A role for leptin and its cognate receptor in hematopoiesis

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Background: Hematopoiesis entails the production of multiple blood cell lineages throughout the lifespan of the organism. This is accomplished by the regulated expansion and differentiation of hematopoietic precursors that originate from self-renewing hematopoietic stem cells. Studies of lineage commitment and proliferation have shown that the cytokine family of growth factors plays an important role in hematopoietic differentiation. However, in hematopoiesis, as in most self-renewing biological systems, the molecules that regulate the stem cells directly remain largely unknown. In this study, we have undertaken a search for novel cytokines that may influence the fate of hematopoietic stem cells.

Results: We have cloned three splice variants of a novel cytokine receptor from human hematopoietic stem cells expressing the CD34 antigen, one of which is identical to the leptin receptor. Expression analysis revealed that the leptin receptor is expressed in both human and murine hematopoietic stem cell populations, and that leptin is expressed by hematopoietic stroma. We show that leptin provides a proliferative signal in hematopoietic cells. Importantly, we demonstrate that leptin provides a proliferative signal in BAF-3 cells and increases the proliferation of hematopoietic stem cell populations. The proliferative effects of leptin seem to be at the level of a multilineage progenitor, as shown by increased myelopoiesis, erythropoiesis and lymphopoiesis. Analysis of *db/db* mice, in which the leptin receptor is truncated, revealed that the steady-state levels of peripheral blood B cells and CD4-expressing T cells were dramatically reduced, demonstrating that the leptin pathway plays an essential role in lymphopoiesis. Colony assays performed using marrow from *db/db* and wild-type mice indicated that *db/db* marrow has a deficit in lymphopoietic progenitors; furthermore, *db/db* mice are unable to fully recover the lymphopoietic population following irradiation insult, and although the levels of peripheral blood erythrocytes are normal in *db/db* mice, spleen erythrocyte production is severely compromized.

Conclusions: We have discovered that leptin and its cognate receptor constitute a novel hematopoietic pathway that is required for normal lymphopoiesis. This pathway seems to act at the level of the hematopoietic stem/progenitor cell, and may well also impact upon erythropoiesis, particularly in anemic states that may require output from the spleen. These findings offer a new perspective on the role of the fat cell in hematopoiesis.

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Background

Hematopoiesis is dependent upon the capacity of hematopoietic stem cells to both replicate and generate an extensive array of differentiated progeny. These tasks are accomplished *in vivo* through complex interactions among the cells that constitute the hematopoietic microenvironment [1]. In the bone marrow, the principal hematopoietic site in adult mammals, this microenvironment consists of a heterogeneous network of cells that includes fibroblasts, endothelial cells, macrophages, adipocytes, and mature and immature hematopoietic cells. To date, an impressive number of molecules with hematopoietic activity have been discovered in this microenvironment [2]. However, with the recent increase in cDNA sequences obtained using expressed sequence tag (EST) approaches, it is possible that many others will be discovered. For example, no molecules that specifically elicit the self renewal of hematopoietic stem cells have been identified.

Many of the events involved in the development and homeostasis of hematopoiesis — such as cell survival, proliferation, differentiation and functional activation — can be modulated by the cytokine family of growth factors [3]. Consequently, in our search for hematopoietic regulators we sought to clone novel cytokine receptors that may be involved in the proliferation of hematopoietic stem cells.

This approach was based upon the structural similarity of the cytokine receptors responsible for transducing biological signals in hematopoietic cells. Notably, the members of this family with predominantly hematopoietic expression — such as the interleukin receptors, the erythropoietin receptor and the thrombopoietin receptor (c-mpl) — have a structural unit in the extracellular region termed the hemopoietin receptor domain. This domain contains the distinctive signature motif Trp–Ser–X–Trp–Ser (WSXWS; X denotes any amino acid) [4–6].

Using amino-acid sequences derived from the hemopoietin receptor domain to probe the EST database, we cloned a novel cytokine receptor bearing the distinctive WSXWS motif from a CD34-expressing ($CD34^+$) human stem cell population. Interestingly, this receptor is identical to the recently cloned leptin receptor that is presumed to transduce signals from the *obese* (*ob*) gene product, leptin [7]. The same cytokine receptor has been cloned independently from murine fetal liver and a yolk sac-derived cell line, and shown to be expressed in primitive hematopoietic cells [8]. Signal transduction through the leptin receptor seems to be one of the major regulators of fat metabolism in mammals, although the mechanism by which this is accomplished is still unclear [7,9]. The expression of the leptin receptor in $CD34^+$ cells is intriguing, given the spatial relationships often seen between cytokine producer and responder cells. Initial results indicate that leptin is made predominantly by fat cells (in both white and brown adipose tissue) [10]. This is relevant to hematopoiesis because, in humans, bone marrow adipocytes occupy most of the marrow cavity [11]. However, despite being a major component of the hematopoietic microenvironment, the role of the fat cell in hematopoiesis has been difficult to define.

A nurturing role for fat cells in hematopoiesis is supported by several lines of evidence; marrow fat cells are preserved during starvation [12,13], but depleted in states of induced anemia [12,14]; the presence of mature adipocytes has always been associated with murine long-term bone marrow cultures that are hematopoietically active [15]; reticulum cells, the prospective progenitors for bone marrow adipocytes, are active in granulopoiesis and lymphopoiesis; pre-adipocyte lines have also been shown to maintain lymphopoiesis [15–18]; similarly, fat cells first appear 7 days after irradiation of the marrow, and this has been correlated with the onset of hematopoietic proliferation [19]. Nevertheless, it remains uncertain whether bone marrow adipocytes play an active role in hematopoiesis, or are only necessary from an architectural standpoint by providing structural support to the marrow [11,20].

In this article, we demonstrate a proliferative role for the leptin signalling pathway in primitive hematopoietic cells. Furthermore, analysis of the *db/db* mice, which only express a truncated leptin receptor, underscores the important role that leptin-mediated signal transduction plays in lymphopoiesis.

Results

Identification of EST T73849

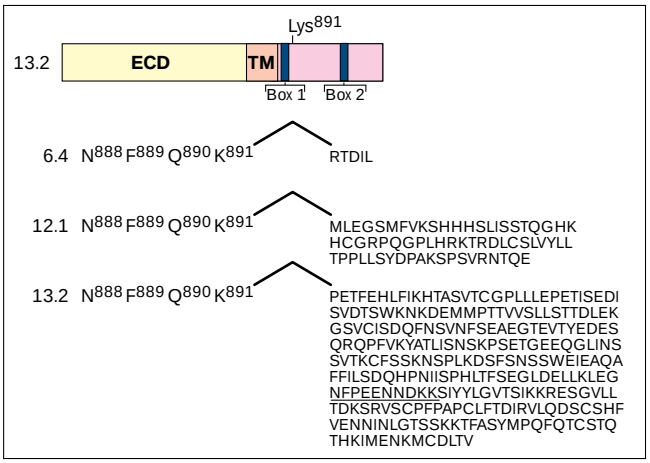
To identify novel cytokine receptors that may be active in hematopoiesis, we carried out a homology search of the EST database using amino-acid sequences of known cytokine receptors. This identified a 416 bp EST (T73849) with homology to human gp130. Within this region of homology, T73849 putatively encodes the cytokine receptor motif WSNWS. To obtain full-length cDNA clones, oligonucleotide probes were designed from the EST sequence and used to screen a human fetal liver library and then a library from the hepatoma cell line Hep3B [21]. Sequence information from the resulting cDNAs allowed the amplification of full-length clones from human $CD34^+$ progenitor cells, using the RACE (rapid amplification of cDNA ends) technique.

