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Differential expression of Bcl-2 protein in non-irradiated or UVC-irradiated murine myeloid leukaemia (ML) cells

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In this work, we examined the expression of Bcl-2 protein in myeloid leukaemia (ML) cells and the effect of UVC-light on the expression level. The protein of bcl-2 oncogene was detected by immunocytochemical method. In spleen cells of healthy RFM donors was detected 34.3% of Bcl-2 positive cells. When leukaemia came to the non terminal phase (NTP) more than 50% of cells expressed this protein. However, in terminal phase (TP) of leukaemia growth, only 24.4% Bcl-2 positive cells was determined. After UVC irradiation, the expression of Bcl-2 protein was significantly higher in spleen cells of healthy donors. However, UVC light did not change the expression of Bcl-2 in cells of both investigated phases of ML growth. Bcl-2 protein may be involved in the resistance of ML cells to UVC light.

Key words: myeloid, leukaemia; UVC light, Bcl-2 protein

Introduction

The *bcl-2* (B-cell lymphoma/leukaemia 2) gene becomes involved in chromosomal translocations in many humans B-cell lymphoma.[1] Chromosomal translocation t (14;18) frequently occurs in non-Hodgkin's B-cell lymphomas. In that case, the *bcl-2* gene moves from its normal location at 18q21 into *cis*-configuration with strong enhancer elements associated with immunoglobulin heavy-chain locus at 14q32,[2] resulting in deregulated *bcl-2* gene expression primarily through transcriptional mechanisms. The altered levels of Bcl-2 protein found in these cells is thought to contribute to the pathogenesis of these B-cell neoplasms.[1] Interestingly, translocations involving *bcl-2* gene have not been described in T-cell leukaemias and lymphomas. Reed and co-workers presume two possible explanations; the conditions contributing to the specific chromosomal breaks and recombinations do not exist in T-cell or *bcl-2* translocations occur at the stage of T-cell differentiation when *bcl-2* fails to confer a selective growth advantage.[1] High levels of Bcl-2 protein production have been also reported in a wide variety of human solid tumours and leukaemias even in the absence of translocation or other gross alterations in the structure of *bcl-2* gene, including adenocarcinomas of the prostate and colon, neuroblastomas, acute myelogenous leukaemias and chronic lymhocytic leukaemias.[2,3]

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The *bcl-2* proto-oncogene encodes a 26 kDa protein, which is localized in the inner and the outer mitochondrial membrane, the nuclear envelope and the endoplasmic reticulum.[4] This protein is structurally and functionally unique in that it bears little or no significant homology with other known cellular proteins.[5] Bcl-2 protein contributes to malignant cell expansion by blocking the normal physiological turnover of cells death (apoptosis), rather than by increasing the rate of cellular proliferation.[6,7] Recent findings are beginning to reveal details of the mechanisms by which Bcl-2 protein suppress cell death. Namely, evidence from cell transfection studies indicated that Bcl-2 might have a membrane transport function, with reported effects on Ca^{2+} flux and protein translocations across some of the intracellular membranes where this protein is localized.[8] The mechanism by which Bcl-2 protein create channels has not been explored in detail.

Apoptosis is a fundamental biologic process which allows the cell to actively participate in its own death. Leukaemia and tumour cells, which expressed *bcl-2* gene, are more resistant to induction of apoptosis by chemotherapeutic drugs, irradiation and other agents. Namely, during the process of apoptosis "megapores" on the mitochondrial membrane were opened and apoptogenic protease activators (cytochrome c and apoptosis-inducing factor, AIF) released from mitochondria (Figure 1). It has repercussions on the generation of oxygen free radicals and the release of mitochondrial proteins into the cytosol in order to activate the caspases, that are the terminal effectors of apoptosis.[9] Overexpression of Bcl-2 protein inhibited mitochondrial permeability, and thus inhibited programmed cell death.[10] Bcl-2, therefore, plays a significant role, not only in the origins of cancer, but also in its treatment.[2]

