NAF- INDUCED APOPTOSIS IN HUMAN BONE MARROW AND CORD BLOOD CD34 POSITIVE CELLS

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SUMMARY: Our previous findings indicated that fluorides could potentially damage human cord blood hematopoietic cells. In the present study our aim was to estimate the induction of apoptosis in human bone marrow (BM) as well as cord blood (CB) $CD34^+$ cells exposed to sodium fluoride (NaF). We employed two different assays to evaluate early and final phases of apoptosis. BM and CB $CD34^+$ cells were exposed to different doses of NaF (0, 1, 10 and 50 mg/L) and stored at 37°C for 24 h. The next day the percent of apoptotic cells was assessed. Apoptotic and necrotic cells were detected after Annexin-V and propidium iodide staining, respectively, and evaluated by FACS (Flow Activated Cell Sorter). Moreover, the cell culture supernatants were evaluated using enzyme-linked immunosorbent assay (ELISA) for quantitative detection of human copper zinc superoxide dismutase (Cu/ZnSOD). Apoptosis was detectable both in BM and CB hematopoietic progenitor cells exposed to different doses of sodium fluoride. High doses of NaF induced a larger number of cells to enter the early phase of apoptotis, especially at the concentration 50 mg/L measured by FACS. We detected even more differences when analyzing cord blood $CD34^+$ cells. We also found that BM as well as CB $CD34^+$ cells exposed to the high dose of NaF (50 mg/L) secreted significantly more Cu/ZnSOD to the medium when compared to equivalent numbers of cells exposed to lower doses of NaF or unexposed cells.

Keywords: Apoptosis, Bone marrow cells, Cord blood cells, Hematopoiesis, Sodium fluoride (NaF).

INTRODUCTION

Production of blood cells is sustained throughout an individual's life. This constant process is based on hematopoietic stem cell (HSC) function. These cells are primitive, noncycling, metabolically quiescent, and have self-renewal capacity.[1] Although HSC is rare (~1/100,000 nucleated marrow cells) and daily demand for newly formed morphotic elements of blood is very high, hematopoiesis operates very effectively.[2,3] More differentiated cells gradually lose their self-renewal potential and acquire the ability to undergo extensive proliferation.

There are many agents and factors that can influence or disturb normal hematopoiesis.[1,4] Negative consequences of fluoride overaccumulation in living organisms are widespread and well known.[5] Fluorides accumulate mostly in the bone tissues.[6-8] Therefore, they could also directly influence the formation of the hematopoietic cells in the bone marrow cavities. Envi-

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ronmental pollution with fluorine compounds has been already connected with the increasing morbidity due to hematological diseases.[9] Our previous findings indicated that fluorides could potentially damage human hematopoietic cord blood progenitor cells.[10]

Accordingly, because early hematopoietic cells (stem and progenitor cells) express the CD34 antigen,[11] we evaluated apoptosis in human bone marrow and cord blood CD34 positive cell populations, after incubation with sodium fluoride, by using two different techniques (FACS – Flow Activated Cell Sorter and ELISA - enzyme-linked immunosorbent assay **-** test).

MATERIALS AND METHODS

Cells: Bone marrow (BM) cells were obtained from 18 brain-dead adult heparinized cadaveric organ donors (HCOD). The cells from the iliac crests were aspirated before disconnecting from the respirator.[12] Umbilical cord bloods (CB) (60-120 mL) were obtained immediately after delivering of placenta from 21 consenting healthy women. In every case the donor's family consent was obtained. A fraction of mononuclear cells was isolated after centrifugation over Gradisol L (Polfa Kutno, Poland) gradient. Normal light-density mononuclear cells (MNC) were depleted of adherent cells and T lymphocytes (A^-T^-MNC), as previously described.[13] $CD34^+$ cells were enriched from the A^-T^-MNC population by an immunoaffinity selection with commercially available $CD34^+$ isolation MACs kit (Miltenyj Biotec, Auburn, CA, USA) according to the manufacturer’s protocol. After the separation, the cells were resuspended in Iscove DMEM + 10% BCS (bovine calf serum). The purity of $CD34^+$ cells was >95%. Their viability was assayed by the trypan blue exclusion test. The cells were counted using a hemocytometer and subsequently used for experiments.

