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REVIEW

DEVELOPMENT OF SUPERACTIVE LEPTIN ANTAGONISTS AND THEIR POTENTIAL USE IN RESEARCH AND MEDICINE

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Abstract

Superactive leptin antagonists (D23L/L39A/D40A/F41A), mutants of mouse, human, rat or ovine leptons, were developed in our laboratory, expressed in *E. coli,* refolded and purified to homogeneity as monomeric proteins. Pegylation of leptin antagonists resulted in a potent and effective long-acting reagents suitable for *in vivo* studies. In the present review we summarize the possible use of leptin antagonists as (*i*) research reagents in the study of bone development, autoimmune diseases, metabolic syndrome and type 2 diabetes mellitus (T2DM) with particular emphasis on creation of a novel, fast and reversible model of metabolic syndrome and T2DM in mice, and (*ii*) possible leptin blockers in various human pathologies such as uremic cachexia, anti-inflammatory and anti-autoimmune diseases, and cancer. In conclusion, the recognition and mutagenesis of D23L of previously developed leptin antagonists (L39A/D40A/F41A) enabled construction of novel compounds that induce potent and reversible central and peripheral leptin deficiency.

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Key words: leptin, antagonist, inflammatory disease, anti-autoimmune diseases, cancer, uremic cachexia, metabolic syndrome, type 2 diabetes

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Development of potent leptin antagonists

Though leptin and leptin receptor (LEPR) were cloned almost 20 years ago (1,2) and leptin's 3D structure has been resolved (3), the mechanism of leptin:LEPR interaction and activation has not yet been elucidated. Nevertheless, several putative models have been proposed (4). The extracellular domain (ECD) of LEPR, as depicted in Figure 1, consists of several subdomains termed (from the N-terminus): cytokine homology region 1 (CHR 1), followed by an immunoglobulin-like domain (IGD), then by another CHR 2 and finally by two consecutive F3 domains. Fong and co-workers (5) localized the leptin-binding domain to the membrane-proximal CHR 2 (~ 200 amino acids) in the LEPR-ECD. This domain from human (6) and chicken LEPRs (7) was subcloned in our laboratory and expressed as a recombinant protein which showed a 1:1 molar interaction with leptin. Over 10 years ago, Tavernier and his group (8) revealed that leptin binding to its receptor resembles the interaction between interleukin 6 (IL-6) and IL-6 receptor (9-11), and they formulated the existence of

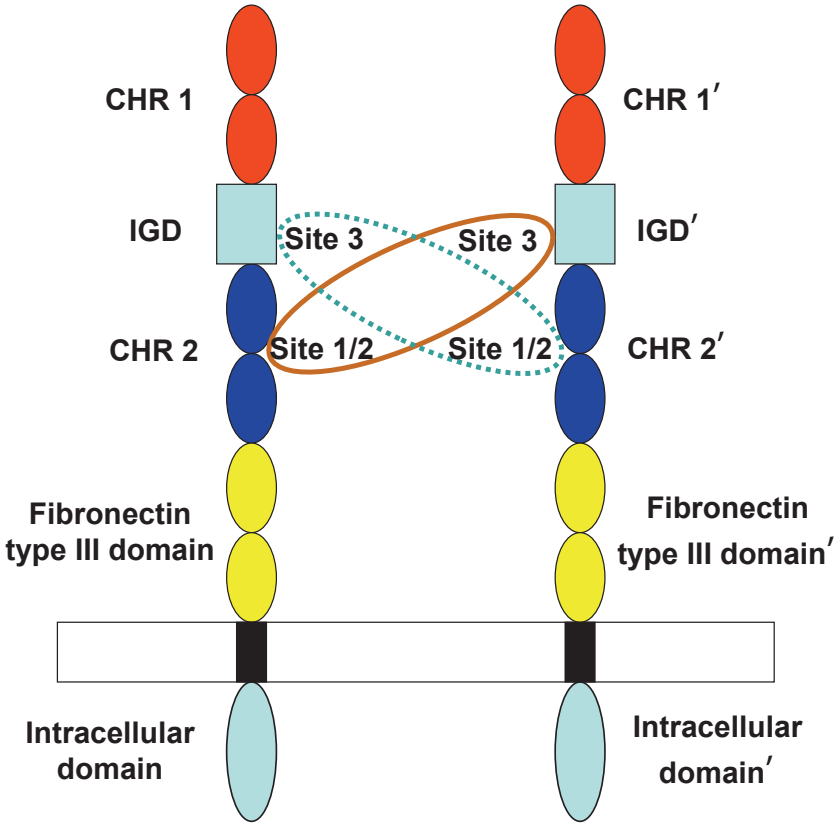


Figure 1. Schematic illustration of the interaction of two leptin molecules with the extracellular domains of two leptin receptors. The first leptin molecule (designed as a brown-solid line oval) interacts through its 1/2 binding site with the cytokine homology domain 2 (CHR 2) of the left receptor and subsequently through its binding site 3 with the immunoglobulin-like domain 2′ (IGD′) of the second receptor. In parallel the second leptin molecule (designed as a green-pointed line oval) interacts through its 1/2 binding site with the CHR 2′ of the right receptor and subsequently through its binding site 3 with the (IGD) of the left receptor. There is no known interaction between the two leptins. Mutations L39A/D40A/F41A abolish the interaction of leptins with the IGDs, preventing receptor activation but not the binding to CHR2 domains and just acts as antagonist. Mutation D23L increases the affinity of leptin (or leptin antagonist) toward CHR-2.

the putative leptin site III as a major site responsible for the formation of active 2:2 or 2:4 leptin:LEPR complex. The IGD located between the distal and proximal CK-F3 domains was clearly documented to be essential for productive dimerization (or even tetramerization) of the LEPR, as its removal attenuated activation, but not binding, of the ligand (8). Schematic illustration of 2:2 leptin:LEPR complex showing interaction of two leptin molecules, each with the CHR 1 of one receptor (through binding site 1/2) and with the IGD of the second receptor (through binding site 3) is presented in Figure 1. This model was further substantiated by the extensive mutagenesis of mouse and human leptins which led to the identification of Ser 120 and Thr 121, located in the N-terminal part of helix D, as contributors to site III (12). Mutation of these amino acids to Ala led to the formation of leptin antagonist. In addition to the N-terminal part of helix D, we have employed a sensitive bidimensional Hydrophobic Cluster Analysis (13) and by comparison to known structures of IL-6-receptor complexes (viral (v) IL-6/gp130) (9) and IL-6/IL-6Rα/gp130 complex (10) in which site III was first identified, we have identified amino acids L39/D40/F41/I42 in the A-B loop in leptin to be responsible for interaction with the receptor that leads to functional dimerization or tetramerization required for receptor activation. To verify this hypothesis and to test its generality, we prepared and purified to homogeneity several ovine and human recombinant leptin Ala muteins in the A-B loop (L39A/D40A, L39A/D40A/F41A or L39A/D40A/F41A/I41A) and documented their activity as potent competitive LEPR antagonists (14). To verify the