In previous work, we showed that ML cells could survive irradiation with very high dose of UVC-light (1280 J/m^2), but normal bone marrow cells died even after small dose (5 J/m^2).[11] Also, we have shown previously that UVC light induced apoptosis in high percent of spleen cells of healthy mice, but in spleen of ML bearing mice induction of apoptosis was weaker and was expressed latter.[12] That was the reason, why we looked at expression of Bcl-2 protein in ML cells and the effect of UVC light on the expression level of Bcl-2. During the ML growth we distinguished two phases, non terminal and terminal, because the expression of some oncogenes are different in different stages of disease.[12,13]

Materials and methods

In experiments RFM/Rij Zgr mice, bread at our Institute were used. ML was induced by sublethal X-irradiation of mice 1965. and since than the leukaemia was transplanted by spleen cells of moribund mice, or cells were kept frozen in liquid nitrogen. Mice injected intravenously with 10^6 cells died with high leucocyte number in the blood, enlarged spleens and livers. Spleen cells were tested 9 (non terminal phase of disease NTP) or 12 days (terminal phase TP) after inoculation of 10^6 ML cells. Cell suspensions of healthy and leukaemic spleen ($8.5x10^6$ cells per ml in Hank's solution without phenol red, in thin layer, 0.2 mm) were irradiated with UVC light by four germicidal lamps (Phillips, 15 watts). Doses of UVC light were 50, 100, 1000 and 50000 J/m^2. Before exposition to UV light, erythrocytes were lysed in hypotonic salt solution. During exposition the suspension was constantly stirred by a magnetic rod.

Bcl-2 protein was detected by immunocytochemical method described by Kranz and co-workers.[14] Ten μl of cell suspension ($6x10^6$ cells/ml) was dropped on slide coated with poly-L-lysine and incubated for 30 min at 4°C. The slide was washed in phosphate-

buffered saline (PBS), pH 7.4, followed by fixation for 7 min at 20°C with freshly prepared 0.05% glutaraldehyde (grade 1; Sigma) in PBS. For the differential staining of endogenous peroxidases, 10 μl PBS containing 0.1% 4-chloro-1-naphthol (Sigma) and 0.015% hydrogen peroxide (Merk) was added to each spot and incubated for 15 min at 20°C, followed by washing the slide in PBS. In order to permeabilized membranes, spots were than incubated for 15 min at 20°C with PBS containing 0.04% polyoxyethylene 10 cetyl ether (Brij 56; Sigma), followed by spots washing. Non-specific binding of primary antibody was blocked by applying MAG solution (MEM with 0.2% albumin and 0.2% gelatine, Gibco). Monoclonal antibody mouse IgG anti Bcl-2 (Oncogene Science), in optimized dilution (1:40) was applied for 30 min at room temperature. After washing the slides in PBS, secondary antibody goat immunoglobulins to mouse immunoglobulins (Jackson Immunoresearch), diluted 1:100 in MAG, was added on each spots and incubated 30 min at room temperature. After that, the slides were washed by simply dipping into PBS. The immunoperoxidase reaction was performed for 25 min at 20°C using a freshly prepared mixture of 94 ml 0.05 M phosphate buffer, pH 6.9, 6 ml dimethyl sulfoxide (DMSO; Merck) containing 0.167% 3-amino-9-ethylcarbazole (Sigma) and 15 μl 30% hydrogen peroxide (Merck). Slides were then stained for 20 sec with Mayer's acid hemalaun, rinsed in tap water, and mounted with phosphate-buffered glycerol. The number of positive or negative cells was evaluated under the light microscope (Reichert, x 502). The nuclei of positive cells were brown, while nuclei of negative cells were blue. Experiments were done three times in triplicate. The positive cells was determined by scoring at least 100 cells on each spot; finally 900 cells were counted for each point showed in results.

Statistical analyses were performed using Model 1 ANOVA to determine whether differences existed among the group means, followed by a paired Student's t distribution to identify the significantly different means ($p = 0.05$).

