Expression of Death Receptors and Associated Regulatory Proteins in Pediatric Acute Lymphoblastic Leukemia: Preliminary Results
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Abstract: Altered regulation of the cell cycle and/or apoptosis are well established events in the process of neoplastic transformation. Fas-mediated apoptosis is regulated by several inhibitory proteins in normal lymphocytes and leukemic cells. Some of these protein molecules may also play a role in the induction of apoptosis through death receptors for a similar molecule, TNF-related apoptosis inducing ligand (TRAIL). The involvement of these anti-apoptotic molecules in leukemogenesis is uncertain. Our preliminary findings indicate decreased expression of pro-apoptotic TRAIL-DR4 receptors in leukemic T cells relative to untransformed T cells. This decrease in expression may represent a potential determinant of poor acute lymphoblastic leukemia (ALL) prognosis, as it may result in a favorable biological situation for escape from apoptosis. Our data also demonstrate decreased levels of expression of pro-apoptotic Fas protein molecules in pediatric patient samples, relative to ALL cell lines. This difference suggests that circulating leukemic cells differ in their susceptibility to Fas-mediated apoptosis. Taken together, our data suggest that ALL cell lines and circulating leukemic cells from pediatric patients possess different regulatory mechanisms in order to attenuate Fas-mediated signaling.

Traditionally, prognostic data for pediatric leukemia patients is obtained from routine physical examination, bone marrow morphology, and peripheral blood counts of pediatric leukemia patients. With recent developments in the understanding of molecular and cellular events in leukemia, information obtained via karyotyping, molecular genetics, and surface immunophenotype has also been useful for determining the optimal course of treatment along with the likelihood of treatment success.1,3 By determining the molecular composition of various leukemic cells, determination of individual prognoses may be more accurate and treatment may be facilitated due to a better understanding of this disease.

Acute lymphoblastic leukemia (ALL) and acute myeloblastic leukemia (AML) are heterogeneous groups of diseases characterized by malignant proliferation and accumulation of immature lymphoid and myeloid cells in the lymphoid organs, bone marrow, and blood. The neoplastic process that leads to leukemogenesis is characterized by cellular and molecular dysregulation leading to uncontrolled cell growth. Malignant cells often result from alterations to the genes that regulate death receptor-mediated apoptosis. These alterations may be due to genetic mutations or altered expression of affected genes.

Fas (CD95, APO-1) is a member of the tumor necrosis factor (TNF) superfamily. Ligation of the Fas receptor with Fas Ligand induces aggregation of Fas molecules, which creates an intracellular region designated a “death domain”. This domain is essential for inducing caspase-dependent apoptosis in affected cells.4 Altered expression of Fas has been shown to render malignant cells less susceptible to killing by immune effector cells. Interestingly, certain chemotherapeutic agents have been shown to increase expression of Fas on malignant cells and cause Fas ligand-independent aggregation of Fas molecules, leading to apoptosis.5,6 TNF-Related Apoptosis Inducing Ligand (TRAIL) is a molecule that has been shown to induce apoptosis primarily in malignant cells7. TRAIL receptors include death receptors R1 and R2. Although chemotherapy sensitizes cancer cells to killing by TRAIL, it is not known whether apoptosis caused by anti-cancer drugs involves TRAIL receptor signaling.8 Given that both ligand-mediated and ligand-independent triggering of apoptosis through Fas and possibly TRAIL-R1 and -R2 are relevant to the success of chemotherapy, it is reasonable to hypothesize that alterations in the expression of molecules that regulate death receptor-mediated apoptosis may affect chemotherapy-resistance in malignant cells. Thus, it is important to examine the expression of regulatory molecules such as Fas-associated phosphatase (FAP)-1 and FLICE (caspase 8)-Inhibitory Protein (FLIP). To date, no studies have examined the expression of FAP-1 or FLIP in leukemias. The involvement of these anti-apoptotic molecules in leukemogenesis is uncertain.

The purpose of this study was to investigate the expression of Fas, TRAIL receptors, FAP-1, and FLIP by pediatric leukemia cells. For these preliminary analyses, leukemic cells were isolated from two pediatric patients suffering from ALL and AML who had not been previously treated for their malignancy. Expression was compared between these leukemic cells and Jurkat and CEM ALL cell lines, as well as normal circulating leukocytes isolated from non-leukemic pediatric patients, which were used as controls. This preliminary study will eventually enroll additional pediatric patients with leukemia.

Methods

Peripheral blood samples were obtained from pediatric patients treated at the Isaac Walton Killam (IWK) Health Centre. The IWK Research Ethics Board approved this study and its consent forms. Pediatric patients suffering from ALL and AML who were to undergo standard induction chemotherapy, but had not been previously treated for their malignancy, were selected for inclusion in the study. Peripheral blood samples from two patients with ALL were analyzed for this study. Blood was collected prior to induction chemotherapy and was processed immediately to prevent potential degradation or alteration of samples. Leukemic cells were isolated by standard procedures. Briefly, heparinized

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blood samples were separated by Ficoll-Hypaque density centrifugation at the time of collection. Purified leukocyte fractions were washed with phosphate-buffered saline (PBS). Normal circulating leukocytes isolated from non-leukemic pediatric patients were used as controls.