preservation and importance of this sequence for activation of LEPRs, we also prepared the corresponding muteins of mouse and rat leptin and documented their antagonistic activity (15). In a subsequent work, we increased the half-life of the leptin antagonist by pegylation, resulting in significant extension of its bioavailability while retaining antagonistic activity (16). In mice, administration of the pegylated antagonist produced rapid and dramatic weight gain, due to enhanced appetite and increased food consumption. The resulting fat was confined to the mesenteric region with no accumulation in the liver. Remarkably, weight changes were reversible upon cessation of leptin-antagonist treatment. The mechanism of severe central leptin deficiency was found to involve inhibition of leptin transport across the blood-brain barrier, as well as accumulation of pegylated antagonist within the central nervous system and direct inhibition of LEPRs in the hypothalamic region.

In view of the potential pharmaceutical uses of leptin antagonists, the general question of how the biopotency of recombinant proteins can be enhanced *in vivo* needs to be explored. One possible approach is to increase the antagonist's affinity for the receptor by increasing k_{on}, or mainly by decreasing k_{off}, and thus prolonging receptor occupation. Theoretical thermodynamic considerations show that if antagonists and agonists exhibit the same affinity, at a 100-fold molar excess of antagonist, 99% of all occupied receptors will be occupied by antagonists. A 100-fold increase in antagonist affinity will lead to similar results at an approximate 1:1 molar antagonist:agonist ratio. Pegylation of such muteins combines both approaches, resulting in a potent and effective long-acting competitive antagonist *in vivo*. To explore this approach, we employed random mutagenesis of mouse leptin followed by selection of high-affinity mutants by yeast-surface display, and discovered that replacing residue D23 with a non-negatively charged amino acid leads to dramatically enhanced affinity of leptin for LEPR. Rational mutagenesis of D23 revealed the D23L substitution to be the most effective. Coupling the D23 mutation with Ala mutagenesis of three amino acids (L39A/D40A/F41A) previously reported to convert leptin into antagonist (14, 15) resulted in potent antagonistic activity (17). Those novel superactive mouse and human leptin antagonists (D23L/L39A/D40A/F41A) termed, respectively, SMLA and SHLA, exhibited over 60-fold increased binding to LEPR and 14-fold higher antagonistic activity *in vitro* relative to the L39A/D40A/F41A mutants. To prolong and enhance the *in vivo* activity, SMLA and SHLA were monopegylated mainly at the N terminus. Administration of the pegylated SMLA to mice resulted in a remarkably rapid, significant and reversible 27-fold more potent increase in body weight as compared to pegylated MLA (Table 1). To test whether the D23L mutations confer increased affinity in other leptin species, we have recently prepared D23L/L39A/D40A/F41A muteins of ovine (18) and rat leptins (G. Solomon and A. Gertler, unpublished results) and found that they also act as potent leptin antagonists.

Table 1. Effect of PEG-MLA and PEG-SMLA on weight gain in male mice determined one and two weeks after initiation of the injections. Each material was injected daily at 20, 6.7, 2.2 and 0.72 mg/kg, n = 8. The results are presented as % of body mass change (mean ± SEM). The initial body weight was 20 to 22 g.

Material injected	mg/kg/day	One week	Two weeks
Control	–	0.5 ± 0.1	0.9 ± 0.6
PEG-MLA	0.73	7.8 ± 0.8[a]	11.3 ± 1.3[a]
"	2.2	10.1 ± 0.9[b]	14.7 ± 1.5[ab]
"	6.7	12.0 ± 1.3[b]	16.6 ± 1.8[b]
"	20.0	18.7 ± 2.0[c]	22.8 ± 1.5[c]
PEG-SMLA	0.73	12.7 ± 2.1[b]	22.0 ± 2.1[c]
"	2.2	19.6 ± 1.7[c]	30.2 ± 2.3[d]
"	6.7	27.3 ± 2.1[d]	42.3 ± 1.8[e]
"	20.0	29.0 ± 2.6[d]	44.7 ± 3.4[e]

All groups in the same column not designated with the same letter are significantly different ($P < 0.05$).

Leptin antagonist-induced rapid and reversible mouse model of metabolic syndrome and type 2 diabetes mellitus

Obesity and its major consequence, type 2 diabetes mellitus (T2DM), has become an epidemic, globally. T2DM accounts for 95% of the diabetes worldwide. One limitation to the development of new diabetes treatments has been a lack of effective animal models to use in research. There are no rodent models that recapitulate the pancreatic β-cell lesions of humans with T2DM. Moreover, animal models of obesity require either overfeeding, which is expensive and takes weeks to months to establish, or specific genetic mutations that cause lifelong metabolic dysfunction. Thus the ability to rapidly induce obesity in healthy rat and mouse strains would constitute a major advance in diabetes and obesity research leading to the development of novel therapies. As shown above PEG-SMLA has strong orexigenic properties, and when given to mice every 24 to 48 h leads to remarkable weight gain. So far, over 15 experiments have been performed over the course of 2 to 12 weeks to test the effects of PEG-SMLA on weight gain in mice, achieving a uniform 25 to 45% weight gain in 14 to 21 days. Some representative data from these studies including long term experiment in female mice started at the age of 4 weeks and ended at the age of 4 months are presented in Table 2. The results clearly show that weight gain is accompanied by elevated glucose, cholesterol and triglyceride levels, and an even more dramatic increase in insulin levels and insulin resistance (HOMA-IR). Interestingly, no hepatic damage was observed, even after 12 weeks of treatment, although morphologically the livers of PEG-SMLA-treated animals seem fatty. PEG-SMLA treatment did not affect other blood parameters such as albumin, globulin, creatinine, urea, calcium, potassium, phosphorus or bilirubin (not shown). An additional experiment was carried out to test the effects of PEG-SMLA, and showed abnormal glucose tolerance (by oral glucose tolerance test) after 3 weeks of treatment. This change, along with others such as weight gain, increased fat content, hyperinsulinemia and hypertriglyceridemia, were fully reversible with cessation of PEG-SMLA injections, disappearing within 10 to 14 days (not shown).

