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THE INFLUENCE OF SODIUM FLUORIDE AND SODIUM HEXAFLUOROSILICATE ON HUMAN LEUKEMIC CELL LINES

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SUMMARY: Although potential toxic effects of sodium fluoride on early progenitor and stem cells have been reported previously, surprisingly few investigations have examined the effects of fluoride on human leukemic cells. To address this need, four different human leukemic cell lines (HL-60, HEL, TF-1, and K562) were exposed to increasing levels (0, 0.24, and 1.19 mM F) of two forms of fluoride: sodium fluoride (NaF) and sodium hexafluorosilicate (Na_2SiF_6). Because of its widespread use in water fluoridation, Na_2SiF_6 was investigated in addition to NaF. The early response effect of Na_2SiF_6 was greater, and in several cases significantly greater, than NaF on clonogenic growth and the induction of apoptosis in all four cell lines. These findings show that human leukemic cells can be influenced and damaged by fluorine compounds.

Keywords: Apoptosis; Clonogenicity; Fluoride toxicity; Human leukemic cells; Sodium fluoride (NaF); Sodium hexafluorosilicate (Na_2SiF_6).

INTRODUCTION

Leukemias originate from hematopoietic stem cells at certain stages of their maturation and differentiation. It is well established that acute leukemias originate from immature hematopoietic stem cells that have the capacity to undergo self-renewal, whereas less aggressive leukemias such as chronic leukemias seem to originate from the more mature committed hematopoietic progenitor cells.[1] Potential toxic effect of sodium fluoride on early progenitor and stem cells has been described previously.[2-5] Surprisingly, few investigations have examined the effects of fluoride on human leukemic cells. According to Kawase *et al*, sodium fluoride (NaF) at a concentration of 0.5 mM for up to four days inhibited proliferation of human promyelocytic HL-60 cells.[6] Anuradha *et al* reported that NaF induced apoptosis in HL-60 cells by caspase-3 activation.[7] Because of the common occurrence of fluorides in the environment, it is desirable that their toxicity be accurately assessed and their mechanism of action be clarified.

To address this issue, we exposed four different human leukemic cell lines to increasing concentrations of two sources of fluoride. Beside sodium fluoride (NaF), sodium hexafluorosilicate (Na_2SiF_6) was also investigated because of its widespread use in water fluoridation. We assessed the effect of

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these compounds on clonogenic growth and the induction of apoptosis in the four cell lines.

MATERIAL AND METHODS

Cells: The four different human leukemic cell lines studied were: HL-60 – M3 subtype of AML in the French-American-British classification (FAB), HEL – M6 subtype of AML in the FAB classification, TF–1–M6 subtype of AML in the FAB classification, and CML cell line – K562 (ATCC, USA). They were maintained in IMDM (Iscove's Modified Dulbecco's Medium) supplemented with 10% fetal calf serum (FCS), 1 mg/mL L-glutamine, and antibiotics. Additional human IL-3 and GM-CSF were necessary for the growth of the TF-1 cells.[8] The viability of cells was assayed by a trypan blue dye exclusion test. Cells were counted using a hemocytometer and subsequently used for further experiments.

Exposure to fluoride compounds. Sodium fluoride (Sigma, USA) and sodium hexafluorosilicate (Na_2SiF_6) (Sigma, USA) were diluted in phosphate-buffered saline (PBS 0.9%; pH 7.3) at a concentration of 1 g/L and were stored at 4°C. A total of $5x10^3$ leukemic cells of each cell line was resuspended in 1 mL of IMDM + 10% FCS and then exposed to different concentrations of NaF: 0 mg/L (control) (0 mM F), 10 mg/L (0.24 mM F) and 50 mg/L (1.19 mM F) or Na_2SiF_6: 0 mg/L (control) (0 mM F), 7.46 mg/L (0.24 mM F) and 37.32 mg/L (1.19 mM F) for 24 hrs at 37°C. The concentrations of Na_2SiF_6 correspond to those of NaF according to the total amount of fluorine (but not F^-) in the solution. After the exposure, the cells were washed twice with PBS and plated in methylcellulose cultures to grow blastic colonies. TF-1 cells required addition of growth factors to *ex vivo* cultures (interleukin-3 [IL-3, 20 U/mL] [Genetics Institute, USA] + granulocyto-macrophagopoietin [GM-CSF, 5 ng/mL]), as mentioned above. In contrast, HEL as well as the other cell lines did not require any addition of growth factors.