Results

Bcl-2 protein was expressed in 34.3% of spleen cells of healthy RFM donors, which were used as control cells (Figure 2). However, spleen cells of leukaemia bearing mice 9 days after inoculation of ML cells (NTP), expressed Bcl-2 protein in significant higher percentage (56.7%) than control cells (Figure 2). Opposite to this, in TP of leukaemia growth the percentage of Bcl-2 positive cells was significantly smaller than in sample of "healthy" spleen cells or spleen cells of NTP of leukaemia (Table 1). In that case, we detected only 24.4% of Bcl-2 positive cells (Figure 2).

Spleen cells suspensions of healthy mice and leukaemia bearing mice of both phases were irradiated with UVC light (doses 50, 100, 1000 and 50000 J/m^2) and than the presence of Bcl-2 protein was determined. After UVC irradiation with all used doses, the number of "healthy" spleen cells which expressed Bcl-2 protein significantly increased (Table 1). The range of positive cells was from 44% to 58.4% (Figure 2).

Different effect was detected after UVC irradiation of leukaemic cells of NTP. Namely, UVC light did not provoke any significant increase the number of Bcl-2 expressing cells (Table 1). In that case the percentage of Bcl-2 positive cells was from 56.7% in unirradiated sample, to 52% or 64% in irradiated samples (Figure 2).

Nearly the same effect was observed after UVC exposition of leukaemic cells of TP. In TP of ML growth this range of positive cells in irradiated samples was from 24% to 29.6% (Figure 2). The exception was at a dose of 100

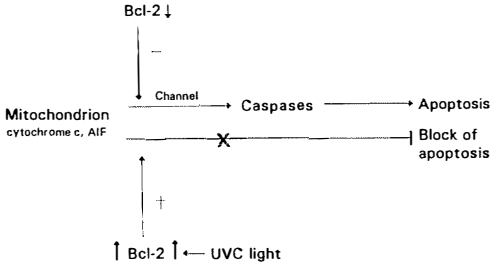

Figure 1. The role of Bcl-2 protein in apoptosis.

J/m^2, where significant increase was detected in the percentage (32.8%) of Bcl-2 expressing cells (Table 1).

Discussion

Since its discovery over ten years ago as an oncogenic protein involved in many human tumours, *bcl-2* gene and their protein are topic of many investigations because of its anti-apoptotic action. Bcl-2 protein can function as channels for ions, proteins or both, across intracellular membranes like the outer mitochondrial membrane, the endoplasmic reticulum and the nuclear envelope.[10] The mechanism by which Bcl-2 create channels in membranes has not been explored in detail, but preliminary indications are that at least aspects of the process may be similar to the bacterial toxins.[10]

Cells undergo apoptosis when exposed to a variety of cytotoxic agents, like a radiation. However, it is known that some leukaemia cell lines are resistant to apoptosis induced by irradiation.[2] This is in connection with our previously work, where we determined apoptosis in spleen cells of healthy and leukaemia bearing RFM donors, 4 or 24 hours after UVC irradiation.[12] Irradiated "healthy" spleen cells died by apoptosis in significantly higher percentage than unirradiated cells, detected 4 as well 24 hours after irradiation. Opposite to healthy spleen cells, unirradiated ML cells did not enter in apoptosis, and we did not find correlation between doses of UVC light and apoptosis of leukaemic cells of TP, tested 4 and 24 hours after irradiation. For that could be responsible Bcl-2 protein and their anti-apoptotic effect.