Determining insulin and glucose levels and HOMA scores can indicate whether mice are normal (euglycemic with normal insulin levels), insulin-resistant, or have developed T2DM according to the following criteria:

Normal: Glucose and insulin are less than 2.5 SD above control average

Insulin-resistant (metabolic syndrome): Insulin more than 2.5 SD above control average but glucose less than 2.5 SD above

Table 2. Weight gain and selected biochemical and hormonal parameters in 8-week-old male and 4-week-old female mice after long-term 4 or 12 weeks IP administration of PEG-SMLA at 12 (male) or 5 (female) mg/kg daily.

	Males[1]		Females[2]			
	after 4 weeks		after 4 week		after 12 weeks	
	Control	PEG-SMLA	Control	PEG-SMLA	Control	PEG-SMLA
Weight gain (%)	15 ± 0.3	65 ±1.2**	20 ± 1	69 ± 7***	39 ± 4	124 ± 6***
Glucose (mg/dl)	97 ± 6	151 ± 8**	178 ± 15	246 ± 21*	131 ± 8	183 ± 10*
Chol (mg/dl)	133 ± 4	173 ± 10**	96 ± 8	147 ± 9**	105 ± 4	153 ± 4**
TG (mg/dl)	63 ± 3	86 ± 4**	63 ± 3	87 ± 4*	79 ± 5	106 ± 8**
Insulin (ng/ml)	Not tested		0.80 ± 0.1	4.1 ± 0.7**	0.31 ± 0.07	1.84 ± 0.08***
HOMA (arbit U)	Not tested		57 ± 9	380 ± 80***	18 ± 4	146 ± 28***
SGOT (IU/ml)	150 ± 19	165 ± 20	120 ± 23	249 ± 54	187 ± 13	173 ± 16
SGPT (IU/ml)	158 ± 37	136 ± 16	74 ± 41	203 ± 67	129 ± 41	163 ± 45
AP (IU/ml)	122 ± 10	136 ± 18	192 ± 6	166 ± 19	124 ± 5	115 ± 6

[1]Initial weight 20.8 g at 8 weeks of age.
[2]Initial weight 11.8 g at 4 weeks of age.
Values are mean ± SEM, *P < 0.05, **P < 0.01, ***P <0.001; male: 4 h post-fasting, females: not fasted.
Chol, cholesterol, TG, triglycerides, SGOT, serum glutamic oxaloacetic transaminase, SGPT, serum glutamic pyruvic transaminase, AP, alkaline phosphatase.

control average

T2DM: Both insulin and glucose more than 2.5 SD above control average.

Using these criteria, we have found in various experiments that close to 100% of PEG-SMLA-treated animals develop insulin resistance in a time-dependent manner within 3 to 6 weeks of PEG-SMLA treatment and 30-60% exhibit symptoms of border-line T2DM. In view of these results, we conclude that PEG-SMLA treatment leads to the appearance of reversible metabolic syndrome and even T2DM phenotype.

In conclusion, administration of PEG-SMLA or even PEG-MLA to mice causes reversible obesity, hyperglycemia and hyperinsulinemia. These findings raise questions as to whether islet β-cells are specifically affected by a LEPR blockade preventing their full compensation for insulin resistance. A second issue raised by these findings is the role of hepatic glucose production and insulin sensitivity in the rapid development of hyperglycemia. Hypothalamic LEPRs have been implicated in the regulation of hepatic glucose production, and direct effects of leptin on hepatocytes have also been demonstrated. The ability to rapidly and directly affect systemic and central nervous system (CNS) leptin signaling with high potency LEPR antagonists provides a powerful means of addressing the breadth of leptin action.

As PEG-SMLA injection drastically changed several metabolic parameters (Table 2), we decided to determine to what extent those metabolic changes affect or may be related to expression of several key genes in adipose tissue, muscle, liver and brain. Over 40 different genes were tested and the results of all genes whose expression was significantly changed are presented in Table 3. Supplemental Table S1 shows all the tested genes along with their full gene bank assignment. In adipose tissue a remarkable increase in leptin expression was already detected after one month and dramatically elevated after 3 months. It was paralleled by initial 5-fold decrease in expression of leptin receptor that was normalized after 3 months. Similar but lesser decrease in leptin receptor was also observed in muscle. In adipose tissue there was a gradual increase in expression of adiponectin, proinflammatory TNFα, and refeeding induced fat and liver factor and immediate and constant elevation of lipoprotein lipase which is also a ligand/bridging factor for receptor-mediated lipoprotein uptake. It was paralleled by a decrease pyruvate dehydrogenase kinase (which down regulates production of AcCoA) all leading to increased fat synthesis. Expression of PPARγ that regulates fatty acid storage and glucose metabolism was elevated in PEG-SMLA treated mice. Genes activated by PPARγ stimulate lipid uptake and adipogenesis by fat cells. SCD1 which is an iron-containing enzyme that catalyzes a rate-limiting step in the synthesis of unsaturated fatty acids was also elevated in both adipose tissue and liver. It is known that SIRT1 is downregulated in cells that have high insulin resistance and inducing its expression increases insulin sensitivity, suggesting the molecule is associated with improving insulin sensitivity. We found such down regulation in both liver and muscle particularly after 3 months of PEG-SMLA treatment. Liver expression of FGF21 stimulates glucose uptake in adipocytes was not changed after one month of PEG-SMLA treatment but after 3 months was drastically reduced possibly reflecting downregulation of SIRT1 known as its positive regulator. Pyruvate kinase in the liver of PEG-SMLA treated mice was elevated indicating increased glycolysis. This parallels to down regulation of PEPCK, the key enzyme upregulating gluconeogenesis in both liver and muscle, contrary to the situation usually occurring in T2DM where gluconeogenesis in elevated. It was suggested that UCP2-mediated mitochondrial uncoupling and reduced ROS formation in brain during seizures could be neuroprotective (19). It seems that leptin blocking downregulated its brain expression at least after one month of treatment. Apolipoprotein E (apoE) that plays a central role in the brain response to injury was also downregulated after 3-months PEG-SMLA treatment. Interestingly NPY a counter-regulatory peptide of leptin in hypothalamus was elevated after one but not after 3 months of PEG-SMLA treatment.