Three different cDNA clones were obtained from $CD34^+$ cells (Fig. 1). The predicted sequence of the 4102 bp cDNA clone 13.2 was identical to that of the leptin receptor recently cloned by Tartaglia *et al.* [7]. Clone 13.2 includes an open reading frame of 1168 amino acids with the hallmarks of a cytokine receptor. The two other clones isolated from $CD34^+$ cells appeared to represent splice variants of the receptor, which differ in the cytoplasmic domain following the lysine residue at position 891 (Lys^{891}). Clone 6.4 stops five amino acids after Lys^{891} and is the human homolog of the short form of the murine receptor [9]. Clone 12.1 diverges from clones 13.2 and 6.4 after Lys^{891}, and encodes a completely different cytoplasmic tail that lacks the putative Janus kinase (JAK)-binding 'Box 2' region found in clone 13.2 [5,22,23].

Expression analysis in stem cell populations

Many cytokine receptors are found to be expressed in early cells of the hematopoietic lineage. Such receptors and their cognate ligands form functional units that regulate the commitment and expansion of these progenitor cells [2] To determine whether the leptin receptor has a role in hematopoiesis, we examined its expression profile in human and mouse stem cell populations. $CD34^+$ cells were isolated from human cord blood [24], and the long-term repopulating murine fetal liver $AA4^+$ Sca^+ Kit^+ stem cell population (flASK cells) was isolated from midgestation fetal liver [25]. Both populations contain a heterogeneous mixture of hematopoietic stem cells and early hematopoietic precursors. Analysis of these populations using the polymerase chain reaction (PCR) showed that mRNAs encoding all three forms of the receptor were present in $CD34^+$ cells (Fig. 2). We detected mRNAs for the long and short forms of the receptor in flASK cells. Thus far, we have not isolated from mouse tissues any

Figure 1



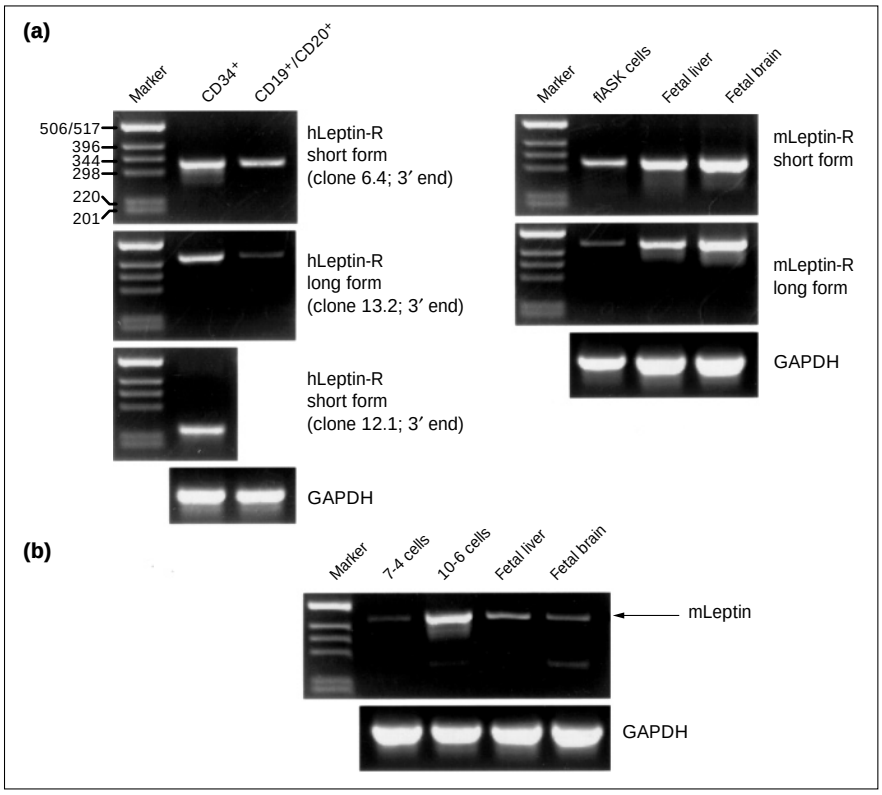
Leptin receptor isoforms present in CD34+ stem cells from human umbilical cord blood. Three splice variants of the leptin receptor were isolated based on the original EST T73849. Clone 6.4 is the human equivalent of the short form of the murine leptin receptor, Ob-Ra [9]. Clone 13.2 is the full-length human leptin receptor, and clone12.1 is a novel form of the leptin receptor. A representation of the full-length 13.2 leptin receptor is shown above. Clone 13.2 contains the putative JAK-binding domains, 'Box 1' and 'Box 2'; the amino-acid sequence of Box 2 is underlined. The splice variants arise after Lys891. ECD, extracellular domain; TM, transmembrane domain.

cDNAs that encode the 12.1 cytoplasmic tail. Human B cells isolated from peripheral blood using anti-CD19/CD20 antibodies were also positive for mRNAs expressing the short (6.4) and long (13.2) forms of the receptor. Using oligonucleotide primers that specifically corresponded to the leptin cDNA sequence, we showed that the mRNA for this ligand was expressed in fetal liver and fetal brain, as well as in two fetal liver stromal cell lines, designated 10-6 and 7-4 (Fig. 2). Both of these immortalized cell lines support myeloid and lymphoid proliferation of stem cell populations [25].

The hematopoietic role of the leptin signalling pathway

Having established that leptin and its receptor were expressed in the hematopoietic compartment, we sought to characterize the potential hematopoietic role of this transduction system. We initially designed antisense oligonucleotides corresponding to several regions adjacent to the initiation methionine of the leptin receptor. Using these oligonucleotides, we performed methylcellulose colony assays in the presence of complete conditioned media containing serum, granulocyte/macrophage colony-stimulating factor (GM-CSF), G-CSF, interleukin-3 (IL-3), IL-6, erythropoietin (EPO) and c-Kit ligand (KL). Under these conditions, the ability of human CD34+ stem cells to form colonies was not inhibited: the number of colonies per 10^3 cells formed in the presence of antisense

Figure 2



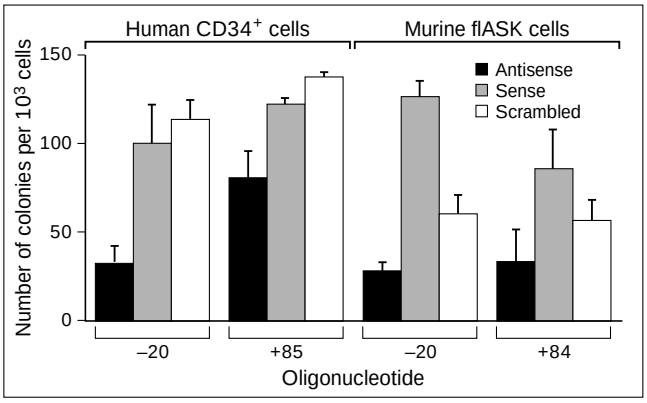
Expression profiles of (a) the leptin receptor variants and (b) leptin in hematopoietic tissues. Human CD34+ stem cells, murine flASK stem cells, human CD19+/CD20+ B cells, and the murine fetal liver stromal cell lines 7.4 and 10.6 were isolated, and mRNA was prepared as described in Materials and methods. We synthesized specific primers corresponding to the nucleotide sequences of the human cDNA clones 6.4, 13.2 and 12.1, the short and long forms of the mouse leptin receptor (mLeptin-R) and mouse leptin (mLeptin), and performed PCR analysis as described in Materials and methods. Primers specific for GAPDH were used as controls. The specificity of the amplified bands was confirmed by Southern blot analysis (data not shown). The oligonucleotide probes used for this analysis were as follows: clone 6.4, a 47mer beginning at nucleotide 2809; clones 12.1 and 13.2, a 28mer beginning at nucleotide 2838; murine leptin receptor short and long forms, a 49mer beginning at nucleotide 2671; murine leptin, a 49mer beginning at nucleotide 391. The leptin receptor mRNA could not be amplified from the stromal cell lines 10-6 and 7-4, and leptin mRNA could not be amplified from the stem cell populations (data not shown). For all cell populations, control reactions were run in the absence of reverse transcriptase, where no specific amplification was observed.

