Overexpression of leptin mRNA in mesenteric adipose tissue in inflammatory bowel diseases

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SUMMARY

Background — Leptin, a protein with a cytokine-like structure, is produced predominantly by adipocytes. It appears to play a key role in immune responses by increasing the secretion of Th1 and pro-inflammatory cytokines. As fat-wrapping is a characteristic feature of Crohn’s disease (CD), and as increased leptin levels have been reported in animal models of intestinal inflammation, this study investigated whether mesenteric adipose tissue could be a source of leptin in human inflammatory bowel disease (IBD).

Aim — To quantify the expression of leptin mRNA in mesenteric adipose tissue of patients with CD or ulcerative colitis (UC).

Methods — Specimens were obtained from mesenteric white adipose tissue of patients with CD or UC and, for controls, from apparently healthy mesentery of patients operated for carcinoma of the right colon. The expression of leptin mRNA was assessed by reverse transcription-competitive polymerase chain reaction.

Results — Leptin mRNA levels were significantly higher in mesenteric adipose tissue of CD and UC patients than in controls (P < 0.05). In CD and UC, concentrations were not significantly different in mesenteric fat specimens, whether contiguous to macroscopically normal or grossly abnormal intestine.

Conclusions — This study provides the first evidence of a novel abnormality of the mesentery of patients with IBD. Overexpression of leptin mRNA in mesenteric adipose tissue may contribute to (a) the inflammatory process, (b) enhancement of mesenteric TNFα expression in CD (as recently reported), and/or (c) the anorexia frequently reported during flares of IBD.

Leptin (the protein product of the ob gene) is an adipocyte-secreted hormone that regulates the size of adipose tissue mass [1]. It acts on specific neural targets in the hypothalamus and thereby controls body weight homeostasis, reducing food intake and increasing the metabolic rate [2, 3]. Moreover, clear relationships exist between leptin and cytokines. The primary sequence of leptin is compatible with a cytokine-like structure [4, 5]. The long isoform of the leptin receptor Ob-Rb bears homology to members of the cytokine receptor superfamily [6-8]. Accordingly, in vivo and in vitro studies have indicated that leptin is implicated in immune responses. Leptin enhances the secretion of pro-inflammatory cytokines (TNF-α, IL-6 and IL-12) by monocytes and macrophages [9, 10] and promotes CD4+ helper T-cell (Th) activity in vitro, which orchestrates most immune responses. In particular, leptin increases Th1 (IFN-γ) cytokine production [11].

In Crohn’s disease (CD), mesenteric adipose tissue displays altered morphology, such as thickening, stiffness, hypertrophy and fat-wrapping [12, 13], and has recently been shown to express high levels of TNFα [14]. An abnormal expression pattern has been described for PPARγ [14], a member of the nuclear hormone receptor family which is predominantly expressed in adipocytes and involved in adipogenesis [14, 15]. As PPARγ is also a well-known regulator of leptin gene expression [16-18], we hypothesised that leptin could be involved in mesenteric abnormalities. In fact, our previous studies in models of intestinal inflammation in rodents showed a transient increase of plasma leptin levels during the early stages of inflammation [19]. However, controversial results have been published about plasma leptin levels in human inflammatory bowel disease (IBD) [20-22], which suggest that they may be affected by various factors not necessarily reflecting changes in mesenteric adipose tissue. In this context, the present study focused on direct quantification of leptin mRNA expression in the mesentery of patients with either CD or UC (ulcerative colitis) and in control patients.

Patients and methods

Patients and samples

All patients gave their informed consent, and the study was approved by the Ethics Committee of the Hôpital Hôtel-Dieu de Lille, France. Study subjects were of normal weight and without diabetes mellitus. The diagnosis of CD and UC was established using previously published criteria [23]. No patients presented with perforating IBD.

Forty-six patients were included in the study. Fourteen with CD (9 females, 5 males; mean age 31 years old, body mass index (BMI) 22 kg/m²) underwent right ileocolonic resection for symptomatic ileitis (or Crohn’s disease of the right colon), seven subjects had colonic resection for carcinoma of the right colon (4 females, 3 males; mean age 75 years old, BMI 22 kg/m²) after failure of a short course of steroids and/or cyclosporin. Seven subjects with carcinoma of the right colon (4 females, 3 males; mean age 75 years old) served as controls. None of these control subjects was obese, but no data on their height were available for BMI calculations.

