Study of Caspase-3 Level in Relation to Prognostic Risk Factors in Children with Acute Lymphoblastic Leukemia

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Abstract:

Apoptosis refers to a biochemically regulated process of automated cell death mediated through a highly organized network of interacting proteases. Malignant transformation of hematopoietic progenitors into leukemic cells results in defects in the cell cycle regulation; including defects in apoptosis. Multiple mechanisms may account for why leukemic cells become resistant to chemotherapy. One common cause includes defects in the cell inherent programmed cell death (apoptosis). This study was conducted on 20 children with newly diagnosed ALL and 10 controls. Our aim was to evaluate the level of caspase-3 among them and relate it to prognostic factors. It can be concluded that caspase-3 level after induction therapy proved to be a significant predictor of remission stage especially for B-ALL. CD10 positive cases also showed a significant difference between caspase-3 level before and after induction therapy. The same was demonstrated with low risk cases. We can also conclude that failure of caspase-3 level to increase after induction is associated with poor prognosis.

Introduction:

Malignant transformation of hematopoietic progenitors into leukemic cells results from aberrations in the control of cell cycle regulation and proliferation, differentiation, and the abrogation of cell suicide, or apoptosis. Acute leukemia is a neoplastic disease characterized by a rapid accumulation of primitive hematopoietic cells. Acute leukemia is subclassified as myeloid or lymphoid depending on the origin of the malignant cell. Acute lymphocytic leukemia (ALL) is predominantly a disease of childhood with 75% of all cases occurring in patients younger than 15 years of age. In contrast, acute myeloid leukemia (AML) is more common in adults with an incidence that increases with age. Both AML and adult ALL are aggressive diseases with generally poor prognosis.

Multiple mechanisms may account for why leukemic cells become resistant to chemotherapy. One common cause includes defects in the cell's inherent programmed cell death (apoptosis) pathways. These defects may impair the ability to achieve remission and cure with chemotherapy. In an attempt to overcome drug resistance and improve clinical outcomes, attention is turning to developing therapeutic agents that overcome defects in the apoptosis pathways. Although mostly in preclinical stages of development, these efforts offer a look into the future of agents that may eventually be available for treating leukemia. Apoptosis in particular refers to a biochemically regulated process of automated cell death mediated through a highly organized network of interacting proteases, caspases, and their inhibitors in response to noxious stimuli from either inside or outside of the cell. Progression of apoptosis is characterized by nuclear condensation, DNA fragmentation, dismantling of cell ultrastructure, membrane blebbing, formation of apoptotic bodies and subsequent phagocytosis. At least 14 members of the caspase family were identified, a subset of which participates in apoptosis. The remainder likely to be involved in the processing of pro-inflammatory cytokines. Caspases play a central role as both initiators and executioners of pathways that abrogate the effect of nuclear DNA repair enzymes such as poly(ADP-ribose) polymerase and other nuclear and cytosolic proteins, thereby inducing the changes characteristic of apoptotic cell death. Caspases are synthesized as inactive pro-enzymes and undergo cleavage to produce the active enzyme containing two subunits. Caspases are able to cleave themselves; they can also be activated by other caspases. At present, at least 2 major pathways of caspase activation have been revealed: (1) the receptor-mediated apoptosis pathway where the TNF family of death receptors activate upstream caspase-8 and (2) the mitochondrial-mediated apoptosis pathway where cytochrome c is released from the mitochondria and activates upstream caspase-9. Both pathways share in the activation of a major downstream effector caspase; caspase-3. The caspase-3 gene has been mapped to chromosome 4q33-q35.1. The protein assumes a prominent role as a down-stream-acting caspase, largely responsible for proteolytic cleavage of key proteins such as poly(ADP-ribose) polymerase. It is highly expressed in cells of...
lymphocytic origin. A third minor pathway of caspase activation involving granzyme B has also been revealed. Granzyme B is a serine protease synthesized in cytotoxic T lymphocytes via the pore-forming protein, perforin, cytotoxic T lymphocytes inject granzyme B into target cells. Granzyme B directly cleaves and activates several caspases including procaspase-3. As such, it bypasses both the mitochondrial-mediated and receptor-mediated pathways of caspase activation. At a basic level, chemoresistance and treatment failures in acute leukemia represent a failure of the malignant clone to undergo apoptosis in response to chemotherapeutic agents. Resistance to apoptosis in acute leukemia is often due to aberrant gene expression. Chromosomal translocations are classic examples of aberrant gene expression in acute leukemia. Defects in the apoptosis pathway render leukemic blasts resistant to multi-agent chemotherapy. These blocks translate clinically into reduced rates of remission, higher rates of relapse, and reduced overall survival. Studies have identified specific blocks in the receptor-mediated and mitochondrial-mediated pathways that confer prognostic information in adult patients with acute leukemia.