DNA was 323 ± 3, with sense DNA there were 287±5 colonies, and with oligonucleotides in which the sequence was scrambled the number was 289 ± 7. However, when these assays were performed in the presence of fetal calf serum and either KL alone, or KL, IL-3 and IL-6, we observed a significant inhibition of colony formation, particularly with the –20 antisense oligonucleotide (Fig. 3). Similar results were obtained using flASK cells and appropriately designed oligonucleotides based on the sequence of the mouse leptin receptor. The inhibitory effects of antisense oligonucleotides in these experiments implies the presence of leptin in the assay system. We could not detect any mRNA for leptin within the stem cell populations that we used, and we consider fetal calf serum to be the most likely source. The concentration of leptin in bovine serum has not been reported but, for comparison, very recent studies have indicated that human serum from non-obese individuals contains ~5–10 ng ml^{-1} of leptin, with levels reaching 50–100 ng ml^{-1} in obese states [26].

These results indicated that the leptin receptor transduces a proliferative signal to the hematopoietic cell. We confirmed this hypothesis by analyzing thymidine incorporation in the IL-3-dependent cell line BAF-3. We first carried out experiments in the absence of leptin by constructing fusion proteins [27] consisting of the extracellular domain of the human growth hormone receptor (hGHR) fused to the cytoplasmic domain of the leptin receptor encoded by clone 13.2 or clone 12.1 (hGHR–hLeptin-R13.2 and hGHR–hLeptin-R12.1, respectively). Transfection the BAF-3 cell line with plasmids encoding these constructs allowed us to test thymidine incorporation following stimulation with hGH. These experiments showed that hGHR–hLeptin-R13.2, but not hGHR–hLeptin-R12.1, could trigger an increase in thymidine incorporation, which is a probable indication of a proliferative response (Fig. 4a). These results were confirmed by using leptin to stimulate thymidine incorporation in BAF-3 cells transfected with the full-length 13.2 clone (Fig. 4b). Interestingly, the expression of hGHR–hLeptin-R12.1 in BAF-3 cells resulted in a decrease in the basal level of thymidine incorporation when these cells were removed from medium containing IL-3 (Fig. 4a). It is unclear what the role of the 12.1 variant may be; further study is warranted to determine if it has a role in the differentiation, rather than proliferation, of hematopoietic cells.

To examine the hematopoietic activity of leptin, we performed a variety of *in vitro* assays. In keeping with its ability to transduce a proliferative signal in BAF-3 cells, leptin stimulated a four-fold increase in the number of flASK cells grown in suspension culture in the presence of 25 ng ml^{-1} KL (Fig. 5a). Although the addition of KL at this concentration was sufficient to permit modest expansion of human stem cells, leptin alone was unable to effect their survival. We tested a variety of hematopoietic growth factors in suspension culture assays and found an apparent synergistic effect of leptin on growth with KL, GM-CSF and IL-3 (Table 1). We did not detect preferential expansion of any particular lineage when the resulting cultures were subjected to cytospin analysis. When methylcellulose colony assays were carried out, leptin augmented myeloid colony formation, and dramatically increased the formation of lymphoid and erythroid colonies (Fig. 5b,c). Under myeloid conditions, cytospin analysis revealed that 45 ± 7% of the colonies were CFU–GM (colony-forming units–granulocyte/macrophage), 48 ± 6% were CFU–Mixed, and 6 ± 1 % were BFU–E (burst-forming units–erythrocytes).

Figure 3



Antisense oligonucleotides based on the sequence of the leptin receptor inhibit colony formation in stem cell populations. Human CD34$^+$ stem cells and murine flASK cells were isolated as described in Materials and methods. Methylcellulose assays were performed using sense and antisense oligonucleotides that corresponded to the cDNA sequence of the leptin receptor, starting at position –20 and +85 with respect to the initiation methionine codon; see Materials and methods for details. Sense and scrambled oligonucleotides were used as controls. The number of colonies formed per 10^3 cells was assessed after 14 days in culture. Assays were performed in quadruplicate and the results confirmed in three independent experiments.

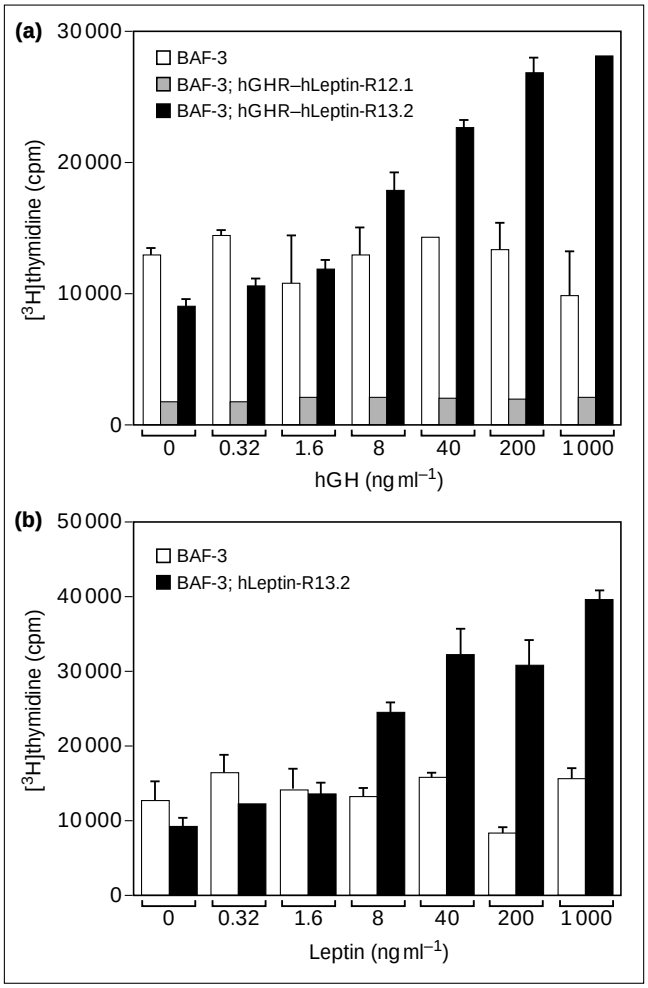
Table 1

Growth factor-induced proliferation of flASK cells.

