The K562 chronic myeloid leukemia cell line undergoes interferon-α-apoptosis in response to interferon-α

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Abstract
Background and Objective. The K562 cell line, derived from a chronic myeloid leukemia (CML) patient and expressing B3A2 bcr-abl hybrid gene, is known to be particularly resistant to apoptotic death. IFN-α treatment of CML patients impairs malignant cell clone, apparently protecting from progression to terminal blast crisis. The mechanisms underlying this kind of cell deletion are analyzed here by multiple technical approaches.

Design and Methods. K562 cells, variably treated with IFN-α, were examined by agarose gel DNA electrophoresis, light and electron microscopy. The presence of bcr-abl rearrangement was revealed by RT-PCR.

Results. At 4 day treatment both DNA ladder and apoptotic nuclear changes were identified, consistently in the presence of bcr-abl expression.

Interpretation and Conclusions. Even cells expressing bcr-abl, such as K562, can be triggered to apoptosis. Therefore, this genetic condition, commonly preventing PCD, does not prevent IFN-α-mediated apoptosis. PCD seems thus to be the mechanism underlying IFN-α-treated K562 cell deletion and it could be the basis of malignant clone reduction in IFN-α treated CML patients.

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Key words: K562, CML, IFN-α, bcr-abl, apoptosis

Apoptosis, also named programmed cell death (PCD), is a form of cell death aimed at the control of cell differentiation and proliferation, thus regulating organogenesis and adult tissue turnover.1 Morphologically, apoptosis is characterized by chromatin margination around the nuclear membrane, followed by its condensation in cup-shaped dense masses.2 Micronuclei formation and appearance of apoptotic bodies represent the final stage of the process. These aspects are usually associated with internucleosomal and/or genomic DNA fragmentation.3 PCD has been shown to be regulated by several genes, particularly p53, c-myc,4,5 bcl-2, bad, bak,6 and bcr-abl. The bcr-abl hybrid gene results from a reciprocal translocation between chromosome 9 and 22 where, the abl proto-oncogene, usually localized on chromosome 9, is transferred and fused to the breakpoint cluster region (bcr) of chromosome 22.7,8 Two chimeric mRNA transcripts are derived from the fusion of either bcr exon 2 or 3 to abl exon 2 to give B2A2 and B3A2 bcr-abl junctions, respectively. As a result of this mechanism, the BCR-ABL (p210) fusion protein exhibits increased ABL tyrosine-kinase activity and triggers the development of leukemia.9 K562 is an erythroblastic cell line expressing the typical hallmark of CML, the Philadelphia (Ph) chromosome, and the B3A2 bcr-abl.10 Consequently, it has been extensively studied in order to investigate CML and metabolic pathways underlying its therapy. Moreover, the K562 cell line has been the object of experiments concerning its resistance to apoptosis-inducing drugs.11 IFN-α is a biologically active molecule, extensively used in vitro, in order to study its effects on normal and leukemic cells.12,13 It has been also widely used in vivo, to achieve remission in CML early chronic phase, where it induces a significant reduction of the malignant cell clone, with an apparent protection from progression to terminal blast crisis.13,14 The mechanisms responsible for IFN-α effectiveness remain poorly understood. It has been proposed that IFN-α might interfere with the normal production of cytokines, stimulating IL-10 and, at the same time, inhibiting IL-1 and IL-8, as shown by Aman et al.15 IFN-α effect on K562 cells was thus studied to clarify the involvement of the bcr-abl hybrid gene. Apoptosis was investigated by molecular and morphologic approaches in ten closely comparable experiments. Light microscopy was utilized to identify good experimental conditions, in terms of IFN-α optimal concentration and incubation time. The ultrastructural analysis was performed to describe details of K562 apoptosis. Finally, the presence of bcr-abl rearrangement was revealed by reverse transcriptase polymerase chain reaction.
Materials and Methods

Cell line

Human leukemia cell line K562 was cultured in RPMI 1640 (Seromed, Biochrom KG, Berlin, D) supplemented with 10% heat-inactivated fetal calf serum, 2 mM glutamine, 20 mM Hepes (pH 7.5) and antibiotics (penicillin-streptomycin 100U/100 µg/mL) and maintained in 5% CO₂ atmosphere at 37°C. Cell viability was assessed by the trypan blue exclusion test.

