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

Maryse BARBIER (1), Hubert VIDAL (2), Pierre DESREUMAUX (3), Laurent DUBUQUOY (3), Arnaud BOURREILLE (1), Jean-François COLOMBEL (3), Christine CHERBUT (1), Jean-Paul GALMICHE (1)

(1) Pôle Digestif et CIC-INSERM, CHU Nantes et INRA, 44093 Nantes Cedex 1 ; (2) INSERM U449, Faculté de Médecine Laënnec, 63373 Lyon; (3) Laboratoire de Recherche sur les Maladies Inflammatoires Intestinales, INSERM U539, CHU Hôtel-Dieu, 44035 Nantes Cedex 01.

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 TNF-α 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 close to healthy and inflammatory small intestine and/or colon in 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 TNF-α expression in CD (as recently reported), and/or (c) the anorexia frequently reported during flares of IBD.

L eptin (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 neuropeptide 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 Th1 cytokine production, which orchestrates most immune responses. In particular, leptin increases Th1 (IFN-γ and IL-2) and suppresses Th2 (IL-4) cytokine production [11].

In Crohn’s disease (CD), mesenteric adipose tissue displays well-recognised hallmarks, such as thickening, stiffness, hypertrophy and fat-wrapping [12, 13], and has recently been shown to express high levels of TNF-α [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 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 Huriez Hospital in 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.

Twenty-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 colectomy for perforating IBD or for other indications. Seven subjects with carcinoma of the right colon (4 females, 3 males; mean age 75 years old) and seven subjects with carcinoma or UC (5 females, 3 males; mean age 60 years old, BMI 22 kg/m2) without evidence of active disease served as controls. None of these control subjects was obese, but no data on their height were available for BMI calculations.

All 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 mesenteric close to both 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.

Adipose tissue samples were immediately frozen in liquid nitrogen, and total RNA was prepared from the frozen samples. No patients presented with perforating IBD. Twenty-six patients were included in the study. Forty-four with CD (9 females, 5 males; mean age 31 years old, BMI 22 kg/m2) underwent right colectomy for perforating IBD or for other indications. Seven subjects with carcinoma of the right colon (4 females, 3 males; mean age 75 years old) and seven subjects with carcinoma or UC (5 females, 3 males; mean age 60 years old, BMI 22 kg/m2) without evidence of active disease served as controls. None of these control subjects was obese, but no data on their height were available for BMI calculations.

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Adipose tissue samples were immediately frozen in liquid nitrogen and stored at -80°C for subsequent mRNA analysis.

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

RNA preparation

Adipose tissue samples (about 200 mg of frozen tissue) were pulverised in liquid nitrogen, and total RNA was prepared from the frozen samples...
powder using the RNaseasy total RNA kit (Qiagen, Courtaboeuf, France) as previously described [24, 25]. Total RNA solutions were quantified spectrophotometrically at 260 nm. The absorption ratios at 260 to 280 nm were 1.8 to 2.0 for all preparations. Extraction yield was 2.5 μg of total RNA/100 mg of adipose tissue. Total RNA was stored at −80°C for less than 3 weeks before analysis.

**Quantification of leptin mRNA**

Human leptin was quantified by RT-competitive PCR, as previously described [24, 25]. After a specific reverse transcription reaction, target leptin 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 standardising the exponential amplification process. The initial concentration of target cDNA corresponded to the initial concentration of amplified 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 (RNase Hminus M-MLV reverse transcriptase, Promega, Charbonnières, France) in the presence of 10 mM Tris-HCl (pH 8.3), 90 mM KCl, 1 mM MgCl2, 0.2 mM deoxynucleoside triphosphates, and 13 pmol 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 [24]. 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 qPCR.

For qPCR amplification, the 20 μL of RT medium were added to a PCR master mix (10 mM Tris-HCl (pH 8.3), 90 mM KCl, 0.75 mM MgCl2, 0.2 mM deoxyribonucleotide triphosphates, 5 units of Taq polymerase (Gibco, France), 20 pmol 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 60 s at 55°C, and elongation for 50 s at 72°C.

To improve analysis, sense primers were 5′-labelled with CY-5 fluorescent dye (Europaergen, Sarzago, Belgium), and the amplified products were separated and analysed in 4% polyacrylamide gel electrophoresis using an ALF-Express DNA sequencer (Pharmacia, Uppsala, 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-cPCR assay for leptin mRNAs were previously described in detail [24, 25].

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.

**ABREVIATIONS**

A: amol
B: β
C: control
D: disease
F: factor
H: homology
I: inter för
P: patient
Q: quality
R: result
S: sample
T: tissue
U: unit
W: weight
X: unknown
Z: zero

**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 highest values 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 4.15 vs controls, P = 0.004) and UC (median 2.05, n = 4, vs controls, P = 0.073). 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-qPCR 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 mesentery, a dramatic increase was observed in both CD and UC, with or without intestinal inflammation contiguous to the fat biopsy sample.

The quantitative RT-qPCR 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 mRNA can be quantified with only minimal amounts of sample material, this is the method of choice for investigating the role of leptin mRNA 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 the leptin gene is very low in mesenteric fat in humans. With the same RT-qPCR 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 leptin, if secreted by mesenteric adipose tissue, is unlikely 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 leptin 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 likely 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 effect 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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Fig. 1 – Typical analysis of RT-cPCR products in 4% polyacrylamide gel electrophoresis using an Alf-Express DNA sequencer. a) Each line corresponds to a competitive amplification with a known amount of a specific leptin DNA competitor. The amounts of competitor (in amol) are indicated. After separation, the peaks corresponding to the leptin competitor (Cob, 407 bp) and the leptin cDNA (ob, 487 bp) were integrated, and the area under the peak ratio between competitor and target was plotted against the initial amount of competitor added in the PCR. b) At the equivalence point (log ratio = 0), the initial amount of leptin cDNA corresponds to the initial amount of leptin competitor added. The initial concentration of leptin mRNA can be calculated once dilution factors are taken into account. Open circles correspond to the quantification of leptin mRNA in mesenteric fat of a control subject (1 amol of total RNA), filled circles to quantification in a sample from a patient with Crohn’s disease (4 amol of total RNA), and the square to quantification in a sample from a patient with ulcerative colitis (9.4 amol of total RNA). The analysis on the DNA sequencer in the panel (a) corresponds to the filled circles indicated in the panel (b).

Fig. 2 – Individual values and medians (horizontal bars) of leptin mRNA level (amol/mg total RNA) in mesenteric tissues of 7 controls, 12 patients with Crohn’s disease contiguous to healthy intestine (CDh), 6 patients with Crohn’s disease contiguous to inflamed intestine (CDi), 4 patients with ulcerative colitis contiguous to inflamed intestine (UCi), 3 patients with ulcerative colitis contiguous to healthy intestine (UCh) and 5 patients with ulcerative colitis contiguous to inflamed intestine (UCi): P < 0.05, significant difference between IBD patients and controls.
In summary, this study is the first report of high mRNA levels of leptin in the mesenteries of patients with CD and UC. Further studies are required to determine whether this biological abnormality contributes to the inflammatory process by releasing other cytokines (or enhancing their effect) or is a factor in metabolic disturbances and denutrition.

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