Factor	KL	KL + leptin	Leptin
N/A	128 ± 9	192 ± 13	
G-CSF	131 ± 3	177 ± 8	30 ± 5
GM-CSF	148 ± 4	165 ± 6	134 ± 10
IL-3	189 ± 7	187 ± 4	144 ± 10
IL-6	112 ± 4	198 ± 5	32 ± 3
EPO	121 ± 3	177 ± 8	30 ± 6
IL-3 + IL-6	112 ± 2	198 ± 7	32 ± 7

Cells (2×10^5) were plated in suspension culture with the relevant growth factor combination. Cells were harvested and counted after 5 days. Cell numbers $\times 10^3$ are shown. Assays were performed in triplicate and repeated in two independent experiments. N/A, no addition. Note that 100 ng ml^{-1} KL was added to these cultures.

Figure 4



Thymidine incorporation assays using leptin receptor variants in BAF-3 cells. (a) hGHR–hLeptin-R12.1 or hGHR–hLeptin-R13.2 fusion proteins were expressed in BAF-3 cells as described in Materials and methods. These transfected cells and the parental BAF-3 line were stimulated with hGH, and the incorporation of tritiated thymidine was determined. (b) BAF-3 cells were stably transfected with the cDNA encoding hLeptin-R13.2; thymidine incorporation was then determined in these cell lines following stimulation with human leptin. Assays were performed in triplicate and the results were confirmed in three independent experiments.

The presence of leptin in the myeloid methylcellulose cultures did not give rise to any significant differences in the resulting phenotypes. Importantly, the hematopoietic activity of leptin was not confined to fetal liver stem cells — the mouse bone marrow Lin^{lo} Sca^{+} stem cell population also proliferated in response to leptin (KL, 5-fold expansion; KL and leptin, 10-fold expansion).

Analysis of hematopoiesis in *db/db* mice

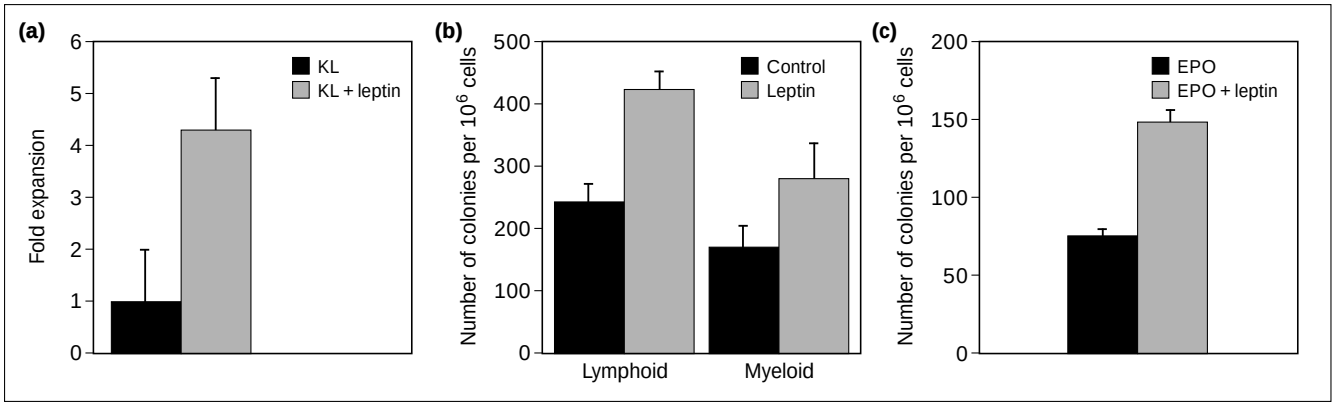
Further hematopoietic analysis of the role of the leptin receptor was facilitated by the recent cloning of the mouse *db* gene [9,28]. Previous data had suggested that the *db* gene encodes the leptin receptor [29,30]. It has been confirmed very recently that *db/db* mice express a truncated splice variant of the leptin receptor, which probably renders it defective in signal transduction [9,28]. We initially examined the hematopoietic defects in this mouse by measuring the proliferative potential of *db/db* homozygous mutant marrow. Under conditions favoring either myeloid [31] or lymphoid expansion [32], the colony-forming potential of the *db/db* marrow was significantly reduced compared to the wild-type control marrow (Fig. 6). This was particularly evident when the comparison was made under pre-B (lymphoid) methylcellulose conditions, where KL and IL-7 were used to drive lymphopoiesis (Fig. 6). Similar analysis of marrow from *ob/ob* mice, which are deficient in the production of leptin [33], also indicated that the lymphoproliferative capacity was compromized in the absence of a functional leptin signalling pathway (Fig. 6). However, this reduction was less than that observed with *db/db* marrow.

Analysis of the cellular profile of the *db/db* and wild-type marrow revealed significant differences between the two. The total number of cells in the *db/db* marrow was unchanged (data not shown), but when we examined various B-cell populations in the *db/db* marrow, we found decreased levels of $B220^{+}$ and $B220^{+}$ $CD43^{+}$ cells. $B220^{+}$ cells represent all B-cell lineages, whereas CD43 is thought to be expressed preferentially on the earliest cells of the B-cell hierarchy [34]. The anti-CD43 antibody recognizes leukosialin [35], a major sialoglycoprotein on myeloid and T lymphocytes, as well as B-lineage cells. It is interesting that the $CD43^{+}$ population in the heterozygous *db*/misty gray mouse was increased, but the implications of this are unclear. We found no differences between the CD4 and CD8 staining profiles of the two groups. Interestingly, the TER 119 (a red cell lineage marker) population was increased in the *db/db* marrow (Fig. 7a).

The spleens of *db/db* mice showed a significant decrease in tissue weight and in the number of cells compared with the homozygous misty gray controls (37 ± 6 mg *versus* 63 ± 9 mg; $4.3 \times 10^7 \pm 1 \times 10^4$ cells *versus* $1.10 \times 10^8 \pm 1 \times 10^5$ cells: $p > 0.05$). The decrease in the number of cells in the *db/db* spleen was reflected by a marked reduction in TER 119 staining (Fig. 7b). This result seems to confirm that leptin and EPO act synergistically, and points to a role for leptin in the regulation of erythropoiesis.

Examination of the hematopoietic compartments of *db/db* mice revealed that the number of peripheral blood lymphocytes was significantly reduced compared with heterozygous or wild-type controls (Fig. 8). Because *db/db* mice fail to regulate blood glucose levels and become diabetic at approximately 6–8 weeks of age, we followed peripheral blood counts as the animals matured. This analysis demonstrated that the number of peripheral

Figure 5



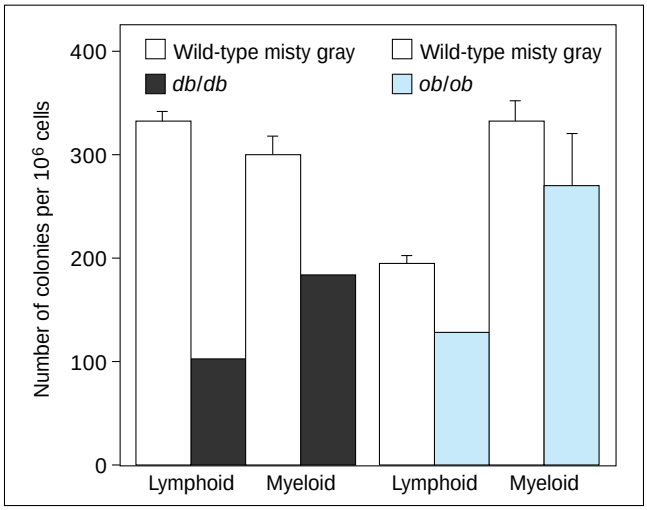
Expansion of murine flASK stem cells in suspension or methylcellulose culture. (a) flASK cells were cultured in liquid medium containing serum, and either 25 ng ml^{-1} KL, or KL and leptin. Cell counts and cytospin analyses were performed 5 days later. (b) flASK cells were seeded into methylcellulose under myeloid or lymphoid conditions, as described in Materials and methods. Colony counts were performed 14 days later. For colonies produced under lymphoid conditions, FACS (fluorescence-activated cell sorting) analysis showed that the vast majority of cells were $B220^+$. (c) flASK cells were seeded into methylcellulose containing KL; EPO, or EPO and leptin, were then added to this basic medium. The resulting colonies were counted 14 days later. FACS analysis showed that approximately 95 % of these colonies were $TER\ 119^+$. All assays were performed in triplicate and confirmed in at least three independent experiments. All stimulations by leptin were statistically significant: $p > 0.05$.

