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 Laennec, 63373 Lyon ; (3) Laboratoire de Recherche sur les Maladies Inflammatoires Intestinales, INSERM-EPI 114, CHU Huriez, 59037 Lille.

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 close to healthy and inflammatory small intestine and/or colon in patients with CD or UC and, for controls, from apparently healthy mesentry 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 patients with CD or ulcerative colitis (UC).

Conclusions — This study provides the first evidence of a novel abnormality of the mesentry 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.

OVEREXPRESSION OF LEPTIN mRNA IN MESENTERIC ADIPOSE TISSUE IN INFLAMMATORY BOWEL DISEASES

RÉSUMÉ

Surexpression de l’ARNm de la leptine dans le tissu adipeux mésentérique des malades atteints d’une maladie inflammatoire chronique de l’intestin

Maryse BARBIER, Hubert VIDAL, Pierre DESREUMAUX, Laurent DUBUQUOY, Arnaud BOURREILLE, Jean-François COLOMBel, Christine CHERbut, Jean-Paul GALMICHE

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Rationnel/objectifs — La leptine est une protéine produite de façon prédominante par les adipocytes et dont la structure est voisine d’une cytokine. Elle joue un rôle important dans les réponses immunitaires, en augmentant la sécrétion des cytokines pro-inflammatoires de type Th1. L’hypertrophie du tissu adipeux mésentérique est assez caractéristique de la maladie de Crohn, et des taux élevés de leptine ont été observés dans des modèles animaux d’inflammation intestinale. Le but de cette étude était de préciser si le tissu adipeux mésentérique pouvait être une source de leptine chez l’homme dans les maladies inflammatoires chroniques de l’intestin.

Méthodes — Une analyse quantitative de l’expression de l’ARNm de la leptine a été effectuée chez des sujets atteints de maladie de Crohn ou de rectocolite hémorragique. Des prélèvements de tissu adipeux mésentérique étaient obtenus, soit au voisinage d’une zone saine, soit au voisinage d’une zone inflammatoire, dans l’intestin grêle et/ou dans le côlon. Des prélèvements témoins ont également été effectués dans des mesentères apparentement sains de malades opérés pour un cancer du côlon droit. L’expression de la leptine a été quantifiée par RT-PCR compétitive.

Résultats — Les niveaux d’expression de l’ARNm de la leptine étaient significativement plus élevés dans le tissu adipeux mésentérique des sujets atteints de maladie de Crohn et de rectocolite hémorragique que chez les témoins (P < 0,05). La concentration n’était pas significativement différente dans les prélèvements voisins de zones macroscopiquement saines et inflammatoires.

Conclusion — Ces résultats montrent pour la première fois l’existence d’une anomalie de l’expression de la leptine dans le mésentère de sujets atteints de maladie inflammatoire chronique de l’intestin. Cette anomalie pourrait contribuer au processus inflammatoire en augmentant notamment la production de TNFα et en contribuant à l’anorexie fréquemment observée au cours des poussées évolutives de ces maladies.

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 neuronal 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
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 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 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 ileocolonic resection because of symptomatic ileal stenosis with transmural inflammation. They had not previously received 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.

Seven patients with CD (3 females, 4 males; mean age 30 years old; BMI 22 kg/m²) underwent ileocolonic resection of a short segment of the right colon due to symptomatic obstruction. None of these patients had a history of UC. None of these control subjects was obese and no data on their height were available for BMI calculations. The construction of the leptin competitor DNA molecule, the sequence of which has been described [24, 25]. After a specific reverse-transcription reaction, target leptin cDNA was co-amplified in competition with a known amount 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 added competitor when the PCR product ratio was equal to 1.

#### 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 powder using the RNasy 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 between 1.8 and 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 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 (RNase H polymerase, Promega, Charbonnières, France) in the presence of 10 mM Tris-HCl (pH 8.3), 90 mM KCl, 1 mM MgCl₂, 0.2 mM deoxyribonucleotide triphosphates and 1.5 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 cPCR.

For cPCR amplification, the 20 µL of RT medium were added to a PCR master mix (10 mM Tris-HCl (pH 8.3), 90 mM KCl, 1 mM MgCl₂, 0.2 mM deoxyribonucleotide triphosphates and 1.5 pmol of leptin antisense primer, in a final volume of 225 µL). Four aliquots (45 µL) were then transfected 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 (Eurogentec, Seraing, 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 molecule, the sequence of the primers for leptin, and the validation of the RT-cPCR assays for leptin mRNA were previously described in detail [24, 25]. The lower limit of the RT-cPCR 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].

### 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 control mesenteric fat, leptin transcripts were quantified spectrophotometrically using a 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 2.6 vs 0.81, P = 0.022). In
patients with UC, similar significant increases were detected in mesenteric adipose tissue close to inflamed intestine (median 2.4, n = 5, vs controls, P < 0.01). 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-cPCR 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-cPCR 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 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-cPCR 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 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 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 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 is 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 respond to a previous course of steroids and immunosuppressive therapy.
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**Fig. 2** – Individual values and medians (horizontal bars) of leptin mRNA level (amol/µg 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 healthy intestine (UCd) and patients with ulcerative colitis contiguous to inflamed intestine (UCi): *P*<0.05, significant difference between IBD patients and controls. 

Values individuelles et médianes (barres horizontales) des concentrations d’ARNm de la leptine (amol/µg d’ARN total) dans le tissu mésentérique de sujets témoins (contrôles) et de malades atteints de maladie de Crohn (CD) et de sujets atteints de rectocolite hémorragique (UC). Si les tests ont été réalisés, soit en zone saine (CDd ou UCh), soit en zone inflammatoire (CDi ou UCI). La présence d’un astérisque indique une valeur significative (*P* ≤ 0.05) entre les malades et les témoins.

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 overexpression in the mesentery of IBD patients may contribute to the inflammatory process. In fact, leptin itself may be a factor in increased TNFα secretion, as indicated in vitro studies [9, 10]. Moreover, a dysregulation of the balance between Th1 and Th2 cytokines is usually considered as one of the primary events in IBD. *In vitro*, leptin increases Th1 (IFN-γ and IL-2) and suppresses Th2 (IL-4) cytokine production [11]. In view of the known regulatory effects of these cytokines, it would appear that leptin may help amplify selected proinflammatory responses. On the basis of these data, it seems likely that overexpression of leptin in the mesenteric fat of CD patients could contribute to overexpression of TNFα in this tissue. Finally, in another study carried out in our laboratory [41], inhibition of leptin secretion during the early stages of experimental intestinal inflammation in rats attenuated colonic inflammation dramatically, which suggests that leptin plays an active pro-inflammatory role in the development of acute intestinal inflammation in rats. This has been recently confirmed by a reduced disease severity in acute and chronic colitis induced in leptin-deficient ob/ob mice, accompanied by a reduced stimulation of intraepithelial lymphocytes and lamina propria mononuclear cells [42].

In summary, this study is the first report of high mRNA levels of leptin in the mesentery 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.

**REFERENCES**


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