Patients underwent surgery after an overnight fast. The first step in the surgical procedure consisted of taking biopsy specimens from mesenteric white adipose tissue. As far as possible, fat samples were obtained from mesentery close both to inflamed and non-inflamed segments. In patients with carcinoma, mesenteric fat samples were obtained in front of normal intestine at a sufficient minimal distance from tumour.

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RNA analysis by reverse transcription-competitive polymerase chain reaction (RT-cPCR)

RNA preparation

Adipose tissue samples (about 200 mg of frozen tissue) were pulverised in liquid nitrogen and total RNA was prepared from the frozen

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rescent dye (Eurogentec, Seraing, Belgium), and the amplified products of variation of less than 10% [24].

RNA. Several determinations of leptin mRNA in the same adipose tissue assays for leptin mRNAs were previously described in detail [24, 25]. leptin mRNA was determined at the competition equivalence point, as described [25]. After a specific reverse-transcription reaction, target cDNA was co-amplified with known amounts of a specific leptin DNA competitor molecule added in the same PCR tube. The competitor (or internal standard) was co-amplified in competition with the target, thereby standardizing the exponential amplification process. The initial concentration of target cDNA corresponded to the initial concentration of added competitor when the PCR product ratio was equal to 1. The RT reaction was performed from 0.1 μg of tissue total RNA using 2.5 units of a thermostable reverse transcriptase (random hexamers, Promega, Charbonières, France) in the presence of 1.0 mM Tris- HCl (pH 8.3), 90 mM KCl, 1 mM MgCl2, 0.2 mM deoxyribonucleoside triphosphates and 1.3 μM of leptin antisense primer, in a final volume of 20 μl. The medium was overlaid with mineral oil and incubated for 3 min at 60 °C and then 15 min at 70 °C in the thermocycler (Minicycler PTC150, MJ Research, Watertown MA). The efficiency of single-strand cDNA synthesis from leptin mRNA was optimal under these conditions [25]. Reaction was stopped by heating for 5 min at 99 °C. After chilling on ice, 4 μl of water were added to the RT medium, from which 20 μl were sampled for cDNA-quantification by PCR.

For PCR amplification, the 20 μl of RT medium were added to a PCR master mix (10 mM Tris- HCl (pH 8.3), 100 mM KCl, 0.75 mM EDTA, 5% glycerol) containing 0.2 mM deoxyribonucleoside triphosphates, 5 μl of the primer (Gibco, Belgium), 30 μM of leptin antisense primer and 45 pmol of leptin sense primer, in a total volume of 225 μl. Four aliquots (45 μl) were then transferred into microtubes containing 5 μl of leptin DNA-competitor at four different known concentrations. After 120 s at 94 °C, the PCR mixtures were subjected to 40 cycles of PCR amplification with a cycle profile, including denaturation for 40 s at 95 °C, hybridisation for 40 s at 95 °C, hybridisation for 50 s at 65 °C and elongation for 50 s at 72 °C. To improve analysis, sense primers were 5'-labelled with CY-5 fluorescent dye (Eurogentec, Seraing, Belgium), and the amplified products were separated and analysed in 4% polyacrylamide gel electrophoresis using an ALF-Express DNA sequencer (Pharmacia, Upsala, Sweden) and Fragment Manager software (Pharmacia) [26]. The concentration of leptin mRNA was determined at the competition equivalence point, as previously described [25].

The construction of the leptin competitor DNA molecules, the sequence of the primers for leptin, and the validation of the RT-PCR assays for leptin mRNAs were previously described in detail [24, 25]. The lower limit of the RT-PCR assay was 0.05 amol/μg of tissue total RNA. Several determinations of leptin mRNA in the same adipose tissue sample indicated the high reproducibility of the assay, with a coefficient of variation of less than 10% [24].

Statistical methods

Leptin mRNA values are shown as absolute concentrations in amol/μg of tissue total RNA and as medians. Comparisons were analysed by the non-parametric Mann-Whitney test. Differences were considered statistically significant when the P value was <0.05.