blood lymphocytes was significantly reduced at all time points compared with control animals, and that the peripheral lymphocyte population in *db/db* mice does not change significantly with age or with the onset of diabetes (Fig. 8). FACS analysis revealed that the decrease in the lymphocyte population resulted from a decline in the number of $B220^+$, $CD4^+$ and $CD8^+$ cells; the number of erythrocytes and platelets were at wild-type levels throughout all time periods examined. Interestingly, the number of peripheral blood lymphocytes in *ob/ob* homozygous mutant mice was the same as in the wild-type controls, suggesting that a compensatory mechanism operates in *ob/ob* mice, but not in *db/db* mice. The difference in the number of lymphocytes may be attributable to the different background strains of the *ob/ob* and *db/db* mice, or it may result from the presence of a locally acting alternative ligand for the leptin receptor.

Because hematopoietic analysis of *db/db* mice can be complicated by the onset of diabetes, we examined the impact of high glucose levels on lymphopoiesis by comparing the peripheral blood profiles and blood glucose levels in *db/db* (and control) mice and in two other diabetic models — glucokinase heterozygous knockout mice [36] and IFN-α transgenic mice [37]. The results showed that there was no relationship between blood glucose levels and peripheral lymphocyte counts (Fig. 9). These data therefore suggest that the lymphoid defects seen in *db/db* mice are directly attributable to the hematopoietic function of the leptin signalling pathway. However, at this time we cannot rule out secondary events that may result from the eventual diabetic state of the *db/db* mouse.

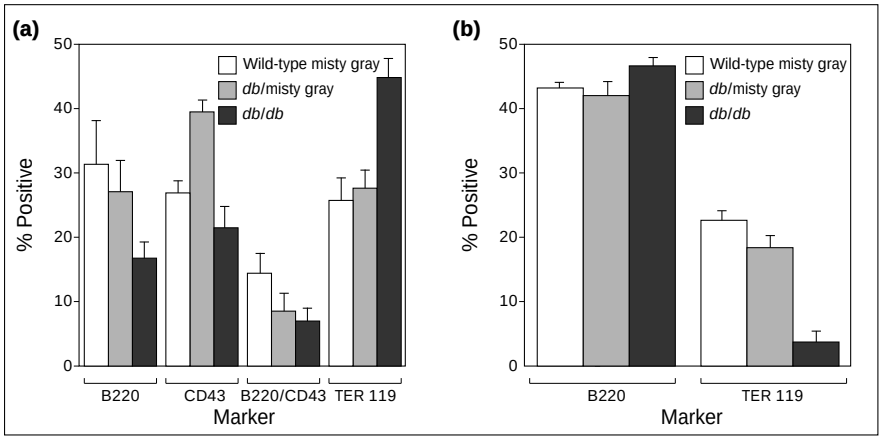
To test the ability of the *db/db* hematopoietic compartment to respond to challenge, we subjected *db/db* mice and controls to a sub-lethal dose of γ irradiation (Fig. 10). The kinetics of hematopoietic recovery were then followed by

Figure 6



Methylcellulose assays to determine the colony-forming potential of *db/db*, *ob/ob*, and the corresponding wild-type marrow. Bone marrow cells (1×10^5) were seeded into methylcellulose, and the resulting colonies were counted after 14 days. Assays were performed in triplicate under myeloid and lymphoid conditions, and the experiments were repeated a minimum of three times. Differences between *db/db* and wild-type mice under both culture conditions, and *ob/ob* and wild-type mice under lymphoid conditions were statistically significant: $p > 0.05$.

Figure 7



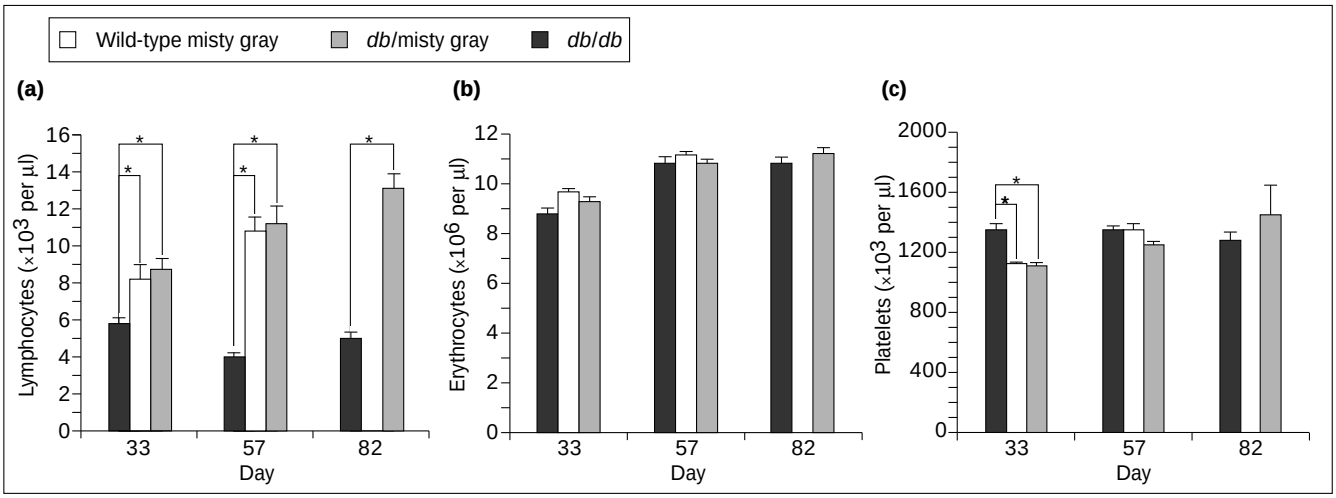
Bone marrow cellular profiles in wild-type misty gray homozygotes, *db*/misty gray heterozygotes, and *db/db* homozygotes. The overall number of cells in the *db/db* marrow was unchanged compared to controls. (a) Cellular profiles were determined using anti-B220, anti-CD43 (clone S-7), and anti-TER 119 antibodies. (b) Cellular profiles of the spleens from the above groups.

monitoring the peripheral blood during the recovery phase. This illustrated the inability of the *db/db* hematopoietic system to fully recover the lymphopoietic compartment of the peripheral blood 35 days after irradiation. Platelet levels in these mice followed the same recovery kinetics as controls; however, the reduction in the number of erythrocytes in *db/db* mice lagged behind controls by 7–10 days. The increased TER 119$^+$ population found in the *db/db* marrow may explain this intriguing finding (Fig. 7a).