**Subjects and Methods:**

This study was conducted on 20 children admitted to the Hematology/Oncology unit, Alexandria University Children Hospital, with newly diagnosed ALL. Ten apparently healthy, age and sex matched children were included as a control group. The aim of this work was to evaluate the role of Caspase 3 among children with newly diagnosed ALL and to try to relate it to prognostic risk factors. All subjects were subjected to thorough medical examination and history taking. A diagnosis of ALL was based on morphological analysis of peripheral blood, marrow aspirates according to guidelines published by the French-American-British Cooperative Group. Diagnostic evaluations included cytochemical staining with myeloperoxidase, and immunophenotyping by flow cytometry. ALL was diagnosed if blasts were morphologically lymphoid, myeloperoxidase-negative, and positive for at least two T-cell markers, or for CD19, CD20, or CD10 (common acute lymphocytic leukemia antigen).

Active Caspase-3 estimation in cell extracts of peripheral blood was carried out using a quantitative sandwich enzyme immunoassay. This was done initially at first diagnosis and after 4 weeks induction therapy. Ten milliliters of blood were withdrawn on EDTA, left to sediment for 30 minutes to collect theuffy layer. This buffy layer was pelleted at 1000 x g, washed three times using PBS; then cells were resuspended at a concentration of 10^10 cells/ml. For induction of apoptosis, 1 μl staurosporine was added to the cell suspension and incubated at room temperature for one hour. After incubation, 2 ml of 5 mM biotin-ZVKD-fmk were added per ml cell suspension to label active caspases in cells, incubated for one hour at room temperature. An extraction buffer containing a group of protease inhibitors was used to avoid in vitro caspase activation. Samples were then stored in the extraction buffer overnight at 4°C. Cell extracts were diluted using a diluent before proceeding with the ELISA. Inactive Caspase-3 zymogen was not modified by the biotin-ZVKD-fmk inhibitors and hence was not detected. After determining the Caspase-3 concentration from the standard curve, the relative amount of Caspase-3 in cells was calculated, we divided the concentration by the cell number per milliliter in the diluted sample.

**Results:**

Peripheral blood specimens from 20 patients (10 males and 10 females) with newly diagnosed ALL were analyzed to quantitate caspase-3. Normal controls were obtained from children attending the outpatient clinic suffering minor conditions. All subjects ranged in age between 2–14 years with a mean of 6.6 ± 4.16 years. Pallor was the most common presenting complaint (13 cases), then fever in 50 % of cases, arthralgia (7 cases), bruising (5 cases), weight loss (5 cases) and active bleeding in 10% of cases. Splenomegaly was the most common physical finding (14 cases), hepatomegaly in 9 cases and lymphadenopathy in 8 cases. Two cases had central nervous system infiltration at diagnosis: one presented by paraplegia and was confirmed by CSF pathology and MRI dorsolumbar spine, while the other was proven only by CSF pathology. One case had a mediastinal mass proven by X-rays.

ALL cases had an initial WBC count ranging between 2600 – 436000 / cmm. Day 14 bone marrow examination gave a blast count of 0 – 5 % with a mean of 2.15 ± 1.46; while their initial bone marrow blasts were 77 – 97 % with a mean of 88.05 ± 6.0 %. The mean value of caspase-3 among controls was 1.38 ± 0.96 ng/10^6 cells, while among patients before treatment it was 1.13 ± 0.87 ng/10^6 cells. No statistical difference was found between them although some other groups found a statistically significant difference between patients and the control.