Effect of leptin antagonists on bone metabolism and function

The putative role of leptin in bone metabolism was first demonstrated in *ob*/*ob* (obese) or *db*/*db* (diabetic) mice which lack leptin or its receptor (20). Both mutant mice have an increased bone formation leading to high bone mass. This phenotype was dominant, independent of the presence of fat, and specific for the absence of leptin signaling. No leptin signaling in osteoblasts was observed but intracerebroventricular infusion of leptin caused bone loss in leptin-deficient and wild-type mice, thus identifying leptin as a potent inhibitor of bone formation acting through the CNS. In sheep, central application of leptin decreased bone formation by 70% and mineralizing surface (MS/BS, from 39.4 to 16.1 %, ($p < 0.01$), indicating that the central regulation of bone formation is not limited to rodents, but is also found in large animals (21). No similar results have been so far reported for humans. In contrast, in leptin deficient child the leptin-treated patient continued to gain height and accrue bone mass in keeping with predicted growth for age. Bone mineral content increased over the same period in keeping with age-related growth. Bone age was advanced at time of diagnosis (8 yr, 10 months at a chronological age of 5 yr), and remained advanced after 48 months of therapy (11 yr at a chronological age of 9 yr); however, the gap between bone age and chronological

Table 3. Gene expression at the end of the first and the third months in adipose, liver, muscle and brain tissues of control and PEG-SMLA-treated mice. The results were quantified by qPCR and normalized to GAPDH mRNA levels. Only the differences that were significantly changed either after one or three months or both are shown[1].

Adipose Tissue

	1st Month				3rd Month			
Gene[2]	Control	PEG–SMLA	%	p<	Control	PEG–SMLA	%	p<
Leptin	1.01	6.43	643	0.01	1.53	23.35	1526	0.01
LepR	1.10	0.23	20.9	0.05	1.09	0.94	86.2	NS
Adiponec	1.01	1.10	110	NS	1.10	2.91	264.5	0.05
TNFα	1.25	1.17	93.6	NS	1.18	5.69	482.2	0.05
UCP2	1.04	2.42	232.6	0.05	1.05	1.21	115.2	NS
LPL	1.05	2.29	218	0.05	1.14	3.78	331.5	0.05
SCD1	1.01	1.28	126.7	NS	1.15	2.62	227.8	0.05
PPARγ	1.01	4.85	480	0.05	1.11	2.17	195.4	0.05
PDK1	1.04	0.77	74	NS	1.23	0.51	41.4	0.05
RIFL	1.08	2.57	238	NS	1.21	2.76	228	0.05

Liver

	1st Month				3rd Month			
Gene	Control	PEG–SMLA	%	p<	Control	PEG–SMLA	%	p<
SCD1	1.04	3.23	310.5	0.05	1.01	4.70	465.3	0.05
SIRT1	1.05	0.64	60.9	NS	1.00	0.78	78	0.05
PEPCK	1.04	0.50	48	0.05	1.02	0.80	78.4	NS
PK	1.00	1.29	129	NS	1.01	1.65	163.3	0.05
FGF21	1.25	1.67	133.6	NS	1.48	0.27	18.2	0.05

Muscle

	1st Month				3rd Month			
Gene[2]	Control	PEG–SMLA	%	p<	Control	PEG–SMLA	%	p<
LR	1.06	0.88	83	NS	1.02	0.67	65.6	0.05
UCP3	1.12	3.48	310.7	0.05	1.05	1.09	103.8	NS
PDK1	1.0	1.12	112	NS	1.01	0.68	67.3	0.05
GLUT1	1.04	1.08	103.8	NS	1.01	0.7	69.3	0.05
PEPCK	1.75	0.73	41.7	NS	1.27	0.23	18.1	0.05
SIRT1	1.02	0.93	91.1	NS	0.93	0.27	29	0.05

Brain

	1st Month				3rd Month			
Gene[2]	Control	PEG–SMLA	%	p<	Control	PEG–SMLA	%	p<
UCP2	1.01	0.58	57.4	0.05	1.04	0.82	78.8	NS
apoE	1.00	1.01	101	NS	1.00	0.76	76	0.05
NPY	1.00	1.64	164	0.05	1.01	0.94	93	NS
SIRT1	1.02	1.35	132.3	NS	1.00	0.63	63	0.05

[1]The full list of all expressed genes and their bank numbers are listed in Supplemental Table 1.
[2]Genes in which no significant difference was found are not listed in this Table.

Supplementary Table 1. List of all genes tested for expression along with gene access number

Full name	Short name	Accession no.
Leptin	Lep	NM_008493
Leptin receptor	LR	NM_146146
Adiponectin	Adipo	NM_009605
Tumor necrosis factor-alpha	TNFα	NM_013693
Inhibitor of nuclear factor kappa-B kinase	IKKβ	AF026524
Uncoupling protein 2	UCP2	NM_011671
Uncoupling protein 3	UCP3	NM_009464
Caspase 3	CASP3	NM_009810
Bcl-2–associated X protein	BAX	NM_007527
Lipoprotein lipase	LPL	NM_008509
Lipase	Lipe	NM_010719
Fatty acid synthase	FASN	NM_007988
Sterol regulatory binding protein 1	SREBF1	NM_011480
Sterol coenzyme A desaturase 1	SCD1	NM_009127
Silent information regulator 2 sirtuin protein	SIRT1	NM_019812
Peroxisome proliferator-activator receptor	PPARγ	NM_001127330
Insulin dependent glucose transport 4	GLUT4	NM_009204
Glucose transport 1	GLUT1	NM_01140
Prohibitin	PHB	NM_008831
Protein disulfide isomerase associated 3	PDIA3	NM_007952
Pyruvate dehydrogenase kinase	PDK1	NM_172665
Phosphofructokinase	PFK	NM_021514
Refeeding induced fat and liver	RIFL	NM_001080940
Transcription factor E2F1	E2F1	NM_007891
Apoptosis signal-regulating kinase 1	ASK1	NM_008580
Apolipoprotein E	apoE	NM_009696
Pro-opiomelanocortin	POMC	NM_008895
Neuropeptide Y	NPY	NM_023456
Agouti related protein	AgRP	NM_007427
Silent information regulator (sirtuin)	SIRT1	NM_019812
Phosphoenolpyruvatecarboxy kinase	PEPCK	NM_011044
Glucose 6 phosphate	G6Pase	NM_008061
Peroxisome proliferator-activator receptor	PPARδ	NM_011145
Insulin receptor	IR	NM_010568
Aldolase A	AldoA	J05517
Pyruvate kinase	PK	NM_013631
Fructose bisphosphatase 1	Fbp1	NM_019395
2,3-bisphosphoglycerate mutase	Bpgm	NM_007563
Phosphoglycerate kinase 1	Pgk	NM_008828
Fibroblast growth factor 21	FGF21	NM_020013
Liver X receptor	LXR	NM_013839
Pyruvate dehydrogenase phosphatase catalytic subunit 2	Pdp2	NM_001024606

age had decreased significantly (22). In contrast others (23, 24) observed that leptin-deficient mice had lower bone mass than normal mice. Studies in *ob/ob* mice have shown stimulatory effect of very high doses of intraperitoneal leptin administration on bone tissue, specifically, a dramatic increase in cortical bone formation (25).