Discussion

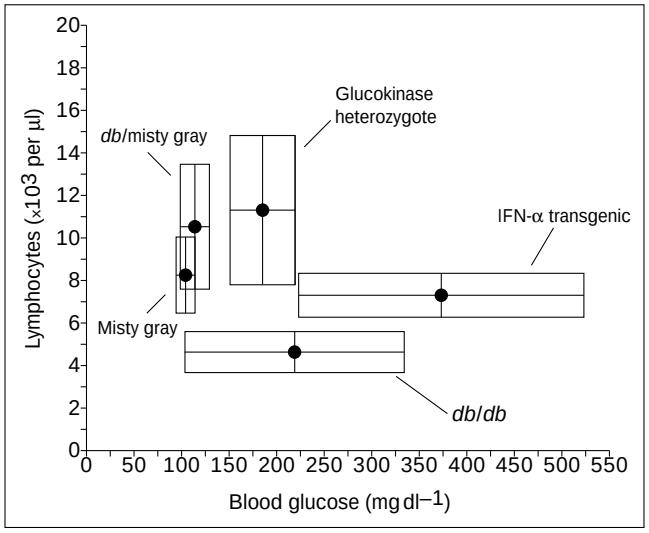
We have isolated a novel cytokine receptor — based upon its homology with gp130 — that is expressed in human CD34$^+$ cells and murine flASK cells. This receptor was found to be identical to the leptin receptor, which was expression cloned using the *ob* gene product, leptin [7,29]. It is thought that the primary physiological role of leptin is to control body weight; this is presumably mediated, at least in part, by signal transduction through the leptin receptor in the hypothalamus [7,9]. However, in keeping with many other cytokines, we show that the leptin signalling pathway also has a role in hematopoiesis. As with other cytokine receptors, the leptin receptor seems to be expressed on progenitor and more mature hematopoietic cells. However, the proliferative effect of leptin on hematopoietic stem cell populations in myeloid, lymphoid and erythroid colony assays demonstrates that leptin can act on very early cells of the hematopoietic lineage. Antisense oligonucleotide experiments, using both human and mouse stem cell populations, demonstrated an inhibition

Figure 8



Time-course analysis of (a) lymphocyte, (b) erythrocyte and (c) platelet numbers in the peripheral blood of *db/db* homozygotes, misty gray/*db* heterozygotes and misty gray homozygotes. Peripheral blood (40 μl) was taken *via* orbital bleed and analyzed on a Serrono Baker system 9018, as described in Materials and methods. The asterisks denote statistical significance: $p > 0.05$.

Figure 9



Comparison of peripheral blood lymphocyte counts and blood glucose levels in misty gray, *db*/misty gray, *db/db*, interferon-α transgenic, and glucokinase heterozygous knockout mice. Blood samples were taken from the orbital sinus and glucose levels were determined as described in Materials and methods. Areas described by the boxes represent the mean ± one standard deviation of the two parameters.

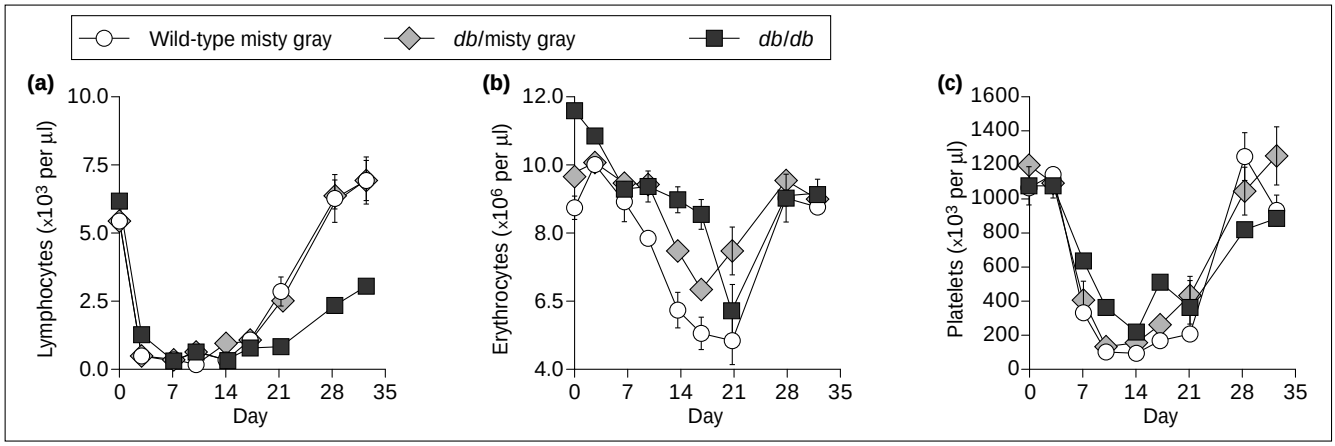
of myeloid colony formation, although the reduction in myelopoiesis could be prevented by the additional inclusion of G-CSF and GM-CSF in the culture medium. These data serve to illustrate the compensatory capacity of cytokine action in the myelopoietic compartment.

Although the myeloid growth-promoting aspects of leptin signalling appear to be redundant, the effects upon lymphopoiesis seem to be more influential. Hematopoietic analysis of *db/db* mice revealed an inherent deficit in lymphopoiesis. In accordance with the proliferative activity of leptin on stem cell populations, this deficit seems to extend to the pro-B cell stage, as indicated by the reduction in B220⁺ CD43⁺ cells in the *db/db* marrow. Impaired lymphopoiesis is further illustrated by the reduced levels of lymphocytes in the peripheral blood, and the inability of this component to fully recover following irradiation.

Our analysis of various models with high blood glucose levels showed no correlation between blood glucose levels and the number of peripheral lymphocytes. This argues strongly that the lymphocyte deficit in *db/db* mice is not a result of the ensuing diabetic state. However, we are further addressing this issue in transplant models of *db/db* marrow, in both wild-type and *scid* mice. Engraftment into *scid* mice should allow us to determine the impact of a non-functional leptin receptor to immune challenge.

The role of leptin and its receptor in erythropoiesis warrants further study. Leptin acts synergistically with EPO and KL to increase erythroid colony production, indicating again that it acts at the level of the progenitor cell. However, analysis of *db/db* mice revealed no peripheral defect in erythropoiesis. Interestingly, the *db/db* spleen shows a dramatic reduction in the number of cells expressing the erythrocytic lineage-specific marker TER 119, while the number of TER 119⁺ cells in the marrow is almost doubled compared to wild-type controls. In the adult mouse, the spleen contributes to normal erythropoiesis, so it is conceivable that the increased number of TER 119⁺ cells in the marrow reflects a compensatory mechanism for decreased splenic output. The increased TER 119⁺ population in the marrow may also explain the decreased susceptibility of *db/db* erythrocytes to radiation.

Figure 10



Recovery kinetics of levels of (a) peripheral blood lymphocytes, (b) erythrocytes and (c) platelets in misty gray homozygotes, *db*/misty gray heterozygotes, and homozygous *db/db* mice subjected to a sub-lethal dose of γ irradiation. Peripheral blood samples were obtained *via* retro-orbital bleeds, as described in Materials and methods.