ABBREVIATIONS

Amol = attomole (10-18 moles)
BMI = body mass index
CD = Crohn’s disease
IBD = inflammatory bowel disease
IL = interleukin
P = drug therapy
RT-PCR = reverse transcription-competitive polymerase chain reaction
UC = ulcerative colitis

Results

As shown in figure 1, the RT-competitive PCR used in this study was able to detect leptin mRNA in the mesentery of all IBD and control patients. In central mesenteric fat, leptin transcripts were expressed at remarkably (and constantly) low levels, with no value exceeding 1.3 amol/μg total RNA (figure 2). In patients with CD, levels of leptin mRNAs were significantly increased in mesenteric adipose tissue contiguous to inflamed intestine as compared with controls (median 2.4, n = 5, vs controls, P = 0.001), although the high increases were observed in CD, no statistically significant differences were detected between CD and UC patients. Increased levels were also measured in samples taken close to apparently normal segments in CD (median 4.1 vs controls, P = 0.004) and UC (median 2.05, n = 4, vs controls, P = 0.027). Leptin mRNA concentrations were not significantly different between mesenteric fat specimens contiguous to normal intestine and those contiguous to the inflamed intestine in CD and UC. Finally, no correlation was found between mesenteric leptin mRNA levels and gender, age or BMI in IBD patients (data not shown).

Discussion

This study using a sensitive quantitative RT-PCR approach enabled us to detect and quantify leptin mRNA expression in mesenteric adipose tissue. Although remarkably low levels of leptin mRNA were found in normal mesenterity, a dramatic increase was observed in both CD and UC, with or without intestinal inflammation contiguous to the fat biopsy sample. The quantitative RT-PCR method used in this study was previously validated and used to study leptin mRNA expression in small samples obtained from abdominal subcutaneous and visceral adipose tissues of lean, obese and type II diabetic subjects [24, 27, 28]. As very low levels of leptin mRNA can be quantified with only minimal amounts of sample material, this is the method of choice for investigating ob gene expression in IBD patients when only small tissue samples can be obtained. It was previously determined that the level of leptin mRNA is about twice as low in omental adipose tissue as in subcutaneous abdominal fat [27, 29, 30]. Our results confirm that the expression level of ob gene is very low in mesenteric fat in humans. With the same RT-PCR methodology, the mean value measured in control mesenteric adipose tissues was about 10 times lower than in subcutaneous abdominal fat of healthy lean subjects [24]. This suggests that, if secreted by mesenteric adipose tissue, it is only likely to increase circulating concentrations of leptin very slightly.

Increased leptin mRNA levels were observed in the mesenteric fat of IBD patients. Obesity, which is known to be a factor in IBD overexpression in subcutaneous and omental fats [27], could be excluded since our IBD patients and control subjects were not obese (BMI ranging from 19 to 25 in both CD and UC). The difference of age between patients and controls is unlikely to play a role as plasma leptin levels are not correlated to age [31]. Interestingly, this overexpression of leptin mRNA appeared to be non-specific since similar increases were observed in both CD and UC. Moreover, increased expression of leptin was observed in tissue samples contiguous to or distant from inflamed segments, which suggests that IBD are associated with a general increase in leptin expression in the mesentery. The effects of previous drug therapy could be excluded because the same findings were observed in patients who did not receive drug therapy and those (usually UC patients) who failed to...
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respond to a previous course of steroids and immunosuppressive therapy.

Although controversial results have been published concerning plasma leptin levels in IBD [20-22], the correlation between BMI and plasma leptin is usually preserved, as in other conditions without bowel inflammation [32]. A lack of correlation between leptin mRNA levels and plasma leptin levels has already been reported [33, 34], which tends to support the approach adopted in our study. The repetition of plasma leptin assessment would not appear to be useful in clarifying the discrepancies in the literature. In our study, mesenteric leptin mRNA levels were not correlated with BMI, possibly because the subjects were all lean. This may also reinforce the notion that leptin produced by mesenteric fat does not increase the plasma level of the hormone but rather contributes to a local paracrine effect in the intestine.

What pathophysiological relevance do our findings have? Overexpression of leptin in mesenteric fat must be interpreted in the context of other abnormalities of mesenteric tissue recently reported in both animal models and humans. Desreumaux et al. [14] found high levels of TNFα in the mesentery of CD patients. Similarly, we observed increased TNFα expression in the mesentery of indomethacin- and TNBS-induced ileitis in rats (unpublished data). This increase in mesenteric TNFα could lead to higher leptin expression in CD (but not in UC), as suggested by many studies [35-40]. Finally, given the interactions between leptin and the immune system, it would appear that leptin produced by mesenteric fat does not increase the plasma level of the hormone but rather contributes to a local paracrine effect in the intestine.

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