Leptin is an adipocyte-derived hormone regulating energy homeostasis and body weight. Leptin also plays a role in hematopoiesis, cell cycle regulation, and in oncogenesis. The leptin receptor is a single transmembrane protein belonging to the superfamily of cytokine receptors, structurally related to the hemopoietin receptor family. The aim of the study was to evaluate bone marrow and peripheral blood leptin level and frequency of distribution of leptin receptor gene polymorphism Gln223Arg in children with acute leukemia. The examined group included 92 children with acute leukemia (83 ALL and 9 AML) and 39 non-leukemic control children. Leptin level was measured by ELISA method at the day of leukemia diagnosis. Genomic DNA was isolated with the use of a column method and the genotyping of DNA sequence variation was carried out by the restriction enzyme analysis of PCR – amplified DNA. The samples were then electrophoresed on 2.5% agarose gel. Leptin level in leukemic children was lower than in healthy children. Bone marrow leptin level was significantly lower than that in the blood in leukemic children with ALL-T and AML. An analysis of frequency distribution of the Gln233Arg polymorphism in the leptin receptor gene in leukemic children showed lack of differences between the patients and controls. There was no difference in the genotype frequencies between the leukemic AML and ALL groups either. The results indicate a possible relation between the leptin level and leukemia development in children. The effector effect of the hormone seems not related to Gln223Arg polymorphism of its receptor.

Key words: Gln223Arg polymorphism, leukemia, bone marrow, leptin
INTRODUCTION

Leptin is a 16-kDa adipocyte-derived hormone regulating energy homeostasis and body weight by adipose tissue (1-3). Leptin serum concentration is proportional to body mass, but may be lowered rapidly by fasting or inflammatory reaction (1-3). Leptin acts via a family of membrane bound receptors (RB) (2, 3). RB is a single transmembrane protein belonging to the superfamily of cytokine receptors related to the hemopoietin receptor family (4-7). RB is predominantly expressed in the hypothalamic regions, but also is distributed ubiquitously in peripheral tissues (4, 6).

Leptin also plays an important role in immunity, hematopoiesis, cell cycle regulation, and susceptibility to infections. It can modulate hematopoiesis by stimulating proliferation and inhibiting apoptosis of myelocytic and primitive hematopoietic progenitor cells (4, 8, 9). This effect may be exerted by cell surface receptors detected also on human hematopoietic cells and cells of other tissues (10). RB is expressed in enriched hematopoietic stem cells and in a variety of hematopoietic cell lines (11). In animal models, leptin is highly expressed in cultured adipocytes isolated from bone marrow (8, 9). Moreover, leptin not only promotes normal hematopoiesis, but also stimulates the growth and viability of leukemic cells, which suggests a role for leptin in the pathogenesis of hematological malignancies (8, 9). Indeed, leptin may induce proliferation and enhance survival of primary leukemic cells from patients with acute myeloid leukemia (AML) (10). The presence of RB was demonstrated on primary AML cells (10). The role of leptin and RB in the pathogenesis of some malignant tumors is debatable. Clement et al (12) reported a mutation in the human RB gene, where at the +1 position of intron 16, transition of glutamine to arginine was observed. Recently, several other polymorphisms have been identified in the leptin receptor genes and the transition of glutamine to arginine is associated with leptin overproduction (13, 14). A hypothesis has been put forward that leptin and RB polymorphism, associated with a higher leptin serum level and a high expression of leptin in adipocytes, may serve as genetic markers for cancer disease (15).

The aim of the present study was to evaluate the level of leptin in bone marrow and in peripheral blood as well as the frequency of distribution of the Gln223Arg polymorphism in the leptin receptor gene in children with acute lymphoblastic (ALL) or myeloblastic (AML) leukemia.