As most of the experiments in mice were carried-out in genetically modified animals we have decided the use the 3-months experiments shown above (Table 2) for testing the effect of blocking leptin on several bone parameters. LV3 and LV4 and tibias were removed after one and 3 months of treatment and analyzed for different bone criteria such as trabecular morphometric analysis of LV3 and LV4, cortical morphometric analysis of tibia and biomechanics 3-point bending test. The results summarized in Table 4 indicated higher bone volume fraction (BV/BT) and increased trabecular thickness (Trb-Th) in LV3 and LV4 of PEG-SMLA treated mice. Visual demonstration of such difference is presented in Figure 2. Cortical morphometric analysis of tibia showed increased cross-sectional and medullary area and increase mean polar moment of inertia without changes in cortical thickness and BMD. Mechanical assay that measures the ultimate load required for fracture, the stiffness and the fracture yield showed significantly higher values in the PEG-SMLA treated mice. Interestingly all the differences appeared already after one month of treatment of 4-weeks old mice. In addition a bone dynamic hystomorphometric analysis was performed by labeling bones on the day 20 of the experiment by injecting Calcein, (15mg/kg) and by injecting Alizarin complexone (10 mg/kg) on day 26. Mean distance between labels/time interval between labels (Fig. 3) indicated that the bone growth in mice injected with PEG-SMLA was (mean ± SEM, n = 5) 0.84 ± 0.05 *versus* control mice 0.63 ± 0.05 ($p < 0.05$). All those results favor the notion indicating that blocking leptin enhances bone formation.

Utilization of leptin antagonists for treatment of uremic cachexia

Most recently, the negative role of leptin in uremic patients has

Table 4. Effect of long-term PEG-SMLA treatment on several bone structural and function parameters (for other details see Table 2).

Parameter tested	Control	PEG-SMLA	Control	PEG-SMLA
	after one month		after three months	
Trabecular morphometric analysis of LV3 and LV4				
LV3-BV/TV (%)	16.5±1.2	21.6± 0.8*	13.8±0.7	17.6±0.4*
LV3-Trb-Th (μ)	0.060±0.001	0.073±0.001*	0.061±0.001	0.072±0.001*
LV4-BV/TV (%)	15.6±1.2	19.7± 0.7*	13.8±0.7	17.2±0.6*
LV4-Trb-Th (μ)	0.070±0.001	0.073±0.001	0.071±0.001	0.073±0.001
Cortical morphometric analysis of tibia				
C.S.Ar (μ^2)	0.57±0.01	0.65±0.01*	0.72±0.03	0.84±0.03*
Med-Ar (μ^2)	0.47±0.01	0.57±0.02*	0.48±0.03	0.57±0.03*
Cor-Th (μ)	0.20±0.002	0.21±0.002	0.22±0.001	0.23±0.003
M-Mlpolar (μ^4)	0.15±0.005	0.22±0.01*	0.24±0.02	0.34±0.02*
BMD (g/cm^3)	1.01±0.03	0.97±0.006	1.06±0.02	0.98±0.008
Biomechanics assay of tibia (3 point bending)				
Fmax (N)	19.2±0.4	21.1± 0.5*	24.3±0.7	27.6±0.8
Stiffness (N/mm)	0.10±0.004	0.12±0.005*	0.10±0.01	0.16±0.01*
AUC (N x mm)	8039±745	10513±758*	9903±0.03	11097±664*
Ffracture (N)	14.7±0.7	15.3± 0.7	18.4±2.5	20.2±1.0
Fyield (N)	14.6±0.4	16.2± 0.4*	16.5±0.7	21.6±0.6*

BV/TV – bone volume/total volume; Trb-Th – trabecular thickness; C.S.Ar – cross-sectional thickness; C.S.Ar – medullary area; Cor-Th - cortical thickness; M-Mlpolar – mean polar moment of inertia; BMD – bone mineral density; Fmax – ultimate maximal load; AUC – area under curve; Ffracture – Fracture load; Fyield – yield load. N – neuton.

* significantly different compared to the respective control ($P < 0.05$).