IFN-α treatment

K562 cells, at a final concentration of 0.5 x 10⁶/mL, were treated with 200, 400 and 1000 IU/mL of IFN-α-2a (Hoffmann-La Roche, Basal, CH) and analyzed after 2, 3, 4 and 5 days of incubation. Untreated control cells were examined at corresponding times.

Gel electrophoresis analysis

Three per 10⁶ cells were lysed in a buffer containing SDS 1%, RNase 200 µg/mL, proteinase k 100 µg/mL (all obtained from Sigma, Poole, Dorset, UK) and incubated overnight at 37°C. Nucleic acids were extracted following standard procedures.16 Two µg DNA/lane were loaded on 1.8% agarose (BIO-RAD, Hercules, CA) gel in tris-borate/EDTA 1X buffer, stained by ethidium bromide (0.5 µg/mL) and visualized on an ultraviolet (UV) transilluminator (BIO-RAD).

Light and electron microscopy

Cell pellets were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.3). They were then post-fixed in 1% OsO₄ in phosphate buffer, ethanol dehydrated and embedded in araldite as described elsewhere.2 Semi-thin sections were stained with a 1:1 mixture of 1% methylene blue and 1% toluidine blue in distilled water. For ultrastructural analysis formvar-carbon coated grids were used as thin section supports. Thin sections, stained with uranyl acetate and lead citrate, were examined with a Philips CM 10 electron microscope at 80 KV.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Apoptotic cells were isolated by means of a Percoll gradient, according to Cotter et al.17 Total RNA was isolated from 2 x 10⁶ cells using RNAzol™ B (Biotex, Houston, TX, USA) following the manufacturer’s instructions. Reverse transcription was carried out as previously described18 using 3 bcr primer (see below). Five µL aliquots of the transcription mixture were used for the amplification reaction, performed by using two oligonucleotide primers designed according to the published bcr-abl sequence: 5 bcr (5'-GGCTGAGGCTTCCCTG-3') and 3 bcr (5'-CCATT TTCGTTGACCACCCATC-3'). The PCR consisted of a 50 µL final volume using, per reaction, 1 x Taq buffer (Promega, Madison, WI), 1.5 mM of MgCl₂, 0.1 mM of each dNTP, 0.25 µM of each primer (5 bcr, 3 bcr) and 1 U of Taq polymerase (Promega). The amplification was carried out on an automated DNA thermal cycler for 45 cycles following an initial denaturation of 1 minute at 94°C. Each cycle consisted of 35 seconds at 94°C, 30 seconds at 60°C, and 30 seconds at 72°C. The PCR samples were analyzed by electrophoresis on a 2% agarose (BIO-RAD) gel containing tris-borate-EDTA buffer and ethidium bromide (0.5 µg/mL) and were photographed on an UV transilluminator.19

Results

IFN-α treatment

As shown in Figure 1, K562 cells were initially treated with different concentrations of IFN-α-2a, in order to find the dose best able to induce apoptosis. Apoptotic cell percentage was evaluated by light microscopy (see below). Our results suggested that at 200-
400 IU/mL of IFN-α treatment the cells mainly underwent PCD, with a higher percentage of apoptotic cells and a lower amount of necrosis at 200 IU/mL. In contrast, at 1000 IU/mL no apoptosis was evident but a high number of necrotic cells appeared.

Simultaneously, the optimal exposure time to induce apoptosis was analyzed by evaluation of the ladder appearance, and light microscopy. Figure 2 indicates that PCD was maximal at 4 days of incubation. Therefore, 200 IU/mL and 4 days of incubation were chosen as the experimental conditions for the subsequent investigation.

**Gel electrophoresis analysis**

Figure 3 shows the gel electrophoresis analysis obtained from samples treated from 1 to 4 days. The DNA ladder is clearly evident at 4 days of treatment (lane H), while it does not appear in lanes E, F and G (1, 2 and 3 days of incubation, respectively) or in control conditions.

