colon cancer but interestingly, possibly only in larger adenomas.

The second step was accomplished using recombinant leptin on colonic epithelial cell lines and showing activation of one of the major mitogenic signal transduction pathway elements, p42/44 MAP kinase.

The third step was achieved by showing increased thymidine incorporation and cell numbers at concentrations of leptin that include those found in the serum of non-obese (below 10ng/ml) and obese (up to 100ng/ml) individuals. As well as being highly significant compared to vehicle control, we have also shown that the magnitude of this effect approaches that of EGF, a known growth factor in HT 29 cells.
Finally, we show that \textit{db/db} hyperleptinaemic mice have higher rates of colonic epithelial cell proliferation than their wild type littermates. The \textit{db/db} mouse, where the long form of the leptin receptor contains a loss of function mutation, is a complex model of hyperleptinaemia as the mice secondarily develop a number of other metabolic and endocrine defects. These include high insulin levels (type II diabetes mellitus), changes in sex hormone levels\textsuperscript{108} and dyslipidaemia,\textsuperscript{109} all of which have been implicated independently as having possible influences on colon cancer progression.\textsuperscript{97 98 110} Thus, while our findings suggest that a primary defect in leptin signalling that results in hyperleptinaemia leads to increased colonic epithelial cell proliferation, it fails to distinguish between the primary and secondary effects of this defect. This is avoided in our second model of hyperleptinaemia where we gave mice a single injection of leptin. This leads to a highly significant increase in colonic epithelial cell proliferation. These findings suggest that our \textit{in vitro} findings are indeed likely to be relevant \textit{in vivo}.

Growth factors are thought to be important in the progression of colon cancer.\textsuperscript{111-113} Insulin like growth factors have been intensively studied and may explain the observed increase on colorectal cancer and adenomatous polyp incidence in patients with acromegaly.\textsuperscript{114} Thus the demonstration that leptin is a growth factor for colonic epithelial cells in mice and cell lines is likely to have relevance in humans. However, we suggest that leptin is not just another growth factor relevant to the colon but may provide a biological explanation for a wealth of epidemiological evidence connecting body mass, physical activity and energy balance with the incidence of colon cancer.