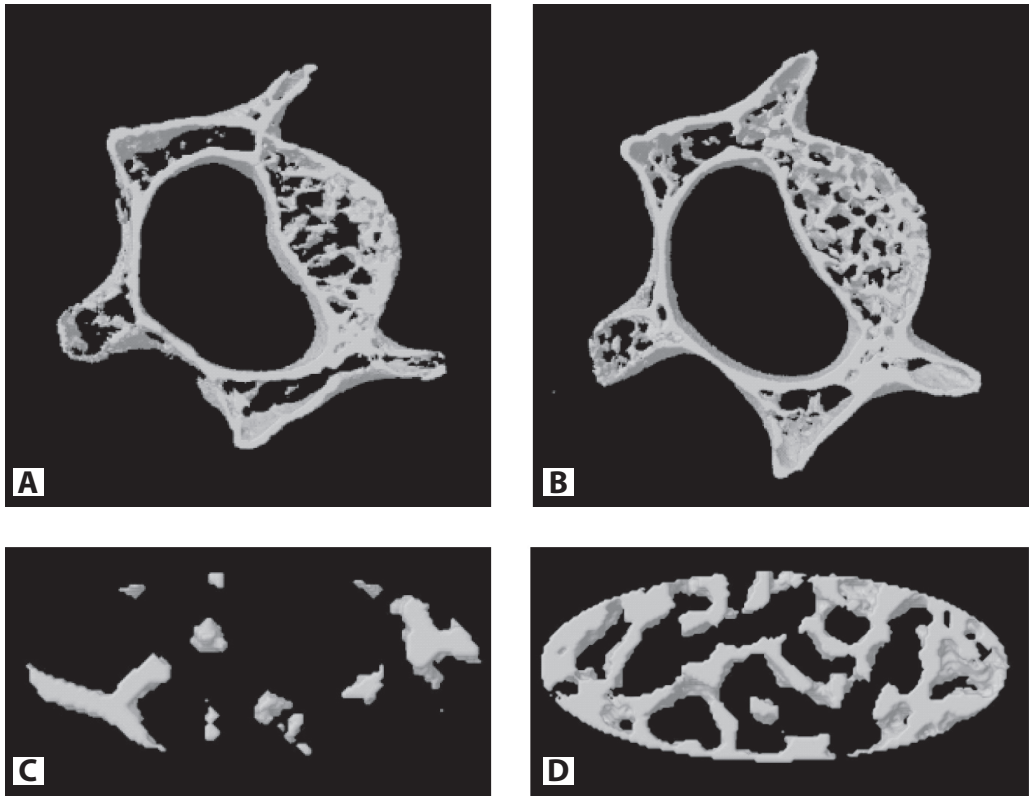
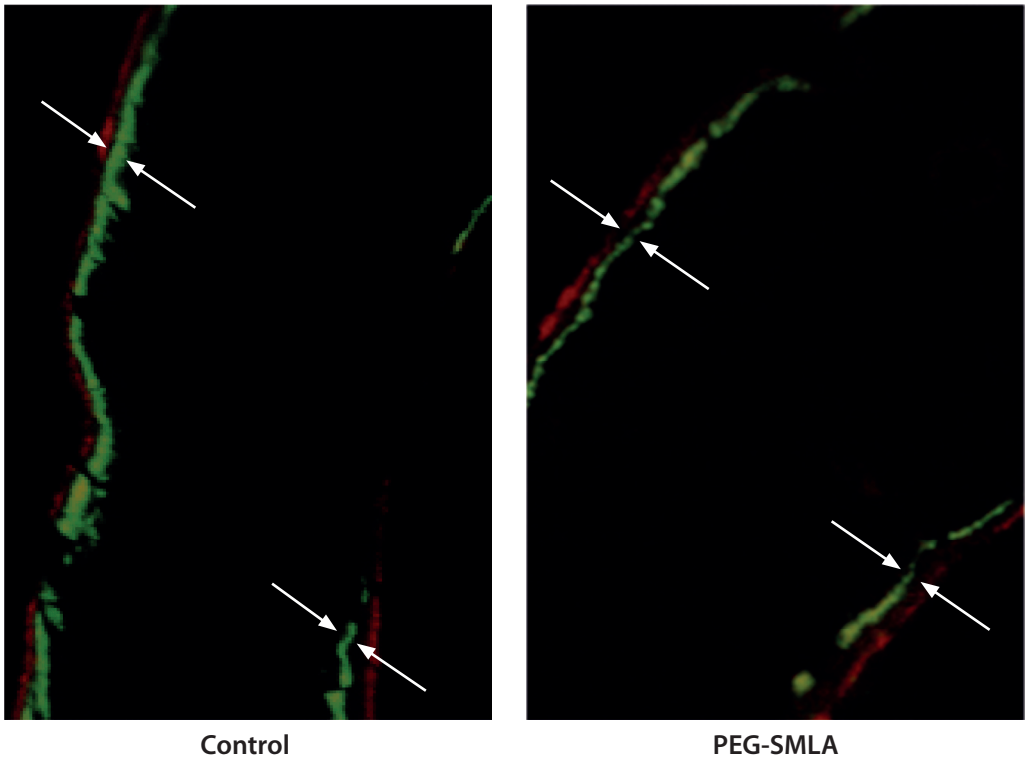


Figure 2. Typical micro-computed tomographic 3D image of trabecular bone Lumber vertebrae 3 (LV3) from the control (A) and PEG-SMLA (B) groups and respective cross-sectional image of trabecular bone Lumber vertebrae 3 (LV3) from the control (C) and PEG-SMLA (D) treated mice.



Control **PEG-SMLA**

Figure 3. Bone Dynamic Histomorphometric analysis of tibia growth was performed by labeling bones on the day 20 of the experiment by injecting Calcein, (15mg/kg) and by injecting Alizarin complexone (10mg/kg) on day 26. Mean distance between labels/time intervals between labels (MAR) was measured. Arrows indicate suitable sites of double labels as used for MAR measurements.

Table 5. PEG-SMLA treatment (28 days) in male mouse (c57BL/6J) model of Cachexia in Chronic Kidney Disease (CKD) [1]

Parameter tested	Sham-operated		Chronic Kidney Disease (CKD)		
	Vehicle None	PEG-SMLA 7 mg/kg/day	Vehicle None	PEG-SMLA 2 mg/kg/day	PEG-SMLA 7 mg/kg/day
n	8	8	12	12	12
Body parameters					
Weight gain (%)	19.2±0.2^{b}	42.7±0.3^{c}	3.0±0.3^{a}	3.7±0.3^{a}	15.1±0.4^{b}
Food intake (g/d)	3.9±0.2^{b}	5.1±0.1^{c}	3.1±0.2^{a}	3.3±0.1^{a}	3.9±0.1^{b}
Fat mass change (%)[2]	8.0±4.2^{c}	234.8±14^{e}	-8.3±0.8^{a}	-4.5±0.5^{b}	24.5±3.0^{d}
Lean mass change (%)[2]	9.9±1.8^{d}	14.8±1.6^{d}	-6.7±0.8^{a}	-3.7±0.4^{b}	2.8±1.0^{c}
BMR (ml/kg/h) x 10^{-3}	3.4±0.08^{b}	3.1±0.04^{a}	3.9±0.06^{c}	3.5±0.10^{b}	3.2±0.08^{a}
Muscle function parameters					
Rotarod at 40 rpm (s)[3]	187±12^{b}	153±13^{ab}	130±3^{a}	140±6^{a}	176±4^{b}
Grip strength (g/100g)[4]	1646±61^{b}	1697±28^{b}	1150±49^{a}	1321±52^{ab}	1568±46^{b}
PAX 3 (expression)[5]	106±6^{c}	98±6^{c}	43±7^{a}	44±6^{a}	59±5^{b}
Atrogin-1 (expression)[5]	92±8^{a}	111±5^{a}	228±7^{d}	178±9^{c}	157±4^{c}
MuRF1 (expression)[5]	107±3^{a}	125±4^{b}	221±6^{e}	177±8^{d} 143±4^{c}	
Pro-inflammatory cytokines expression					
IL-1α (expression)[6]	132±21^{a}	156±24^{a}	468±90^{d}	345±38^{c}	244±20^{b}
IL-1β (expression)[6]	88±12^{a}	97±18^{a}	206±15^{c}	140±19^{b}	99±10^{a}
IL-6 (expression)[6]	78±18^{a}	115±28^{ab}	438±55^{d}	312±31^{c}	187±20^{b}
TNF-α (expression)[6]	99±25^{a}	102±29^{a}	192±17^{c}	175±31^{bc}	133±12^{ab}

[1]CKD was induced by 5/6 nephrectomy in 8-week old male c57BL/6J mice. CKD and sham-operated mice received either PEG-SMLA (7 mg/kg or 2 mg/kg, i.p.) or vehicle, daily. Mice were fed ad libitum for 28 days. All groups in the same row not designated with the same letter are significantly different ($P < 0.05$).