MATERIAL AND METHODS

Study protocol was approved by the Warsaw Medical University Ethics Committee. Written informed consent was obtained during the enrollment visit from the parents of all patients and control subjects. All patients and controls were referred to the Hematology Clinic of the Academic Children’s Hospital of Warsaw Medical University in Warsaw, Poland.
Subjects

The study group consisted of 92 children with acute leukemia of age ranging from 5/12 to 18 years (mean 12.3 ±7.4 years) for girls and 4-9 years (mean 5.7 ±6.6 years) for boys. Eight children (2 girls and 6 boys) with acute lymphoblastic T leukemia (ALL-T), 44 children (18 girls and 26 boys) with acute lymphoblastic B leukemia (ALL-B), 32 children with common ALL and 8 children (2 girls and 6 boys) with acute myeloblastic leukemia (AML) were included in the study. The diagnosis of leukemia was based on bone marrow smear and flow cytometry immunophenotyping.

The control group consisted of 39 healthy children (16 boys, 13 girls) who were free from hematological malignancies. There were no significant differences in the age and sex between the children with or without leukemia. The control children were referred to the Hematology Department and were diagnosed as having benign hematological disorders (anemia, trombocytopenia).

Leukemic and control children were divided into 4 groups according to body mass index (BMI): Group 1 = 11.7-13.9 kg/m$^2$; Group 2 = 14.0-15.1 kg/m$^2$; Group 3 = 15.2-18.4 kg/m$^2$; and Group 4 = 18.5-20.6 kg/m$^2$.

Laboratory assays

Blood and bone marrow samples were collected at the day of the leukemia diagnosis was made, centrifuged and serum or bone marrow supernatants were stored at -40°C until use. The portions of the samples remaining after routine laboratory examinations were used for further immunoenzymatic and molecular analyses.

ELISA test. A commercial immunoenzymatic kit to detect leptin (Quantikine Human Leptin, R & D, Minneapolis, USA) was used. The test is based on a solid double antibody sandwich ELISA. Serum and bone marrow supernatants were added to microwells precoated with leptin. All samples were assayed in duplicates. In the positive cases, antigen-antibody complex reacted with peroxidase-labeled antihuman IgG conjugate. Using H$_2$O$_2$/TMB as substrate, the enzymatic activity of peroxidase was measured at 450 nm with the use of automated reading system Stat Fax 2100 (ALAB, Warsaw, Poland). Standards were provided for the generation of a semi-logarithmic reference curve, which all the results were referred to. Immunoenzymatic assays were performed blindly by a laboratory technician.

PCR assay. Genomic DNA was isolated using Genomic Midi AX isolation kit with ion-exchange membranes (A & A Biotechnology, Gdynia, Poland). One milliter of blood was diluted with saline 1:1 v/v. After incubation for 30 min at 50°C with lysing buffer and proteinase K solution, the mixture was vortexed vigorously. The sample was placed in a column and centrifuged. After washing, the elution buffer was added and the sample was centrifugation twice at 4000 rpm for 2 min. The sample was then centrifuged with isopropanol at 13 500 rpm for 7 min. The supernatant was removed and the pellet was washed with ethanol. After consecutive centrifugation (13 500 rpm for 7 min), the supernatant was removed and the pellet was air-dried for 10 min. Finally, pure DNA was dissolved in 200 µl of sterile water.

Genotyping was done using polymerase chain reaction – restriction fragment length polymorphism analyses. Amplification was carried out in a 50 µl volume containing 300 ng genomic DNA, 0.1 µM of each primer (forward 5’ AAA CTC AAC GAC ACT CTC CTT 3’ and reverse 5’ TGAg ACT GAC ATT AGA GGT GA 3’) (DNA - Gdańsk, Gdańsk, Poland), 200 µM of each dNTP (DNA -Gdañsk, Gdańsk, Poland), 3 mM of magnesium chloride (Applied Biosystems, Warrington, UK), and 0.2U of Taq Gold polymerase (Applied Biosystems, Warrington, UK).
Thirty five cycles were conducted in a thermocycler, Mastercycler personal (Eppendorf, Hamburg, Germany), under the following conditions: initial denaturation at 94°C for 4 min, denaturation at 94°C for 30 s, annealing at 55°C for 35 s, extension at 72°C for 60 s, and a final extension at 72°C for 10 min. The amplified PCR product (80 bp) was digested with the addition of 2U *Msp I* (New England BioLabs, Beverly, MA, USA) overnight at 37°C. The digested samples were separated by electrophoresis through a 4% agarose gel. Digestion of the 80 bp product with *Msp I* produced fragments of the following sizes: 80 bp in the homozygote Gln/Gln (A/A); 80, 57, and 23 in the heterozygote Gln/Arg (A/G); 57 and 23 in the homozygote Arg/Arg (G/G).