Detection of apoptosis: Sodium fluoride (Sigma, USA) was diluted in a phosphate-buffered saline at a concentration of 1 g/L and was stored at 4°C. Two different assays evaluating early and final phase apoptosis (Annexin V and ELISA tests respectively) in bone marrow and cord blood hematopoietic cells were employed. For this purpose, 0.5×10^6 BM and CB $CD34^+$ cells were exposed to different doses of NaF (0, 1, 10 and 50 mg/L) and stored at 37°C for 24 hours in Iscove’s Modified Dulbecco’s Medium (IMDM) supplemented with 20% bovine calf serum. On the next day the percent of apoptotic cells was assessed. Apoptotic and necrotic cells were detected after Annexin-V and propidium iodide staining respectively (R&D, Minneapolis, USA) and evaluated by FACS (Flow Activated Cell Sorter) (FACS Calibur, Becton-Dickinson, USA) according to the manufacturer’s protocol. Moreover, the cell culture supernatants were evaluated using enzyme-linked immunosorbent assay (ELISA) for quantitative detection of human copper zinc superoxide dismutase (Cu/ZnSOD) according to the manufacturer’s protocol (Bender MedSystems, Austria).

Statistical analysis: Arithmetic means and standard deviations were calculated on an IBM computer using MS Excel v. 97. Data were analyzed using the Student's t-test for unpaired samples. Statistical significance was defined as $p<0.05$.

RESULTS

The table shows, expressed in percent, the effect of incubation of $CD34^+$ cells, derived from bone marrow and cord blood, with increasing doses of NaF on induction of apoptosis, which was assessed in the early phase. Apoptotic and necrotic cells were detected after Annexin-V and propidium iodide staining, respectively, by FACS (Figure 1).

As seen in the table, higher doses of NaF caused an increase in the early phase of the apoptotic process, especially at the concentration of 50 mg/L ($p<0.05$). In the case of cord blood the differences were also significant.

Table. Percent of apoptotic cord blood and bone marrow $CD34^+$ cells, after incubation with increasing doses of NaF, evaluated by Flow Activated Cell Sorter

	Control	1 mg/L	10 mg/L	50 mg/L
Cord blood	0.8 ± 0.2	0.6 ± 0.1	1.2 ± 0.1	2.2 ± 0.1
Bone marrow	4.0 ± 3.0	4.0 ± 2.1	4.0 ± 2.8	6.4 ± 3.4

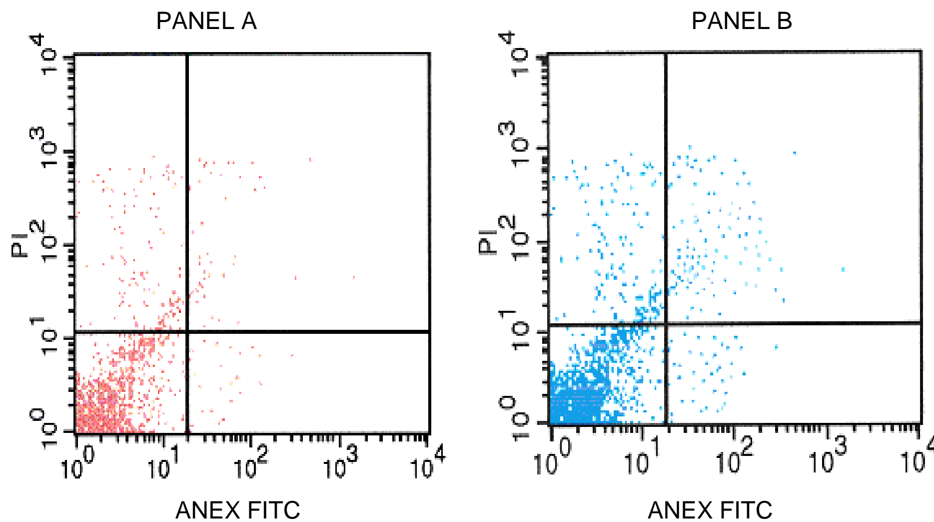

Figure 1. A representative study for detection of the number of apoptotic (x axis – Fluorescein Isothiocyanate-Annexin V) and death cells (y axis – propidium iodide) in the bone marrow samples of the control group (panel A) and the group exposed to NaF at the concentration 50 mg/L (panel B).

Figure 2 shows the effect of incubation of $CD34^+$ cells, derived from bone marrow, with increasing doses of NaF on induction of the final phase of apoptosis, which was assessed by the ELISA test. The cells were exposed to NaF at 37°C for 24 hours. After the incubation the supernatant was collected, and copper zinc superoxide dismutase (Cu/ZnSOD) was assessed by ELISA. Induction of Cu/ZnSOD expression resulting in elevated levels of Cu/ZnSOD in human cell culture supernatants is of diagnostic value for measuring the final phase of apoptosis.

