Case report:

Apoptotic Molecules In Chronic Leukemia

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Abstract

Background: Chronic myeloid leukemia (CML) is characterized by the spread of malignant cells that exhibit resistance to caspase-mediated cell death (apoptosis) and this mechanism is proposed to play an important role in myeloid cell growth. However, the extent to which caspase-mediated cell death plays a crucial role in the regulation of myelopoiesis remains controversial. ***Objectives & Proceedure****:* The objectives of this study were to examine whether or not caspase-mediated cell death-related proteins take part in the development of CML and to also to detect the relationship between Fas, p53 and caspase-mediated cell death protease activating factor (Apaf-1) in 5 patients with CML using the real-time quantitative polymerase chain reaction. We demonstratedthatp53 and Apaf-1 messenger ribonucleic acid (mRNA) expression was moderately elevated (up to 5 fold, $p<0.05$) in 4 out of 5 CML patients. One patient with a p53 point mutation, exhibited a far greater elevation of p53 mRNA expression throughout their blast crisis, but in contrast, displayed a significant reduction in levels of Apaf-1 mRNA and Fas mRNA. ***Conclusion****:* Our results show in-vivo linkages between Fas, p53 and Apaf-1 transcription parameters suggesting that the key genes involved in the caspase-mediated cell death might contribute to CML disease development.

Bangladesh Journal of Medical Science Vol. 15 No. 04 October'16

Introduction

Chronic myeloid leukemia (CML) (ref1) disease develops as a clonal hemopoietic stem cell expansion, characterized by a chronic phase (CP), an accelerated phase followed by a blast crisis (BC) phase. Understanding the key cellular and molecular mechanisms within the BC phase of CML, represent are important as once the BC phase is established, treatment becomes problematic with a consequent decline in a positive prognosis. It is well-established that the appearance of the breakdown point cluster region-Abelson (BCR-ABL) fusion protein is associated with anti-apoptotic defects, high levels of proliferation, insensitivity to negative regulators and defects in adhesion mechanisms[1-2]. These defects are likely to be contributing factors to CML myeloid expansion. Caspase-mediated cell death is a complex, tightly controlled, active cellular process that is critically important for the survival of multicellular organisms by disposing of harmful, damaged or infected cells that may interfere with hemostasis[3]. A key factor in caspase-mediated cell death machinery is the caspase-mediated cell death protease activating factor (Apaf-1), which is released with cytochrome-c from the mitochondrial membrane, activating its oligomerization into a heptameric complex that binds pro-caspase-9, to create a multi-protein structure known as the "apoptosome" leading to downstream

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events that result in apoptotic cell death[4].

The regulation of caspase-mediated cell death in hemopoiesis might afford a mechanism for regulating stem and progenitor cell population size. Suppression of caspase-mediated cell death as a mechanism for treating myeloid expansion in CML remains contentious and is the subject of active research worldwide. This study was conducted between January 2015-Dec 2015 at the Leukemia Research Unit, PSAU, Al-Kharj, Saudi Arabia. We, therefore considered it important to examine expression of Fas, p53 and Apaf-1 through the course of progression from diagnosis through to the BC phase in 5 patients with CML.

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The level of expression of p53, Fas and Apaf-1 was established using real-time quantitative PCR. The expression of p53 was moderately elevated (up to 5 fold, $p<0.05$) in 4 out of 5 CML patients. A very high level of p53 expression (12 fold, $p<0.05$) was observed during BC in patient 5. In contrast, none of the5 patients exhibited Fas expression. Amongst the 4 CML patients who exhibited a moderate p53 elevation, a rise in Apaf-1 expression was also detected (3-7 fold, $p<0.05$). Furthermore, a marked reduction (8 fold) in Apaf-1 during BC was recorded in patient 5 who had a very high level of p53. We intended to additionally examine p53 for mutations and deletions in chronic and BC samples from all 5 patients and performed PCR amplification on DNA extracted from all 5 patients at the CP and BC phases with primers specific for each p53 exon using DNA cycle sequencing system (Promega, Madison, USA) according to the manufacturer's instructions. During the CP phase of the disease, no mutations or deletions were found.