**Light and electron microscopy findings**

The light microscopy analysis provides clear evidence of apoptosis in the treated samples.

Figure 4a,b,c of semi-thin sections show several K562 cells presenting typical apoptotic features, consisting of characteristic chromatin condensation close to the nuclear envelope, associated with micronuclei formation and cytoplasmic vacuolization. Figure 4d illustrates the control, non-apoptotic, cell morphology.

The ultrastructural analysis (Figure 5) also shows evidence of major nuclear modifications. The characteristic chromatin margination (Figure 5a) appears, forming cap-shaped compact structures towards the nuclear periphery. An initial cytoplasm hydration, probably preceding a final secondary necrosis, is shown in Figure 5b. Progressively electron dense micronuclei (Figure 5c) can also be detected. These are mostly surrounded by a defined double membrane, externally covered with ribosomes.

In addition, peculiar changes can occasionally be seen in nucleolar organization of non-apoptotic cells. In fact, the dense filamentous component appears scattered throughout the nucleoplasm, although the chromatin is distributed normally (Figure 5d).

**RT-PCR analysis**

As expected, a 468 bp PCR product, corresponding to the B3A2 transcript, characteristic of the K562 CML cell line, was found by RT-PCR analysis (Figure 6). No significant differences in the presence and level of B3A2 transcript were noticed between K562 cells left untreated or treated for 3-4 days with IFN-α.

**Discussion**

Previous studies have shown that the K562 erythroleukemia cell line, derived from a CML patient and expressing the bcr-abl hybrid gene, is particularly resistant to apoptosis induced via a number of chemical and biological agents. Differently, numerous cell types which do not express bcr-abl, are easily and massively triggered to apoptosis. In order to increase the K562 sensitivity to apoptosis, McGahon et al. investigated the effects of antisense oligonucleotide treatment corresponding to the translation start of the bcr-abl gene and suggested that antisense pretreatment could improve the cellular response to apoptosis-inducing agents. These data indicate that the bcr-abl
Apoptotic action of interferon-α hybrid gene could function as a suppressor of the apoptotic process, as does bcl-2.23 IFN-α is a biologically active molecule produced by leukocytes in response to viral infections24 and a number of other stimuli.25 It is currently widely used in therapeutic protocols. In approximately 30% of CML patients, IFN-α induces a significant reduction of the malignant Ph-positive cell clone, which apparently prevents the progression to terminal blast crisis.12,13

Figure 4a,b,c,d (left). Semi-thin sections of 4 day IFN-α treated K562 cells (a,b,c) and controls (d). Patterns of chromatin margination and micronuclei (▲) appear (a). Late apoptotic stages, with vacuolized apoptotic bodies (▲), are also present (b,c). A well-preserved, non-apoptotic morphology appears in control untreated cells (d). a, ×800; b, ×1000; c, ×800; d, ×1000.

Figure 5a,b,c,d (above). Electron microscopy of IFN-α-treated cells. a: apoptotic cells showing areas of compact and diffuse chromatin; b: initial necrotic features in a late apoptotic cell; c: three progressively condensing micronuclei, surrounded by double membranes, are visible. Nucleolar components (▲), scattered throughout the cytoplasm, also appear; d: nucleolar segregation, as well as disgregation of nucleolar components, are evident in a non-apoptotic cell. a, ×19,000; b, ×17,000; c, ×20,000; d, ×20,000.

hybrid gene could function as a suppressor of the apoptotic process, as does bd-2.23 IFN-α is a biologically active molecule produced by leukocytes in response to viral infections24 and a number of other stimuli.25 It is currently widely used in therapeutic protocols. In approximately 30% of CML patients, IFN-α induces a significant reduction of the malignant Ph-positive cell clone, which apparently prevents the progression to terminal blast crisis.12,13

The real mechanism of the IFN-α effect is not yet known, although several studies suggest a role in the
regulation of cytokine production, possibly through the control of cell proliferation. It has been reported that IFN-α treatment might correct defective adherence of CML-derived hematopoietic progenitors to bone marrow stromal cells following an inappropriate expression of cytokines such as IL-1, G-CSF and TNF.26,27 This phenomenon might contribute to the unbalanced expansion of the malignant clone, which can probably be interrupted by the action of IFN-α on cytokine cascade regulation.28

Aman et al.15 investigated the effect of IFN-α on the expression of IL-10 mRNA and protein synthesis in human monocytes and CD4+ T cells. In purified monocytes, IFN-α treatment induced an overexpression of IL-10 after prolonged incubation periods and a similar effect was observed in CD4+ T cells activated with CD3 and CD28 monoclonal antibodies. This would explain the anti-inflammatory effect of IFN-α.