[2]Determined by DXA

[3]The rotarod performance test is a performance test based on a rotating rod with forced motor activity being applied, usually by a rodent. The test measures parameters such as riding time (seconds) or endurance.

[4] Grip strength test ia an easy way to objectively quantify the muscular strength of rodents (mouse and rat), and to assess the effect of drugs, toxins, muscular (i.e. myopathy) and neurodegenerative diseases on muscular degeneration**.**

[5]PAX3 a member of the paired box (PAX) family of transcription factors that was shown to contribute to early striated muscle developmenAtrogin-1 is an E3 ubiquitin ligase that mediates proteolysis events in response to a variety of catabolic states in muscle; RING-finger protein-1 (MuRF1) is an ubiquitin ligase that has been proposed to trigger muscle protein degradation during pathophysiological muscle wasting. The expression was measured in muscle and normalized to expression of GAPDH (= 100).

[6]The expression was measured in liver and normalized to expression of GAPDH (= 100).

been reviewed (26). Leptin has been defined as a true uremic toxin, and reducing leptin levels in uremic patients, particularly those suffering from uremia-related cachexia, may have potential beneficial effects. This suggestion is based on the finding that plasma leptin is associated with reduced energy intake and protein-wasting in uremic patients (27). In a murine model of uremic cachexia, application of pegylated superactive leptin antagonist (Table 5) prevented lean and fat mass loss in chronic kidney disease (CKD) mice, reversed anorexia, normalized basal metabolic rate and muscle function. PEG-SMLA inhibited also protein degradation pathway genes and pro-inflammatory cytokines in CKD mice. Those results implicate that blocking leptin may be a suitable strategy for treatment of CKD patients.

Leptin antagonists as anticancer agents

The epidemiological evidence linking obesity and breast, cervical, colon, rectal, esophageal, gall bladder, kidney, liver, ovarian, pancreatic, prostate, stomach and uterine cancers is well-established (28-33); the high level of leptin in obese subjects thus raises the notion of a possible link between leptin and cancer. Leptin is an anti-apoptotic molecule in many cell types and its possible role in obesity-linked cancers originates from its known pro-angiogenic, pro-inflammatory and mitogenic activities. Furthermore, leptin was defined as a growth factor not only because of its proliferative activity but also due to its effect on cell motility and migration (34). Leptin is overexpressed in at least 80% of cases of human skin melanoma (35), breast (36), ovarian (37) and prostate (38) cancers. In most of those cases, LEPR is overexpressed as well (39). Interestingly, cancer stem cells, and particularly breast cancer stem cells, also overexpress LEPR, thus sensitizing those cells to leptin action (40). In one such cell type, termed triple-negative breast cancer cell, leptin attenuated the inhibitory effect of cisplatin on cell proliferation and viability (41).

Leptin-induced modulation of colorectal cancer (CRC) has been reported in several *in vitro* and *in vivo* studies (reviewed in 48). Leptin's stimulation of proliferation and inhibition of apoptosis have been shown in several human epithelial colon cancer cells, such as HT-29, CACO-2, DDL-1, SW480, HCT116, LS174-T and Lovo (42-44). However the *in vivo* role of leptin in CRC remains unclear. In spontaneous CRC models, such as in ApcMin/+ mice, leptin did not increase and even decreased intestinal tumorigenesis in ApcMin/+ mice, a mutation that predisposes the animal to tumor development in the intestine and colon (43, 45). In contrast, inflammation-induced CRC induced by Dextrane Sodium Sulphate (DSS)-axoxymethane was attenuated in *ob/ob* and *db/db* mice as compared to wild-type mice (46). Note that leptin's effect may be indirect as leptin is known to induce inflammatory cytokines in colonic tissue that are implicated in colon carcinogenesis, such as IL6, IL1β and CXC (47). Likewise the impact of leptin on colon cancer in humans remains unclear. While some reports show increased leptin expression with progressing tumorigenesis, other have failed to confirm such observations (48). However, it should be noted that serum leptin levels may not be indicative of local leptin levels. Putatively, blocking localized leptin secretion by leptin antagonists endocscopically applied to colon cancer cells may be considered as a possible future treatment option.

The role of leptin in hepatocellular carcinoma (HCC), the third leading cause of cancer death in the world, is also not clear. Patients with cirrhosis resulting from hepatitis B or C infection were found to exhibit increased leptin levels which were suggested to represent a negative prognostic factor (49), but again, studies have reported contradictory findings (50). Another major factor leading to HCC is non-alcoholic fatty liver disease (NAFLD) ranging from simple steatosis to non-alcoholic steatohepatitis (NASH). In an animal model of NAFLD, leptin was suggested to contribute to insulin resistance and steatosis, and leptin injections increased the expression of procollagen-I, TGF-β1 and smooth muscle actin (all markers for HCC), and led to increased liver fibrosis (51). In another study without leptin signaling, neither fibrosis nor HCC developed in the rat NASH model (52). However, contradictory to other reports exogenous leptin significantly decreased tumor size and increased survival rate in an HCC mouse model (53). In human studies, leptin levels were significantly higher in NASH patients and correlated with severity of hepatic steatosis (54), but in another study no differences were found (55). Furthermore, high leptin expression correlated with better survival in HCC patients (56).

More recently, attention has been given to the role of leptin in glioblastoma and is reviewed elsewhere (34). Though extensive *in vivo* studies are still lacking and the information linking leptin to glioblastoma stems mainly from studies with glioma cell line C6 (57,58), the authors strongly suggest leptin's role not only in cell proliferation and inhibition of apoptosis but also in leptin-enhaced cell migration (59). Moreover, indirect leptin actions such as promotion of angiogenesis and augmentation of VEGF levels may also play an important role in leptin promotion of glioblastoma and other cancers (60-63).

One of the most interesting findings connecting leptin to susceptibility to cancer in mouse models of melanoma and colon cancer, related to environmental enrichment, showed that mice kept in an enriched environment express higher levels of brain-derived neurotrophic factor (BDNF), which in turn activates sympathetic nerve fiber innervation of white adipose

tissue, resulting in decreased leptin secretion, cancer inhibition and remission (64). Thus, although the role of leptin in tumor promotion is controversial and may depend on tumor type, at least in those cases in which leptin plays a negative role, leptin antagonists may be potentially used as both important research tools and potential therapeutic modalities.