On analysis of BC phase samples, we established a heterozygote point mutation in patient 5, revealing an exchange of arginine-serine (Arg-Ser) at codon 281 of the p53 protein (data not shown). We also cultured colony-forming unit granulocyte (CFU-GMs) in methylcellulose supplemented with fetal bovine serum and recombinant human cytokines as previously described(ref5). Colonies comprising of 50 cells or more were counted with an inverted microscope by a blinded investigator. From CML patient 5 we recorded a substantial increase in myeloid clonogenic cell frequency from the bone marrow (**Figure 1**).

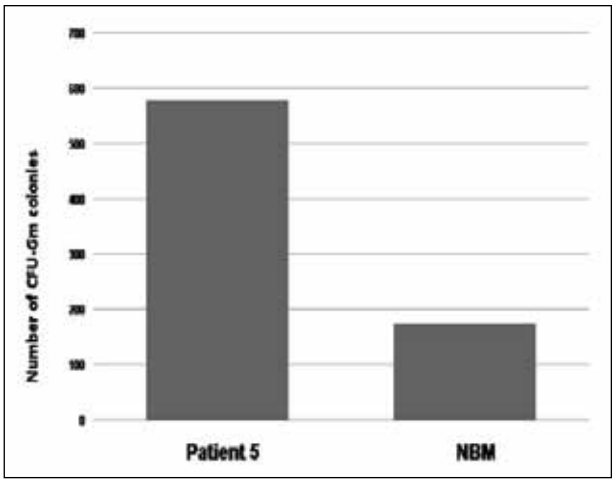

Figure-1: Number of CFU-GM colonies per 105 bone marrow MNC derived from patient 5 grown in methylcellulose containing 10% FCS and supplemented with recombinant human cytokines (IL-3, G-CSF, GM-CSF and SCF) and showing a dramatic difference between normal controls and patient no.5.

The Fas cDNA was re-established into CML CFU-GM by utilizing retroviral-mediated gene transfer technology. The occurrence and correct orientation of the Fas fragment were confirmed by PCR and the successful expression Fas cDNA in CML CFU-GM restored Fas function. Importantly, the Fas-transduced CML CFU-GM colonies demonstrated a substantial and significant increase in the apoptotic rate when compared with the CML CFU-GM transduced with the empty vector (Figure 2).

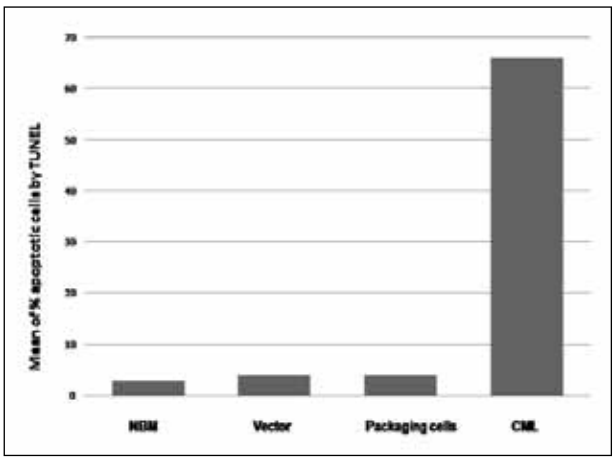

Figure-2: Apoptosis percentage of Fas-transduced CML CFU-GM cells. There is a dramatic increase in apoptosis in Fas-transduced colonies compared to those transduced with empty vector alone, those mock-transduced with GP-E-86 cells, and those of untransduced controls.

Further assays using immunostaining and flow cytometry demonstrated that CML CFU-GM cells did not express Fas receptors. Following transduction of CML CFU-GM cells with Fas, the cell surface expression of Fas was confirmed by flow cytometry (Figure 3).

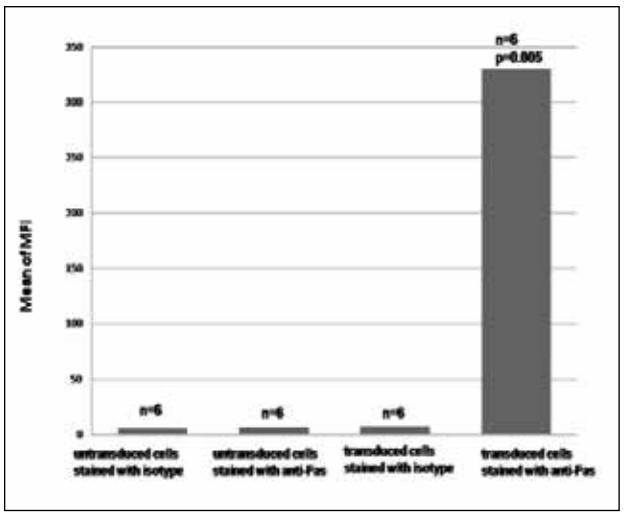

Figure-3: Flow cytometry data showing the expression of Fas on CFU-GM cells. There is a significant difference in the level of Fas expression on the transduced cells stained with FITC conjugated human anti-Fas Mab, compared to untransduced cells (Wilcoxon Signed-rank test

To further confirm that the transduced Fas was functional, the transduced CML CFU-GM cells were removed from the transwells and resuspended in 300 ml of minimal essential medium (MEM) containing a monoclonal antibody (Mab against Fas, or soluble Fas ligand (sFasL), or both. Apoptotic cells were quantitated using terminal deoxynucleotidyltransferase deoxynucleoside-triphosphate (dNTP) scratch end marking (TUNEL). There were higher rates of apoptotic cells when Fas-transduced CFU-GM cells were treated with human anti-Fas Mab, or sFasL, or both, versus the control untransduced CFU-GM colonies(Figure 4).

Discussion

Albrecht et al and other workers[6-8] have previously demonstrated that cells from CML patients or healthy controls exhibit comparable sensitivity to apoptosis inducing signals. The transformed p53 found in CML patient 5, who also exhibited a large decrease in levels of Apaf-1 mRNA, suggested that there is a related, linkage between Fas, p53 and Apaf-1 that might contribute to the development of CML. It could be interpreted that this in vivo linkage between Fas, p53, and Apaf-1 with the genes required in caspase-mediated cell death are also required in CML development. Caspase-mediated cell death is not atypical of CML cells and it has been suggested that the BCR- ABL oncoprotein promotes CML cell resistance to caspase-mediated cell death in these conditions[9-10]

We did anticipated a reduced expression levels of Apaf-1 and p53 in BC but were surprised to observe that p53 expression was exceptionally elevated in all BC phase tests when compared with the chronic phase CML. Interestingly, whilst reasonably high p53 levels were for the most part found in conjunction with elevated Apaf-1 expression, a decrease in Apaf-1 expression was observed when p53 levels were particularly high, suggesting an inhibition of the in the p53 pathway. We acknowledge that improved expression of these pro-apoptotic genes might corresponded with BC changes, or the response to treatment, or both[11]. Indeed, Apaf-1 is upregulated, and apoptosome assembly is mediated, by some oncoproteins, such as E2F[12].

In addition, Kannan et al[13] demonstrated that p53 is an upstream controller of Apaf-1 and is additionally changed, deleted, or enhanced, or both, in 25% of BC myeloid leukemias[14]. It could be deduced that the interplay between the pathways managing both the cell cycle and caspase-mediated cell death will help establish a harmony between the rate of cell division and caspase-mediated cell death of any cell population in vivo, with any loss of function potentially prompting an increment in their self-replication[15]. The retroviral tests demonstrated that myeloid development in the fifth CML patient might be a response to insufficient Fas expression. In this single a clinical case, the presence of an inadequate Fas expression combined with an elevated, p53 expression with diminished Apaf-1 expression that may have contributed to the progression to the CML BC stage.

Acknowledgement

This project was supported by a research grant from the deanship of scientific research at the Prince Sattam bin Abdulaziz University, SAUDI ARABIA (ref no: RU-2015-101).

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