Previous studies reported that IFN-α inhibits the proliferation of progenitor cells, such as myeloid, erythroid and megakaryocytic cells differently, according to their sensitivity.29 In the attempt to clarify the mechanism related to this inhibition, Tarumi et al.30 studied the consequence of IFN-α treatment on highly purified human peripheral blood erythroid burst-forming units (BFU-E) and provided evidence that the inhibitory effect of IFN-α on erythroid progenitors was related to apoptosis.

Despite these preliminary data, little is known about the IFNs’ (α, β, γ) role in apoptosis, and the influence of IFNs on apoptosis seems to depend on cell lineage and maturation level.

Milner et al.31 reported that IFN-α triggered a bcl-2 independent protective response and prevented apoptosis of freshly isolated cells from patients with Burkitt’s lymphoma. Holder et al.32 stressed that IFN-α provided a weak survival signal for human germinal center B cells. Calotta et al.33 reported that IFN-γ interferes with apoptosis of mature circulating polymorphonuclear cells; this differs from biological evidence that IFN-γ inhibits non-erythroid progenitors. In the framework of these considerations, we decided to investigate IFN-α action on the K562 cell line.

Typical apoptotic patterns appeared in IFN-α treated K562 cells after 4 days of incubation.

DNA fragmentation was indeed demonstrated by the appearance of a ladder, after agarose gel electrophoresis, and characteristic apoptotic nuclear features were apparent by light and electron microscopy analysis. It could be argued that the presence of the DNA ladder pattern in K562 cells has been frequently reported to be hardly identifiable or completely absent.22 This would suggest a lack or a decrease of endonuclease activity in these cells. In our case this hallmark of the apoptotic process was present, even if not consistently, indicating that a certain heterogeneity of the endonuclease production level could be found inside the same K562 population and that the experimental results could vary depending on the cell cycle phase. However, it is becoming increasingly clear that some cell types can show morphologic features typical of apoptosis even in the absence of inter-nucleosomal DNA breakage.34-36 Moreover, the characteristic apoptotic features were shown by optical and electron microscopy. Progressive chromatin margination into cap-shaped dense masses was present, followed by the formation of membrane bound micronuclei. Cytosol underwent an evident condensation correlated to diffuse vacuolization, as frequently occurs in apoptotic cells. Secondary necrosis phenomena also appeared, characterized by progressive water influx causing nuclear and cytoplasmic swelling.1,2

Further studies are necessary to investigate the nucleolar changes, even if these can be considered the expression of altered protein synthesis activity, evidently present also in non-apoptotic cells.

A relevant point of this study is the demonstration of the presence of equal amounts of bcr-abl transcripts in IFN-α incubated and untreated K562 cells. The presence of bcr-abl in cells undergoing apoptosis is intriguing and suggests that IFN-α induces apoptosis in K562 cells without interfering with the activity of the bcr-abl gene product.37-39

Figure 6. Amplification of bcr-abl mRNA by RT-PCR.
Lane A, F: size marker (αX174/Hae III).
Lane B: K562 control cells: bcr-abl B3A2 amplification.
Lane C, D: K562 treated cells at 3 and 4 days, respectively: bcr-abl amplification.
Lane E: negative control.
Taken together, our data indicate that even cell lines expressing bcr-abl, such as K562 cells, can be triggered to apoptosis, and that bcr-abl expression, commonly considered a condition preventing PCD appearance, such as K562, does not prevent IFN-α-mediated apoptosis. Moreover, PCD seems to be the mechanism underlying K562 cell deletion after IFN-α treatment and this could represent the basis of malignant clone reduction in IFN treated CML patients.  

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