**Statistical analysis**

Results of bone marrow and serum leptin level are expressed as means ±SD. The results of leptin level in different groups were compared by the U Mann-Whitney test. Statistical significance was accepted at P<0.05. The Pearson test was used for the analysis of the correlations between the BMI and leptin concentration. Frequency distribution analysis was performed with chi square test. A commercial Statistica package was used for all statistical data elaboration.

**RESULTS**

The blood serum and bone marrow leptin levels in leukemic children were lower than those in the control children (*Table 1*). The level of leptin in bone marrow was significantly lower than that in the serum of both control BMI Group 4 and leukemic ALL-T and AML children (*Table 1*). Significant differences in the leptin serum level were observed between the leukemic ALL-T and AML groups and the control BMI Group 3 (*Table 1*).

Stars indicate significant differences between the corresponding leptin concentration in bone marrow and peripheral blood at P<0.05. The P values in the right hand column indicate significant differences between the leukemic and control children in a given group.

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**Table 1.** Leptin level in peripheral blood and in bone marrow of leukemic children compared with control children.

<table>
<thead>
<tr>
<th>PERIPHERAL BLOOD</th>
<th>n</th>
<th>Leptin concentration (µg/ml)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls - BMI Group 3</td>
<td>33</td>
<td>28.5 ±11.81</td>
<td></td>
</tr>
<tr>
<td>Leukemic - ALL-B</td>
<td>15</td>
<td>23.8 ±20.86</td>
<td>NS</td>
</tr>
<tr>
<td>Controls - BMI Group 4</td>
<td>9</td>
<td>189.9 ±83.0</td>
<td></td>
</tr>
<tr>
<td>Leukemic - ALL-T</td>
<td>5</td>
<td>66.9 ±105.7</td>
<td>0.05</td>
</tr>
<tr>
<td>Leukemic - AML</td>
<td>5</td>
<td>53.3 ±8.07</td>
<td>0.004</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>BONE MARROW</th>
<th>n</th>
<th>Leptin concentration (µg/ml)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls - BMI Group 3</td>
<td>13</td>
<td>19.6 ±6.45*</td>
<td></td>
</tr>
<tr>
<td>Leukemic - ALL-B</td>
<td>15</td>
<td>9.8 ±10.7*</td>
<td>0.05</td>
</tr>
<tr>
<td>Controls - BMI Group 4</td>
<td>9</td>
<td>93.5 ±77.37</td>
<td></td>
</tr>
<tr>
<td>Leukemic - ALL-T</td>
<td>5</td>
<td>71.8 ±112.9</td>
<td>NS</td>
</tr>
<tr>
<td>Leukemic - AML</td>
<td>7</td>
<td>20.5 ±27.07*</td>
<td>NS</td>
</tr>
</tbody>
</table>
In the control group comprised of healthy children, there was a significant positive correlation between BMI and blood serum (Fig. 1A) or bone marrow leptin level (Fig. 1B). Such a correlation was not seen in leukemic patients (data not shown). In this group, however, the leptin concentration in blood serum was higher than that in bone marrow in all the BMI groups studied (Fig. 2A). In the group of leukemic children, leptin concentration in peripheral blood was compared with that in bone marrow only in relation to the type of acute leukemia, as these children belonged to the BMI Group 3 and Group 4 (Table 1). The leptin concentration was higher in the blood in children with ALL-B and ALL-T, whereas it was comparable in both blood and bone marrow in children with AML (Fig. 2B).

An analysis of frequency distribution of the Gln223Arg polymorphism in the leptin receptor gene in leukemic children is presented in Fig. 3. In the lymphoblastic leukemia patients, the G/A allele was more frequent, although the difference did not achieve statistical significance. The wild type G/G was more

![Fig. 1. Correlations between body mass index (BMI) and leptin concentration in blood serum (Panel A) and in bone marrow (Panel B) in control, healthy children.](image1)

![Fig. 2. Leptin concentration in peripheral blood and in bone marrow in relation to BMI in control, healthy children (Panel A) and to the type of acute leukemia in leukemic children (Panel B).](image2)
frequent in the AML patients, but likewise the difference was not significant. There were no differences in the genotype frequencies between the AML and ALL-B groups. Nor were there any appreciable differences between the leukemia and control groups. There was no correlation between the leptin level and the type of polymorphism in the control group.