When we compared the level of caspase-3 before and after induction therapy between cases, we found that the level of caspase-3 in B-ALL was 1.148 ± 0.77.
before induction therapy and 2.02 ± 1.14 after induction. This difference was found to be statistically significant (*p = 0.025*).

The level of caspase-3 after induction treatment was 1.74 ± 1.2 ng/10^5 cells. The caspase level after treatment was higher, yet statistically non significant (table II).

**Table I: Level of Caspase-3 before induction therapy of children with acute lymphoblastic leukemia and their control**

<table>
<thead>
<tr>
<th>Caspase level (ng/10^5 cells)</th>
<th>Level before treatment</th>
<th>Control</th>
<th>Test of significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Minimum</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Maximum</td>
<td>3.1 ± 3.0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>1.13 ± 1.38</td>
<td>1.38</td>
<td>t = 0.7, p = 0.48</td>
</tr>
<tr>
<td>S.D</td>
<td>0.87 ± 0.96</td>
<td>0.66</td>
<td></td>
</tr>
</tbody>
</table>

**Table II: Level of Caspase-3 after induction therapy of children with acute lymphoblastic leukemia and their control**

<table>
<thead>
<tr>
<th>Caspase level (ng/10^5 cells)</th>
<th>Level after treatment</th>
<th>Control</th>
<th>Test of significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Minimum</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Maximum</td>
<td>3</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>1.74 ± 1.38</td>
<td>1.38</td>
<td>t = 0.82, p = 0.41</td>
</tr>
<tr>
<td>S.D</td>
<td>1.20 ± 0.96</td>
<td>0.96</td>
<td></td>
</tr>
</tbody>
</table>

**Table III: Level of Caspase-3 before and after induction therapy of children with acute lymphoblastic leukemia**

<table>
<thead>
<tr>
<th>Caspase level (ng/10^5 cells)</th>
<th>Level before treatment (ng/10^5 cells)</th>
<th>Level after treatment (ng/10^5 cells)</th>
<th>Paired t test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>1.13 ± 0.77</td>
<td>1.74 ± 1.14</td>
<td>t = 2.49, p = 0.025*</td>
</tr>
<tr>
<td>SD</td>
<td>1.07 ± 0.65</td>
<td>2.02 ± 1.14</td>
<td></td>
</tr>
<tr>
<td>Paired – t test</td>
<td>1.02</td>
<td>1.20</td>
<td>p = 0.084</td>
</tr>
</tbody>
</table>

**Table IV: Level of Caspase–3 in children with ALL by disease subtype**

<table>
<thead>
<tr>
<th>Type of ALL</th>
<th>Level before treatment (ng/10^5 cells)</th>
<th>Level after treatment (ng/10^5 cells)</th>
<th>Paired t test</th>
</tr>
</thead>
<tbody>
<tr>
<td>B-ALL (n=16)</td>
<td>1.148 ± 0.77</td>
<td>2.02 ± 1.14</td>
<td>t = 2.49, p = 0.025*</td>
</tr>
<tr>
<td>T-ALL (n=4)</td>
<td>1.06 ± 1.32</td>
<td>1.62 ± 0.75</td>
<td>t = 0.54, p = 0.625</td>
</tr>
</tbody>
</table>

*Significant = p < 0.05

In T-ALL the level of caspase-3 before induction therapy was 1.06±1.32 and it became 0.62±0.75 after induction therapy; this was statistically non significant (p = 0.62).

Bone marrow immunophenotyping revealed that 16 cases were CD19 positive, the mean level of caspase-3 among these was 1.27 ± 0.86 before induction therapy and 1.8 ± 1.26 after. The difference was statistically non significant. CD 10 positive cases (13 cases) showed a significant difference between levels before and after induction therapy. The level of caspase-3 was 1.15 ± 0.85 before and 2.02 ± 1.06 after (p=0.04) induction therapy. CD 34 positive cases were 3 out of 16 B-ALL cases and showed no significant difference between caspase-3 level before and after induction therapy; 0.9 ± 0.78 and 2.1 ± 0.85 respectively. CD7 positive cases; 4 T-ALL cases also showed a non significant difference between the two levels; 0.56 ± 0.71 and 1.5 ± 1.08 respectively.