It was the reason why we investigated the presence of Bcl-2 protein in spleen cells of healthy and in ML bearing RFM mice. During the ML growth was observed two phases, non terminal and terminal. The spleen of NTP of leukaemia has a few leukaemic cells, while in TP of disease most of cells are leukaemic. We supposed that, the presence of oncogenic proteins (like Bcl-2 protein) was also different at the different stages of disease, as we showed earlier for c-Myc protein.[15] Thus, in the early period of leukaemia growth (NTP) only 14.3% of c-myc positive

Table 1. The number of Bcl-2 positive cells of healthy RFM mice (control) and leukaemia bearing mice of non terminal (NTP) and terminal (TP) phase. Experiments were done in triplicate. For each point showed in Table 1 and Figure 1, 900 cells were counted

Dose (J/m^2)	Control cells mean ± se (%)	Leukaemic cells of NTP mean ± se (%)	Leukaemic cells of TP mean ± se (%)
0	34.3 ± 4 (34.3)	56.7 ± 1.4 (56.7)	24.4 ± 2.1 (24.4)
50	48.5 ± 2.1[a] (48.5)	60.0 ± 1.8 (60.0)	28.0 ± 1.4 (28.0)
100	58.4 ± 1.5[a] (58.4)	52.0 ± 1.9 (52.0)	32.8 ± 1.6[b] (32.8)
1000	54.4 ± 1.7[a] (54.4)	64.0 ± 1.9 (64.0)	29.6 ± 1.7 (29.6)
50000	44.0 ± 1.5[a] (44.0)	60.0 ± 1.4 (60.0)	24.0 ± 1.8 (24.0)

[a] = significant difference relative to non irradiated control sample
[b] = significant difference relative to non irradiated sample of ML of TP

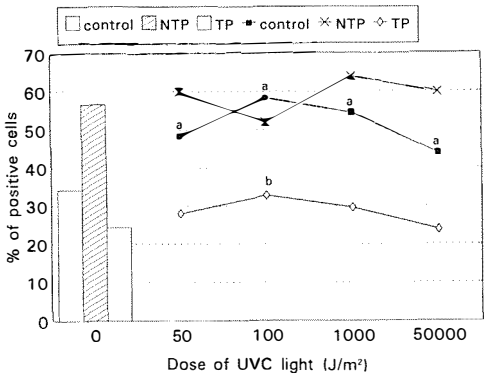

Figure 2. The presence of Bcl-2 protein in spleen cells of healthy mice (control) and leukaemic bearing mice of non terminal (NTP) and terminal phase (TP) of disease.
[a] = significant difference relative to value of non irradiated control sample
[b] = significant difference relative to value of non irradiated sample of ML of TP

cells was found, as opposed to the terminal phase of leukaemia (TP) when even 89.7% of c-myc positive cells were detected. From literature is also known, that alterations of gene expression is in connection with disease progression. For example, inactivation of *p53* gene is quite rare in the chronic phase of chronic myeloid leukaemia, while relatively frequent (around 25%) in the acute phase.[13]

In case of Bcl-2 protein, we found the greater expression in NTP of leukaemia, than in "healthy" spleen cells or in cells of TP of leukaemia. We suppose that, like other oncogenes, auto-regulation of Bcl-2 protein synthesis was lost in this malignant cells,[16] and that is reason of different expression of Bcl-2 protein. However, when leukaemia came to the terminal phase, the mice died during a few hours and ML cells were destroyed.

It is well known that different kind of radiation (as well as UVC light) are carcinogens which can activate different classes of oncogenes.[17] The actively transcribed genes which possessed damage in DNA are preferentially repair by mechanism of DNA repair which exist in all mammalian cells. Today, increased exposure to environmental UVC is a result of depletion of atmospheric ozone. In our experiment we used UVC light doses from 50 to 50000 J/m^2. So wide range of UVC doses we used in our earlier experiments, where the high resistance of ML cells was detected. Also, we examined the ability of UVC light to activate acellular factor, which is possible to induced the malignant transformation of bone marrow cells.[18] There is no doubt that UVC light activated *bcl-2* gene expression in control spleen cells (in comparison to unirradiated cells). But, UVC light did not change the expression of Bcl-2 protein in myeloid leukaemia cells (of both phases). These results agree with our previous finding and our opinion that ML cells could not die by apoptosis. Therefore, leukaemic cells accumulated in spleen and liver causing splenomegaly and hepatomegaly, typically symptoms of leukaemia disease.

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