DISCUSSION

Although there is a lot of evidence about the potential role of leptin in myeloproliferative disorders, the relation between leptin and leukemia is unclear. Total leukocyte counts in healthy humans correlate positively with both body fat and leptin (16) and increasing BMI correlates with the diagnosis of acute promyelocytic leukemia (APL) (17). Furthermore, leukemic blast cells can express a functional leptin receptor (10, 18, 19). In our study, we detected a high leptin level in bone marrow of leukemic children, although it was lower than that in control children. These results are in accord with the data of Gaja et al (20). Those authors examined leptin concentrations in the plasma from peripheral blood and in bone marrow in relation to BMI in patients with lymphoproliferative diseases. They showed that bone marrow and peripheral blood leptin levels significantly correlated with each other as well as with BMI. The authors also demonstrated a relation between blood and bone marrow leptin, on the one side, and the percentage of bone marrow fat, on the other side. In addition, they found a negative correlation between blood and bone marrow leptin and bone marrow malignant infiltration (20). In the control group of our study, a significant correlation between BMI and serum or bone marrow leptin level also was found. This effect was not seen in leukemic patients, which suggests that the mechanisms controlling leptin level in leukemic and non-leukemic persons are different. Adipocytes are the potential source of leptin in

Fig. 3. Frequency distribution of the Gln223Arg polymorphism in the leptin receptor gene in control and leukemic children.
bone marrow. These cells are the prevalent stromal cell type and they play a role in hematopoiesis.

A positive correlation between adipocyte differentiation of stromal cells and the ability to support the growth of lymphoid cells has been documented (21). The molecular basis for the ability of adipocytes to support hematopoiesis is unknown. Tabe et al (22) demonstrated that leptin produced by mesenchymal stem cell (MSC)-derived adipocytes controls survival of APL cells (22). The authors investigated the effects of leptin produced by BM adipocytes on APL cells using a co-culture system with MSC–derived adipocytes. They observed antiapoptotic effect dependent on direct cell-to-cell interactions, associated with phosphorylation of signal transducer and activator of transcription-3 (STAT3) and mitogen-activated protein kinase (MAPK) (22). Alternatively, leptin secretion by BM adipocytes in the vicinity of leukemic cells could play a major role in the proliferation and survival of APL cells through paracrine interactions in the marrow microenvironment (22). More recent data have indicated that the development and progression of nonsolid tumors, such as leukemias, also may depend on the generation of new blood vessels from the pre-existing vasculature (23-25). Inhibition of angiogenesis reportedly retarded leukemic growth in mice (26, 27). Among its pleiotropic actions, leptin may function as an angiogenic factor in various in vitro systems as well as in rodent models of angiogenesis (28, 29). Inhibition of the angiogenic process in hematopoietic tissues by targeting leptin activity might, therefore, represent a novel therapeutic option in leukemia.

The presence of leptin RB receptors on primary AML cells, with the highest expression of RB long isoform on acute promyelocytic leukemia (APL) cells, has been observed (10). RB has recently been detected on human hematopoietic progenitor cells expressing CD34 antigen, and leptin was reported to induce proliferation and differentiation in these cells (4, 8). Multiple isoforms of RB have been identified (5, 6). In the present study, we analyzed the frequency of leptin receptor gene polymorphism in leukemic and control children. We failed to detect significant differences in the polymorphism of RB between the non-leukemic and leukemic groups. No differences in the genotype frequency were observed between the AML and ALL groups. This polymorphism is strongly associated with obesity (12-14), but it does not seem to be as much associated with leukemia development. Other authors did find the association between leukemia development and increased RB expression (10). Thus, RB/leptin interactions may play a pathophysiological role in leukemia.

In conclusion, the results of this study indicate a possible relation between the leptin level and acute leukemia development in children. However, the effectory function of the hormone seems not to be related to the Gln223Arg polymorphism of its receptor.
REFERENCES


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