As seen in Figure 2, we found that the BM $CD34^+$ cells exposed to the highest dose of NaF (50 mg/L) secreted significantly more Cu/ZnSOD to the medium when compared to equivalent numbers of cells exposed to lower doses of NaF or unexposed cells ($p<0.05$). We obtained similar results in the case of cord blood (not shown).

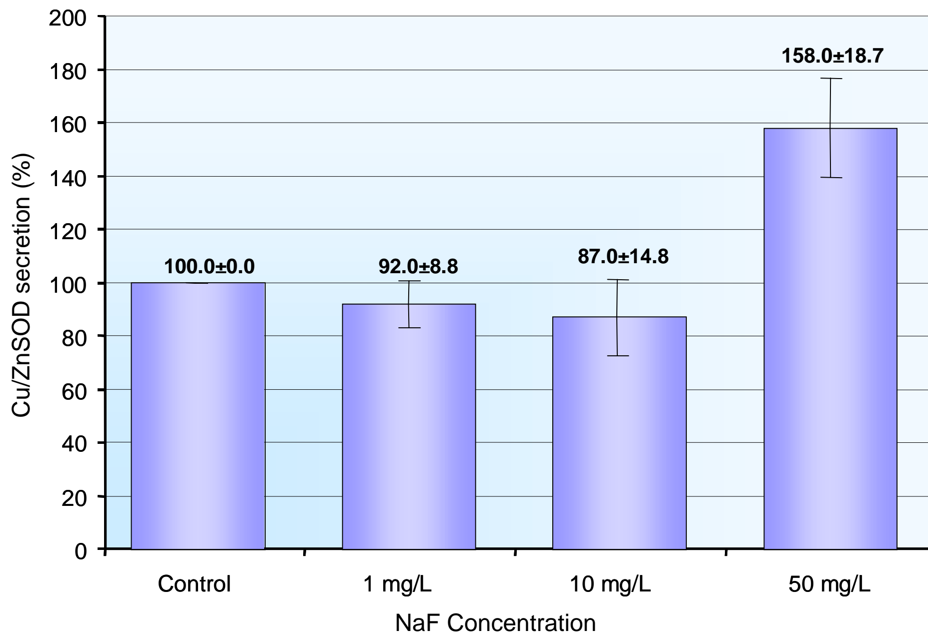

Figure 2. The effect of incubation of bone marrow CD34+ cells with increasing doses of NaF on induction of apoptosis evaluated by enzyme-linked immunosorbent assay (ELISA). The amount of Cu/ZnSOD secreted by cells of the control group is taken as 100%.

DISCUSSION

In living organisms fluorine is relatively common, but low-concentration, component of every tissue and fluid. Fluoride can easily penetrate cell membranes (e.g., blood cells) by simple diffusion and can cause adverse effects on tissue metabolism.[14] The influence of fluorides on living cells is wide and has variety of aspects. Inhibition of protein synthesis and disturbance of enzyme activity are among the most important.[15] Such effects were also found in certain types of blood cells.[16] Our earlier studies indicated that sodium fluoride affects human hematopoietic progenitor cells and significantly decreases their clonogenic growth.[10]

In the present work we employed two different assays to evaluate early as well as final phases of apoptosis in human bone marrow and cord blood early hematopoietic cells. Apoptosis was detectable in BM and CB hematopoietic cells, exposed to different doses of sodium fluoride. The most evident effect was found when the highest dose of NaF (50 mg/L) was used. We also noticed that the percentage of apoptotic cells was slightly decreased at 1 and 10 mg/L in what might reflect a small paradoxical concentration effect.[17]

As is well known, the most primitive early hematopoietic progenitors possess few mitochondria and show much lower metabolic activity than more mature hematopoietic cells.[1] Moreover, they express phospho-glycoprotein on the surface, which is responsible for active elimination of toxic substances from the cytoplasm.[18] Here we found that a relatively high concentration of sodium fluoride (50 mg/L) was able to induce the process of apoptosis. These findings generally confirm that stem and early progenitor cells are not so sensitive to toxic effects of sodium fluoride as perhaps more mature hematopoietic cells.

In our view, exposure to relatively high doses of sodium fluoride is likely to damage human cells involved in hematopoiesis. Knowledge of the influence of fluorides on hematopoiesis may have important theoretical and practical applications, especially in the regions of endemic fluorosis, but this hypothesis needs further and more intensive studies.

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