Our cases were divided into high and low risk groups according to certain criteria; B-ALL, initial WBC count less than 100,000/cmm, age between 2 and 10 years and clearing of bone marrow by day 14 classify as low risk. The difference between caspase-3 level before; 1.23 ± 0.88 and after; 2.25 ± 0.96, induction therapy in the low risk group showed a statistically significant value (p = 0.047). Comparing the values of caspase-3 between cases entering into remission (n=15) and those that die or fail to enter into a remission, we found that there was a statistically significant difference between caspase-3 level before; 1.09±0.85 and after; 1.89 ± 1.15 induction therapy (p=0.038). In cases that failed to enter into remission or died the values were 1.23 ± 1.01 before and 1.3 ± 1.39 after induction therapy with no statistical significance (p=0.94).
Discussion:

Dysregulation of apoptosis is an important mechanism in leukemogenesis. Caspases are cysteine proteases that play a major role in the activation of apoptotic pathways and chemotherapy-induced cell death. The demonstration that many anti-neoplastic agents induce apoptosis in susceptible cells raised the possibility that factors affecting caspase activation and activity might be important determinants of anticancer drug sensitivity. High levels of inactive, uncleaved caspase 2 and caspase-3 have recently been associated with poor survival in patients with acute myelogenous leukemia. We hypothesized a similarly significant role for caspase-3 in patients with acute lymphoblastic leukemia. Inappropriate activation or inhibition of apoptosis has been implicated in many human diseases. A series of recent studies have demonstrated that most, if not all, chemotherapeutic agents exert their tumoricidal effects by inducing apoptosis in target cells and tissues.

Early identification of patients with chemoresistant acute leukemia who are destined to fail to achieve remission after induction chemotherapy is important, so that they may be offered more aggressive or alternate therapy at disease onset.

Our study included 20 cases of newly diagnosed ALL and 10 apparently healthy controls. We measured the cleaved (activated) caspase 3 level in these patients. Caspase-3 level before induction therapy showed no statistically significant difference from controls. These findings contradict those presented by the M.D. Anderson cancer centre group, who demonstrated that median values of caspase-3 were lower in normal controls than in patient samples. The difference may be due to the fact that they studied adult ALL, while we studied pediatric ALL. Also, they measured uncleaved caspase-3 whereas we measured activated (cleaved) caspase-3. According to the levels of caspase 3 presented by the M.D. Anderson group, our control values of caspase-3 are high; this could be due to caspases originating from different cells.

The level of caspase-3 in our B-ALL cases showed a higher level after induction therapy than before therapy and this difference was statistically significant. This was not the case with T-ALL. CD10 positive case, in this study, showed a higher level of caspase-3 after induction therapy than before, and this was statistically significant. This agrees with the study of Cutrone et al., who considered CD10 a favorable prognostic marker for ALL children, as CD10 positive blasts had a great propensity to apoptosis. Whether or not CD10 positivity is related to better response to chemotherapy needs to be further studied. This finding was not demonstrated with CD19 positive cases nor with CD34 positive cases.

We found that cases that achieved remission showed a statistically significant higher caspase-3 level after induction therapy than before therapy. Also, the low risk group of patients showed the same trend of increasing caspase-3 after treatment. These findings agree with Stefan Faderl et al., who suggested a critical cutoff level for caspase-3 (> 0.37) above which the probability of achieving a complete remission was significantly higher. It was also demonstrated by a group of researchers that high levels of the inactive (uncleaved) form of caspase-3 correlated with decreased survival, whereas high levels of cleaved, activated caspase 3 correlated with better survival.

Because of the limited power (n = 20) of our study, the significance of caspase-3 on overall survival remains to be validated by further investigations. Fractionation of blasts from other cell populations could be useful in further studies for comparisons to be valid as, for example, T-lymphocytes are known to express low caspase levels and they constitute most of the peripheral mononuclear cells. Further studies are needed in this area to try to improve and refine ALL risk classification schemes in order to precisely tailor therapeutic approaches to the biology of the tumor and the response of the patient, as defects in the apoptosis pathway render leukemic blasts resistant to multi-agent chemotherapy, these blocks translate clinically into reduced rates of remission, higher rates of relapse and reduced overall survival.

References:
