

# A close link between Fas, p53 and Apaf-1 in chronic myeloid leukemia

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## ABSTRACT

In chronic myeloid leukemia (CML) proliferation is increased and resistance to apoptosis has been proposed as a mechanism accounting for myeloid cell expansion. There is still controversy on whether apoptosis plays an important role in the regulation of myelopoiesis. This study aims to investigate whether apoptosis-related proteins play a role in the evolution of CML and to identify the relationship between Fas, p53 and apoptosis protease activating factor (Apaf-1) in CML. We found increased p53 and Apaf-1 messenger ribonucleic acid (mRNA) in patients with CML. However, one patient, who had a p53 point mutation, showed a massive elevation of p53 mRNA during blast crisis yet, conversely, a considerable reduction in Apaf-1 mRNA and Fas mRNA. Our results show an in-vivo linkage between Fas, p53 and Apaf-1 transcription regulation. This suggests that key genes involved in apoptosis are also involved in CML disease progression.

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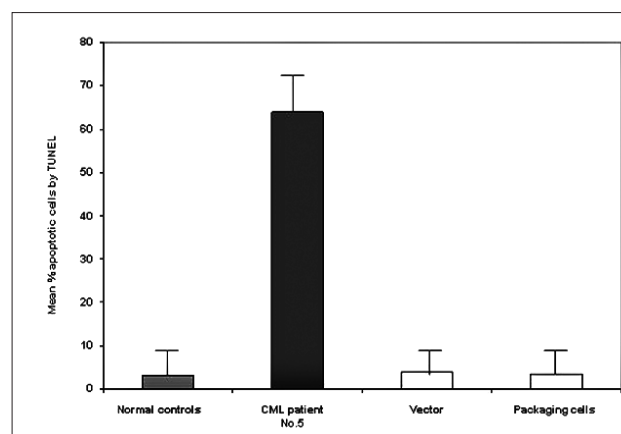
Chronic myeloid leukemia (CML)<sup>1</sup> is an acquired clonal chemopoietic stem cell disease, with a chronic phase (CP), an accelerated phase, and a blast crisis (BC) phase. Understanding the key cellular and molecular mechanisms involved in the BC phase of CML represents a vital goal in patient management because, once BC has been reached, treatment becomes extremely difficult, and prognosis poor. Anti-apoptotic defects, high levels of proliferation, insensitivity to negative regulators and defects in the adhesion mechanisms are considered to result from expression of the break point cluster region-Abelson (BCR-ABL) fusion protein.<sup>2</sup> These defects are therefore, likely to be responsible for CML myeloid expansion. Apoptosis is a complex, tightly regulated, active cellular process. Its role is to remove harmful, damaged, or unwanted cells.<sup>3</sup> A key factor in apoptotic machinery is the apoptosis protease activating factor (Apaf-1), which is released with cytochrome-c from the mitochondrial membrane, triggering its oligomerization into a heptameric complex (that binds pro-caspase-9), to form a multi-protein structure known as the "apoptosome", and thereby causing apoptotic cell death.<sup>4</sup> Regulation of apoptosis in hemopoiesis could potentially provide a mechanism for regulating stem and progenitor cell population size. Suppression of apoptosis as a mechanism for treating myeloid expansion in CML is still controversial, and currently the subject of active research in many laboratories worldwide. We thus, considered it important to assess Fas, p53 and Apaf-1 expression through the course of the progression of CML from diagnosis through to the BC phase.

**Case Report** Real-time quantitative polymerase chain reaction (PCR) assays were developed to determine the level of expression of p53, Fas and Apaf-1. The p53 expression was moderately elevated in 4/5 CML patients (up to 5 fold,  $p < 0.05$ ). During BC in patient 5, we noted a very high level of p53 expression (12 fold,  $p < 0.05$ ). On the contrary, Fas expression was undetectable in all 5 patients. Among the 4 CML patients who exhibited moderate p53 elevation, an increase in Apaf-1 expression was also observed (3-7 fold,  $p < 0.05$ ). In contrast, patient 5, with very high levels of p53 had a marked reduction (8 fold) in Apaf-1 expression during BC. We further examined p53 for mutations and deletions in chronic and BC samples from all 5 patients. We performed PCR amplification on DNA extracted from all patients at the CP and BC phases with primers specific for each p53 exon using DNA cycle

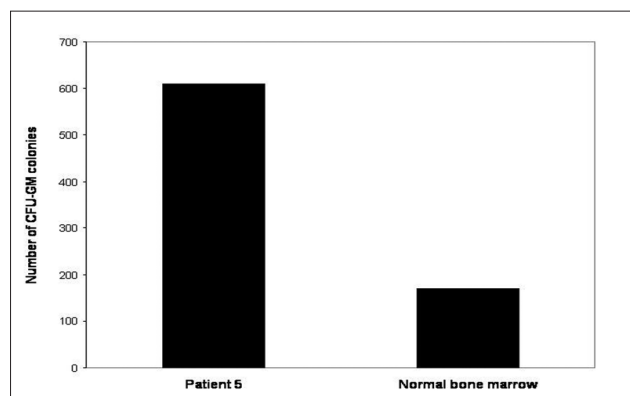
sequencing system (Promega, Madison, USA) according to the manufacturer's specifications. No mutations, nor deletions, were found during the CP of the disease. However, when we analyzed BC phase samples, we found a heterozygote point mutation in patient 5, revealing an exchange of arginine-serine (Arg-Ser) at codon 281 of the p53 protein (data not shown). The CML colony-forming unit granulocyte-macrophage (CFU-GMs) were grown in methylcellulose containing fetal calf serum and supplemented with recombinant human cytokines. Colonies consisting of 50 cells or more were scored under an inverted microscope. There was a substantial increase in myeloid clonogenic cell frequency in the bone marrow from CML patient number 5 (Figure 1). The Fas cDNA was restored into CML CFU-GM using the retroviral-mediated gene transfer technology.<sup>5</sup> The presence and orientation of the Fas fragment was confirmed by PCR. Our report found, that the retrovirus-mediated transduction of Fas cDNA restored Fas function (namely, it is capable of activating the apoptotic process). The Fas-transduced CML CFU-GM colonies showed a dramatic elevation in the apoptotic percentage compared to the untransduced CML CFU-GM, and the CFU-GM transduced with empty vector (Figure 2). Preliminary experiments showed that CML CFU-GM cells do not express Fas receptors when examined by flow cytometry. After transduction of CML CFU-GM cells with Fas, the cell surface expression of Fas was confirmed by flow cytometry (Figure 3). In order to confirm that the transduced Fas was functional, the transduced CML CFU-GM cells were taken from the transwells and resuspended with 300 ml of minimal essential medium (MEM) with anti-Fas monoclonal antibody (Mab), or soluble Fas ligand

(sFasL), or both, and apoptotic cells were quantitated by terminal deoxynucleotidyl transferase deoxynucleoside-triphosphate (dNTP) nick end labelling (TUNEL). There were higher percentages 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 groups (Figure 4).

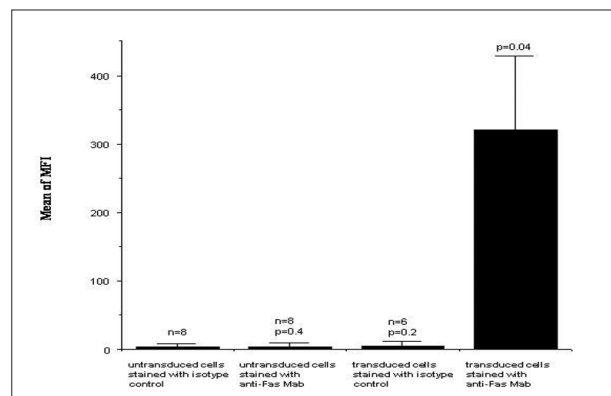
**Discussion.** Albrecht et al and others<sup>6-8</sup> showed that CML patients and normals are equally sensitive to apoptotic stimuli, and concluded that defective



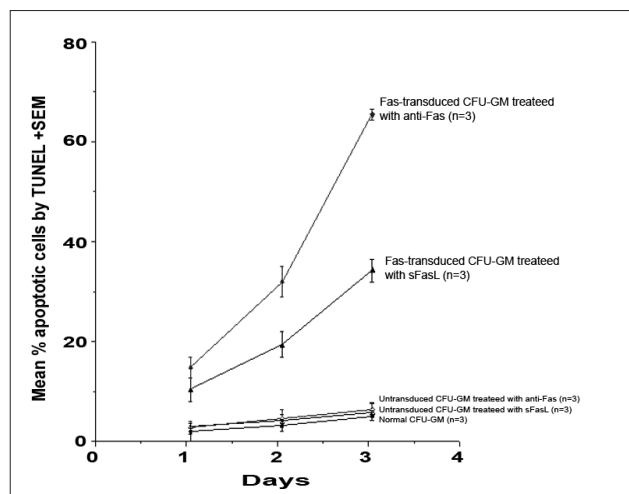
**Figure 2** - Apoptosis percentage of Fas-transduced chronic myeloid leukemia colony-forming unit granulocyte-macrophage cells. There is a dramatic increase in apoptosis in Fas-transduced colonies compared to those transduced with empty vector alone, those mock-transduced with GP-E-86 cells, and those of untransduced controls. TUNEL - terminal deoxynucleoside-triphosphate nick end labelling



**Figure 1** - Number of colony-forming unit granulocyte-macrophage colonies per  $10^5$  bone marrow mononuclear cells derived from patient 5 grown in methylcellulose containing 10% FCS and supplemented with recombinant human cytokines (interleukin-3, granulocyte colony-stimulating factor, granulocyte/macrophage colony stimulating factors and SCF) and showing a dramatic difference between normal controls and patient no. 5. CFU-GMs - colony-forming unit granulocyte-macrophage



**Figure 3** - Flow cytometry data showing the expression of Fas on colony-forming unit granulocyte-macrophage cells. There is a significant difference in the level of Fas expression on the transduced cells stained with FITC conjugated human anti-Fas monoclonal antibody (Mab), compared to untransduced cells (Wilcoxon Signed-rank test).



**Figure 4** - The transduced human colony-forming unit granulocyte-macrophage cells were taken from the transwells and resuspended with 300 ml of minimal essential medium (MEM) with sFasL or human anti-Fas monoclonal antibody (Mab). The percentage of apoptotic cells was scored using the terminal deoxynucleoside-triphosphate nick end labelling (TUNEL) assay. CFU-GMs - colony-forming unit granulocyte-macrophage

apoptosis was not a characteristic of CML progenitor cells. However, it has been suggested that the BCR-ABL oncoprotein prevents CML cells from undergoing apoptosis in these conditions.<sup>9,10</sup> Although, we expected to find a decreased expression level of Apaf-1 and p53 in BC, we were surprised by the results. The p53 was highly expressed in all BC phase samples when compared to the chronic phase CML. Interestingly, whilst moderately high p53 levels was generally seen in conjunction with elevated Apaf-1 expression; however, a reduction in Apaf-1 expression was observed only when p53 levels had become particularly high, suggesting a disruption in the p53 pathway. We believe enhanced expression of these pro-apoptotic genes may be correlated with BC transformation itself, or the response to treatment, or both.<sup>11</sup> In fact, Apaf-1 is upregulated, and apoptosome assembly is facilitated, by several oncoproteins, for example, E2F1.<sup>12</sup> Furthermore, Kannan et al<sup>13</sup> showed that p53 is an upstream regulator of Apaf-1 and is also mutated, deleted, or rearranged, or both, in 25% of BC myeloid leukemia.<sup>14</sup> It could be inferred that the presence of fully functional genes regulating both cell cycle and apoptosis will maintain the balance between the rate of cell division and apoptosis of any population in vivo. Therefore, any malfunction or loss of any one of these, may lead to an increase in their self-replication.<sup>15</sup> The retroviral experiments indicated that myeloid expansion in the 5th CML patient may be entirely attributable to deficient Fas expression. We believe this is the first study to demonstrate in a clinical case, the existence of a close correlation between a deficient Fas, elevated p53 and decreased Apaf-1 expression.

In conclusion, we have demonstrated mRNA elevation of p53 and Apaf-1 during the CML BC phase.

The mutated p53 found in one of the CML patients, and in whom we also found a greatly reduced level of Apaf-1 mRNA, suggests that there is a close, apoptotic-related, linkage between Fas, p53 and Apaf-1 that is directly involved in the progression of CML. This in vivo linkage between intact Fas, p53, and Apaf-1 transcription regulation suggests that genes involved in apoptosis are also involved in CML disease progression.

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