Having defined a role for the leptin signalling pathway in the expansion of primitive hematopoietic cells, what are the implications of the presence of fat cells in the hematopoietic microenvironment? From the mRNA expression data presented here, it seems probable that leptin can be produced by hematopoietic stromal cells, as well as by extramedullary fat cells. It is presumed, although not proven, that the marrow adipocyte is stromal in origin. Hence, the relationship between stromal cells and bone marrow adipocytes in the production of leptin may hold the key to understanding the role of the marrow adipocyte in hematopoiesis. It is possible that the inverse relationship between hematopoietic activity and the number of lipid-containing cells seen under certain long-term culture conditions [11,20] is attributable to changes in leptin production upon differentiation of marrow stroma to adipocytes. It is is not yet known whether marrow fat cells can produce leptin, but it is tempting to speculate that the impressive fat cell content of human marrow reflects the requirement for leptin in active hematopoiesis, especially lymphopoiesis. The findings presented here also have implications for a variety of leukemic states in which the role of the fat cell has also been difficult to define. For example, it has been reported that stroma from patients with chronic myeloid leukemia has a reduced capacity to produce fat cells [38], and several reports have indicated reduced fat content in leukemic patients [38–40]. Spontaneous fat cell formation has also been speculated to characterize patients who most readily engraft following bone marrow transplantation [38]. Clearly, the role of the intramedullary fat cell will attract much greater attention now that a mechanistic reason for their existence can be considered.

Conclusions

These findings clearly demonstrate a role for leptin signalling in mammalian hematopoiesis, and show that leptin can act at the level of the hematopoietic progenitor to stimulate multilineage expansion. Furthermore, analysis of *db*/*db* mice indicates that this signalling pathway plays an important role in maintaining the lymphopoietic component of the peripheral blood. These data also suggest a role for leptin in erythropoiesis, where synergy between leptin and erythropoietin is evident. Compensatory mechanisms in the bone marrow of *db*/*db* mice obviate peripheral anemia, but in the spleen, an important site of erythrocyte production in the mouse, leptin signalling seems to be a constitutive component of erythropoiesis. Previous data have indicated that adipocytes are the major source of leptin; our findings will therefore help to determine the role of the bone marrow adipocyte in hemotopoiesis.

Materials and methods

Isolation of hematopoietic stem cell populations

Human $CD34^+$ stem cells were isolated from human umbilical cord blood as previously described [24]. Murine fetal liver flASK stem cells were isolated from the midgestational fetal liver as previously described [25].

Stem cell suspension cultures

Stem cells were isolated as above. Cells (2×10^6) were seeded in individual wells in 12-well plates in DMEM 4.5/F12 media supplemented with 10 % heat-inactivated fetal calf serum (FCS; Hyclone, Logan, Utah) and L-glutamine. Growth factors were added at the following concentrations: KL, 25 ng ml^{-1}; IL-3, 25 ng ml^{-1}; IL-6, 50 ng ml^{-1}; G-CSF, 100 ng ml^{-1}; EPO, 2 U ml^{-1}; IL-7, 100 ng ml^{-1} (all growth factors were from R and D Systems, Minneapolis, Minnesota). Leptin was added at 100 ng ml^{-1} unless indicated otherwise. Recombinant leptin was produced as described [41]. Briefly, mature leptin was expressed in *Escherichia coli* by inserting the leptin coding sequence in frame with the sequence encoding the signal for the secretion of the *E. coli* heat-stable enderotoxin II, downstream of the *E. coli* alkaline phosphatase promoter. After cell lysis, the insoluble fraction was solubilized in 8 M urea buffer (pH 8.35) containing 25 mM dithiothreitol. Reduced leptin was purified by size exclusion and reverse-phase HPLC, and refolded in the presence of glutathione. Refolded leptin was purified by reverse-phase HPLC and subjected to SDS–PAGE, amino-acid and mass spectroscopic analyses.

Methylcelluose colony assays

Methylcellulose assays were performed as previously described [25], using 'complete' methylcellulose or pre-B methylcellulose medium (Stem Cell Technologies, Vancouver, British Columbia) with the addition of 25 ng ml^{-1} KL (R and D Systems). Cytospin analyses of the resulting colonies were performed as previously described [25]. For cells plated under lymphoid or erythroid conditions, colonies were harvested, washed in Hank's balanced salt solution (HBSS) containing 2 % FCS and pooled at a concentration of 5×10^6 per ml. 1×10^6 cells were used for each sample and stained using antibodies directed against B220 or TER 119, or appropriate controls.

Tissue harvest and FACS analysis

Bone marrow, spleens and peripheral blood were harvested from the diabetic mouse strains C57BLKS/J *db/db* (mutant), C57BLKS/J m+/*db* (lean heterozygous control littermate) and C57BLKS/J+m/+m (lean homozygote misty gray coat control littermate), and the obese mouse strains C57BL/6J-*ob*/*ob* (mutant) and C57BL/6J-*ob*/+ (lean littermate control). All strains were from the Jackson Laboratory, Bar Harbor, Maine. A minimum of five animals were used per experimental group. Femurs were flushed with HBSS containing 2 % FCS and a single cell suspension was made of the bone marrow cells. Spleens were harvested and the splenic capsule was ruptured and filtered through a nylon mesh. Peripheral blood was collected through the retro-orbital sinus in phosphate buffered saline (PBS) containing 10 U ml^{-1} heparin and 1 mmol EDTA. The bone marrow, splenocytes and peripheral blood were then stained with monoclonal antibodies directed against the following mouse antigens: B220/CD45R (FITC), TER 119 (R-PE), CD4/L3T4 (FITC), CD8/Ly2,3 (FITC), and CD43 (R-PE); antibodies were conjugated to FITC (fluorescein isothiocyanate) or R-PE (R-phycoerythin), as indicated. All monoclonal antibodies were from Pharmigen, San Diego, California. The appropriate isotype controls were included in each experiment. For methylcellulose colony assays, the bone marrow from five animals in each group was pooled and 1×10^5 cells used for each assay point.

Searching the dbEST database

Protein sequence files for the cytokine receptors IL-6R, IL-3R, LIFR, c-mpl, gp130, IL11-R and GM-CSFR were used to search the dbEST database [42] using the basic local alignment search tool (BLAST) [43].

Cloning of the full-length receptor

We synthesized an oligonucleotide probe, designated WSX.6#1, that was based on the T73849 EST sequence. The WSX.6#1 probe was a 51mer with the following sequence: 5′–GTCAGTCTCCCAGTTCCAGACTTGTGTGCAGTGCAGTCTATGCTGTTCAGGTGCGC–3′. The radiolabeled WSX.6#1 DNA was used to probe 1.2×10^6 clones from a random and oligo dT-primed λgt10 fetal liver library (Clontech, Palo Alto, California). From the initial screen, five individual plaque pure clones were isolated.

To obtain the full-length genes, clone 6.4 (*Nsi–Hind*III fragment) was radiolabeled and used to screen 1.2×10^6 clones from a λgt10 library constructed from a hepatoma Hep3B cell line. This screen resulted in the isolation of 12 independent clones, but it was unclear which clones contained the correct 3′ end of the molecule. We therefore performed 3′ RACE using $CD34^+$ human hematopoietic stem cells. Briefly, poly A^+ RNA was isolated from ~10^6 $CD34^+$ hematopoietic stem cells using the FastTrack method for mRNA isolation (Invitrogen, San Diego, California). Double-stranded cDNA synthesis and subsequent 3′ RACE was performed with the Marathon cDNA Amplification Kit (Clontech) following manufacturer's protocols. 3′ RACE products were generated using a forward hLeptin-R specific primer, corresponding to nucleotides 2534–2563, and a 3′ anchored reverse primer provided by the manufacturer. DNA sequencing was performed with the Taq dye deoxynucleotide terminator cycle sequencing kit on an automated Applied Biosystems DNA sequencer.