Leptin antagonists as potential anti-inflammatory and anti-autoimmune disease treatments

For years, white adipose tissue has been regarded as an inert organ, with the exclusive function of long-term energy storage. With the cloning of leptin in 1994, that notion has changed drastically (1). In subsequent years, a plethora of tissue-specific and nonspecific adipose-secreted factors were discovered and characterized and found to play fundamental roles in multiple aspects of cardiometabolic processes. Moreover, white adipose tissue has been found to dynamically integrate metabolic and immune signals through interactions between adipocytes and a complex network of innate and adaptive immune cells, including neutrophils, mast cells, and mononuclear T and B cell subsets. Through this complex interaction it was suggested that immune components fundamentally affect metabolic processes such as insulin sensitivity, and conversely, adipocyte-secreted factors affect the innate and adaptive immune response through modulation of tissue-resident cells of hematopoietic origin (65, 66).

Leptin, the prototypical adipokine, has been most widely studied for its effects on the immune response. Leptin secretion is inducible upon signaling from inflammatory mediators such as tumor necrosis factor-alpha (TNF-α), IL1, and IL6. Indeed leptin levels have been suggested to increase during infection and chronic inflammation (67). Like leptin itself, LEPRs resemble cytokine receptors (68) and the long functional isoform of LEPR is found on T-cell subsets, monocytes (69), natural-killer (NK) lymphocytes (70), dendritic cells (71), hepatic stellate cells (72) and a variety of bone marrow progenitor cells. Functionally, leptin signaling promotes macrophage proliferation, phagocytosis and cytokine secretion (73), NK cell development, activation and survival (74), MAPK-mediated neutrophil chemotaxis (75), T-cell proliferation and IL2 secretion, especially when co-administered with classical T-cell mitogens (76). Leptin has also been suggested to suppress regulatory T-cell proliferation and inflammation suppressive functions, further contributing to a general pro-inflammatory effect (77).

In vivo, leptin-deficient *ob/ob* mice are extremely vulnerable to the development of systemic infection and lipopolysaccharide (LPS)-induced organ damage, and resistant to several Th1 cells-mediated immune disorders, including experimental allergic encephalomyelitis, concanavalin A hepatitis, experimental arthritis, and autoimmune nephritis (78-80). Leptin replenishment reverses these disorders (81). These mice feature reduced cell density in their bone marrow and thymus, highlighting the importance of leptin signaling in the development of various subsets of the hematopoietic system. Similarly, humans with inherited or starvation-induced leptin deficiency feature thymic atrophy and severe functional alterations in the innate and adaptive immune response, rendering them susceptible to bacterial infection (82, 83).

In humans, the causative correlation between leptin levels and the propensity and severity of autoimmune disease remains controversial. Perhaps the strongest association between leptin levels and autoimmunity has been found in multiple sclerosis (MS), a T-cell-mediated autoimmune disorder of the CNS; *ob/ob* mice are protected from EAE, the most common small animal model of MS, possibly through leptin-deficiency-induced expansion of nTregs cells (84,85). In humans, leptin levels have been suggested to correlate with disease activity and to decrease following successful treatment with interferon beta (INF-β) (86). In other systemic autoimmune disorders, such as rheumatoid arthritis and systemic lupus erythematosus, leptin levels have been suggested in some, but not all, reports to be elevated independently of gender or weight and to possibly correlate with disease severity (87-92). Interestingly, one study suggested that acute fasting promotes improvement in multiple immune parameters in rheumatoid arthritis patients, an effect that may be linked to the reduced leptin levels in these patients (93). Similarly, leptin was suggested to contribute to the pathogenesis of diabetes mellitus in mice, while a mutation in the LEPR of mice with a NOD background ameliorated diabetes severity in these mice (94,95). Yet another example of leptin's effect on the immune response was recently demonstrated by our groups (96), whereby administration of competitive leptin antagonists induced significant amelioration in a model of chronic liver inflammation and fibrosis.

A role for leptin was suggested in the pathogenesis of intestinal autoinflammation. Inflammatory bowel disease (IBD) is a chronic inflammatory disorder affecting 0.3% of the Western population; its pathogenesis is thought to result from loss of tolerance of the intestinal immune system in the presence of constant antigenic stimuli mediated by resident microflora. In recent years, mounting evidence has suggested that IBD pathogenesis is closely related to a combination of abnormal challenges by normal gut microflora, coupled with inherent or acquired aberrations in the intestinal innate immune response. Leptin's central role as a mediator of colonic autoinflammation has been suggested in both animal models and human studies. Leptin-deficient *ob/ob* mice are resistant to acute and chronic experi-

mental colitis (97,98). In those studies, leptin was suggested to augment pro-inflammatory cytokine secretion, including IFN-g, TNF-α, IL-18, IL-1β, and IL-6, and to inhibit inflammation-associated apoptosis of colonic mononuclear cells. Inflamed colonic epithelial cells were also found to express and release leptin apically into the intestinal lumen. Intrarectal administration of leptin induced activation of NF-κB and epithelial wall damage associated with neutrophil infiltration (99). The source of local and/or systemic leptin has not been elucidated, but it has been suggested not to be *lamina propria* T cells themselves. Similarly, the leptin-responsive cells have not yet been identified. Collateral evidence for a possible pro-inflammatory interaction between leptin and *lamina propria* dendritic cells (lpDCs) comes from studies showing that LEPRb is expressed on immature and mature human DCs, and that leptin induces STAT3 and NF-κB activation, production of IL-1, IL-6, IL-12, TNF-α and MIP-1α, an anti-apoptotic effect through Bcl-2 and Bcl-XL, downregulation of IL-10, polarization into Th-1, and increased CD40 expression (100,101). In human IBD patients, leptin levels have been suggested to correlate with the severity of the disease (102-104).

Taken together, a large body of evidence suggests an association between leptin levels and responsiveness in terms of propensity for autoimmunity, autoinflammation and infection. While much of the current data, particularly in humans, is observational and associative, they suggest that leptin may play direct or indirect roles in the modulation and regulation of innate and adoptive immune responses and that excessive leptin signaling might result in a deleterious tendency toward autoinflammation in susceptible populations. As such, inhibition of leptin signaling, as we have recently suggested, may offer a unique therapeutic modality targeting leptin signaling in the autoinflammatory setup.

Leptin antagonists in other pathologies

The role of leptin signaling in myocardial hypertrophy, heart diseases associated with metabolic syndrome, endothelial dysfunction, arterial hypertension, and neurodegenerative diseases (e.g., Alzheimer's and Parkinson's) are beyond the scope of the present review. However, in all these conditions, leptin's negative effects can be potentially antagonized by leptin antagonists.

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