RNA Isolation
mRNA was isolated from 4 x 10^6 leukemic cells and control peripheral blood lymphocytes. Total RNA was isolated from T-cells using TRIzol (Gibco BRL) as per the manufacturer’s instructions.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis
Reverse transcription of mRNA was carried out in a 20 ml volume containing 200 U Moloney murine leukemia virus reverse transcriptase, 1 mg random hexanucleotide primers, and 0.5 mM dNTPs. The reaction mixtures were incubated at 37°C for 1 hour, and then at 95°C for 10 minutes. The final volume was adjusted to 200 ml with pyrogen free water. cDNA in a total volume of 50 ml, along with 2.5 ml Taq DNA polymerase, 0.2 mM dNTPs, and 50 mM of each primer were used for subsequent polymerase chain reaction (PCR). The PCR reaction mixture was covered with 100 ml of mineral oil to prevent evaporation. The following primers were used for PCR (Table 1):

PCR amplification was achieved using the following protocols:
• b-actin: 26 cycles, denaturation 94°C 30s, annealing 57°C 30s, synthesis 72°C 60s
• Fas: 29 cycles, denaturation 94°C 30s, annealing 57°C 30s, synthesis 72°C 60s
• TRAIL DR4: 29 cycles, denaturation 94°C 30s, annealing 57°C 30s, synthesis 72°C 90s
• TRAIL DR5: 29 cycles, denaturation 94°C 30s, annealing 57°C 30s, synthesis 72°C 90s
• FLIP: 32 cycles, denaturation 94°C 30s, annealing 57°C 30s, synthesis 72°C 120s
• FAP-1: 34 cycles, denaturation 94°C 30s, annealing 57°C 30s, synthesis 72°C 90s

PCR products were visualized by electrophoresis on ethidium bromide stained 1.8% agarose gels. Relative abundance of PCR products was determined by densitometric analysis of gel scans. The number of PCR cycles selected for amplification of cDNA was previously determined based on the generation of a PCR product during the exponential phase. For example, in order to determine the appropriate number of PCR cycles for determination of relative abundance of mRNA coding for b-actin, Fas, TRAIL DR4, TRAIL DR5, FLIP, and FAP-1, the amplification curve of each PCR product was determined by examining a range of cycles from 20 to 41. The appropriate number of cycles was determined based on the number of cycles required to amplify a product during the exponential phase without reaching a plateau of amplification. RT-PCR performed under these conditions allows for semi-quantitative analysis of mRNA levels and the detection of twofold or greater differences in expression.9

Results
Cell viability is similar in malignant and normal lymphocytes
In order to assess whether there was a difference in the apoptotic-status of untreated normal and malignant cells, cell viability was measured by trypan-blue exclusion. Jurkat ALL cells, CEM ALL cells, normal circulating lymphocytes, and circulating leukemic cells exhibited similar cell viability (Figure 1).

Altered expression of regulatory genes FAP-1 and FLIP in ALL
Following the determination of the optimal number of PCR cycles (data not shown), mRNA expression of three apopto-

Table 1: The primers used for PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Product Size</th>
<th>Primer Direction</th>
<th>Primer Sequence</th>
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<tbody>
<tr>
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<td>5’-TGGACATCCCGCAAGACCCGTACG-3’</td>
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<td></td>
<td></td>
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<td>5’-TGTCACCTTCACCGTCCAGTT-3’</td>
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<td>Fas</td>
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<td></td>
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<td>5’-AGGCTGACAGAAGGCAAATAC-3’</td>
</tr>
</tbody>
</table>

Figure 1: Viability of normal peripheral leukocytes and leukemic cell lines (CCRF-CEM and Jurkat), as assessed by trypan blue exclusion.
more susceptible to Fas-mediated apoptosis than are circulating lymphocytes. This finding suggests that the ALL cell lines may be more sensitive to normal circulating lymphocytes, but was increased in ALL cell lines. Decreased expression of pro-apoptotic receptors such as Fas and TRAIL-DR4 could conceivably contribute to the development of drug-resistance in malignant cells. The data presented in this study indicate that expression levels of transcripts of Fas, TRAIL-DR4, and FAP-1 were altered in ALL cell lines and leukemic cells isolated from pediatric patients, relative to normal circulating lymphocytes. Since these molecules are involved in the regulation of the TRAIL- and Fas-mediated apoptotic pathways and certain chemotherapeutic agents induce apoptosis through Fas (and possibly TRAIL receptor) aggregation, a given leukemic cell clone may be more or less susceptible to chemotherapeutic measures based on its expression of these regulatory molecules. Decreased expression of pro-apoptotic receptors such as Fas and TRAIL-DR4 could conceivably contribute to the development of drug-resistance in malignant cells.

Fas expression was decreased in patient samples relative to normal circulating lymphocytes, but was increased in ALL cell lines. This finding suggests that the ALL cell lines may be more susceptible to Fas-mediated apoptosis than are circulating lymphocytes and leukemic cell lines (CCRF-CEM and Jurkat).

**Figure 2:** RT-PCR analysis of mRNA expression for FAP-1, FLIP, Fas, and TRAIL-DR4 in normal peripheral leukocytes and leukemic cell lines (CCRF-CEM and Jurkat).

The primary objective of this study was to determine the relative mRNA expression of pro- and anti-apoptotic molecules in leukemic cells, as compared with normal lymphocytes. Results showed that differences observed in mRNA expression between normal circulating lymphocytes, ALL cell lines, and leukemic cells isolated from pediatric patients were not due to differences in the viability of these cells (Figure 1).

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References