Construction of hGHR–hLeptin-R fusion proteins

Recombinant PCR was used to generate the chimeric receptors containing the extracellular and transmembrane domains of the hGHR and the cytoplasmic domain of either hLeptin-R12.1 or hLeptin-R13.2. In short, the cytoplasmic domain of clone 12.1 or 13.2 beginning with Arg at residue 866 and extending down to residue 958 or residue 1165, respectively, was fused in frame, by sequential PCR, to the hGHR extracellular and transmembrane domain beginning with Met^{18} and extending down to Arg^{274}.

RNA isolation and reverse-transcription PCR analysis

All RNAs were isolated using the FastTrack method (Invitrogen). For tissues and cell lines, mRNA was isolated from either 1 g of tissue or 10^8 cells. For cell populations ~3×10^5 cells were used. cDNA was synthesized with Superscript II reverse transcriptase (BRL/Gibco, Bethesda, Maryland), using either oligo dT or random hexamer priming.

Reverse-transcription PCR (RT–PCR) reactions from fetal liver, fetal brain, and 7.4 and 10.6 cells were performed using 20 ng of poly A^+ mRNA. RT–PCR from cell populations was performed with ~$1–3 \times 10^4$ cell equivalents per reaction; 1/5th of the reaction was used for gel analysis. PCR was performed for 40 cycles of amplification with 97 °C denaturation for 20 sec, 60 °C hybridization for 30 sec, and 72 °C extensions for 1 min. Reactions containing no reverse transcriptase were run for each RT–PCR reaction to demonstrate absence of genomic DNA contamination. To further insure against genomic DNA contribution, the primers for the leptin receptor were designed to span the splice/donor acceptor site at Lys^{891}. For the leptin molecule we designed primers to span the splice/donor acceptor site at Gln^{201}, the splice site of the 2G7 clone produced by exon trapping [33].

Oligonucleotide primers (F, forward; R, reverse) used for RT–PCR expression analysis were as follows:

hLeptin-R13.2; 13.2F 5′–GAAGATGTTCCGAACCCCAAGAATTG–3′ (nucleotides 2810–2835); 13.2R; 5′–CTAGAGAAGCACTIGGTGACTGAAC–3′ (nucleotides 3213–3237).
hLeptin-R6.4; 6.4F 5′CCATTGAGAAGTACCAGTTCAGTCTTTACC-3′ (nucleotides 2601–2630); 6.4R; 5′–GGGAAGTTGGCACATTGGGTTCA–3′ (nucleotides 2908–2930).
hLeptin-R12.1; 13.2F 5′–GAAGATGTTCCGAACCCCAAGAATTG–3′ (nucleotides 2810–2835); 12.1R 5′–GGATTTGGCAGGGTCATAGGACAA–3′ (nucleotides 3019–3043).
mLeptin-R (short form); muLep-RaF 5′–ATGCTATCGACAAGAATTG–3′ (nucleotides 2546–2570); mLeptin-RaR 5′–AGTCATTCAAACCATAGTTAGG–3′ (nucleotides 2843–2865).
mLeptin-R (long-form); muLep-RbF 5′–ACACTGTTAATTTCACACCAGAG–3′ (nucleotides 2629–2651); muLep-RbR 5′–TGGATAAACCCTTGCTCTTCA–3′ (nucleotides 3054–3074).
mLeptin; mObLF 5′–AGTGCCTATCCAGAAAGTCCAGGA–3′ (nucleotides 118–142; mObLR 5′–CTGTTGAAGAATGTCCTGCAGAGA–3′ (nucleotides 513–536).

Generation of stable cell lines expressing hLeptin-R13.2 and hGHR–hLeptin-R13.2/12.1 fusion proteins

hLeptin-R13.2 cDNA and the sequences encoding hGHR–hLeptin13.2 and hGHR–hLeptin12.1 were subcloned into expression vector pRKtkNeo. The expression constructs were electroporated into Ba/F3 cells and selected and maintained as previously described [25].

Proliferation assays

Cells were deprived of IL-3 for 16–18 h (in RPMI 1640 supplemented with 10 % FCS). Cells were washed in serum-free RPMI 1640 and plated at 5×10^4 cells per well in 0.2 ml of serum-free RPMI 1640 supplemented with the indicated concentration of hGH or human leptin. Cells were stimulated for 24 h and thymidine incorporation was determined as described [25].

Antisense oligonucleotide experiments

Human $CD34^+$ or mouse flASK cells were isolated as described above. We synthesized antisense (AS), sense (S) and scrambled (SCR) versions of each oligonucleotide; + or – indicates the first base of the oligonucleotide relative to the initiation methionine of the leptin receptor:

Human –20 (AS) TACTTCAGAGAAGTACAC; (S) GTGTACTTCTCTGAAGTA; (SCR), GAATCACGGTAACTATCA.
Human +85 (AS) CTTAAATCTCCAAGGAGT, (S) ACTCCTTGGAGATTTAAG; (SCR) AAGTCTTAAGCCAGACTT.
Mouse –20 (AS) TTCTTCAGAGGTGTACAC; (S) GTATACACCTCTGAAGAA; (SCR) ATGCGAGGCTACTTCTAT.
Mouse +84 (AS) TTAAATTTCCAGCGAGAG; (S) CTCTCCCTGGAAATTTAA; (SCR) ATTTGAAGGAGTTAAGCC.

$CD34^+$ or flASK cells were incubated at a concentration of 1×10^4 per ml in 50:50 DMEM/F12 media supplemented with 10 % FCS, L-glutamine, and GIBCO lipid concentrate containing either sense, antisense or scrambled oligonucleotides (70 μg ml^{-1}). After 16 h, a second aliquot (35 μg ml^{-1}) of the respective oligonucleotide was added and the cells incubated for a further 6 h.

Initially in colony assays, following incubation with the oligonucleotides, 1×10^3 $CD34^+$ cells were incubated in complete methylcellulose media (Stem Cell Technologies) containing 25 ng ml^{-1} KL, 25 ng ml^{-1} IL-3, and 50 ng ml^{-1} IL-6, EPO, G-CSF and GM-CSF. In subsequent colony assays, following incubation with the oligonucleotides, 1×10^3 $CD34^+$ or flASK cells were incubated in methylcellulose media containing 20 ng ml^{-1} IL-3, 20 ng ml^{-1} IL6 and 50 ng ml^{-1} KL. All cultures were incubated at 37 °C for 14 d and the resulting colonies counted and phenotyped. All assays were performed in quadruplicate.

Sub-lethal irradiation modes

C57BLKS/J *db/db*, C57BLKS/Jm+/*db*, and C57BLKS/J+m/+m mice were subjected to sub-lethal whole body γ irradiation (750 cGy, 190 cGy min^{-1}) as a single dose from a ^{137}Cs source. Ten animals were used per experimental group.

Procurement of blood samples

Before the experiment, and at time points throughout the study, 40 μl of blood was taken from the orbital sinus and immediately diluted into 10 ml of diluent to prevent clotting. The complete blood count from each blood sample was measured on a Serrono Baker system 9018 blood analyzer within 60 min of collection. Only half the animals in each dose group were bled on any given day; thus, each animal was bled on alternate time points. Blood glucose levels were measured in orbital sinus blood samples using One Touch glucose meters and test strips (Johnson and Johnson).

Accession numbers

The cDNA sequences reported in this paper have been deposited in the GenBank database, and can be accessed using the following numbers: U66495, U66496 and U66497.

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