Detection of apoptosis: In order to obtain more information on the influence of fluoride on neoplasmatic blasts, the induction of apoptosis was estimated in the human leukemic cell lines exposed to NaF or Na_2SiF_6. Two different assays were employed to evaluate the early phase of apoptosis. The cells were exposed to increasing concentrations of the fluorine compounds (0, 0.24 and 1.19 mM F) and stored at 37°C for 24 hr. 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 flow cytometry (FACScan (flow activated cell sorter scan), Becton-Dickinson, USA). Moreover, apoptosis was evaluated using RT-PCR (reverse transcription followed by polymerase chain reaction) method for semi-quantitative detection of human proapoptotic (Bax) and anti-apoptotic (Bcl-

X_L) gene expression.[9,10] Total RNA was isolated from $1x10^6$ leukemic cells using an Rneasy Mini Kit (Qiagen Inc, USA) according to the manufacturer's protocol. The following primers were used:

- Bax primers (forward: 5'-GGGGACGAACTGGA-CAGTAA-3'; reverse:5'-CAGTTGAAGTTGCCGTCAGA-3'),
- Bcl-X_L primers (forward: 5'-TTCAGTGACCTGACATCCCA-3'; reverse:5'-TCCACAAAAGTATCCC-AGCC-3').[11]

Statistical analysis: Arithmetic means and standard deviations were calculated on an IBM computer using MS Excel v. 97. The experiments were performed on each cell line 6 times. The cells were cultured in quadruplicate at each fluoride concentration. Data were analyzed by the Mann-Whitney test. Statistical significance was defined as $p < 0.05$.

RESULTS

Decrease in clonogenicity: The 24-hr incubation of the leukemic cells with the fluoride compounds decreased their clonogenicity (Figures 1 and 2). Significantly lower numbers of blastic colonies were present after incubation with NaF at 50 mg/L (1.19 mM F) and Na_2SiF_6 at 37.32 mg/L (1.19 mM F). The most evident effect was found in the HL-60 cell line, in which significant inhibition ($p < 0.01$) was detected even after exposure to the two fluoride compounds at the lower concentration (0.24 mM F). Moreover, the inhibition of clonogenic growth of HEL cells was significantly more evident after exposure to higher concentration of Na_2SiF_6 (1.19 mM F) compared to NaF ($p < 0.05$).

Apoptosis assays: Apoptosis analyzed by Annexin V and PI staining using flow cytometry is shown in Figure 3. This method is useful to distinguish early from late stages of apoptosis. Slight, but not statistically significant, differences in the percentage of the cells undergoing apoptosis were observed between the control cells (not exposed to fluoride) and the cells exposed for 24 hr to NaF at the concentration 0.24 mM F. The differences were more strongly expressed ($p < 0.05$) at the higher concentration of fluoride (1.19 mM F) (Figure 4). In the case of Na_2SiF_6 the differences were more evident and statistically significant ($p < 0.05$) even after exposure to fluoride at the lower concentration (0.24 mM F) (Figure 5). Interestingly, a significantly higher percentage of apoptotic HL-60 and HEL cells was found on exposure to the higher concentration of Na_2SiF_6 (1.19 mM F) than to NaF.

The expression levels of the proapoptotic-Bax and anti-apoptotic-Bcl-X_L genes in the cells after 24-hr incubation with fluoride were also investigated using the RT-PCR method. No differences were observed in the expression

of the mRNA of the Bcl-X_L gene between the control cells and the cells exposed to fluoride (not shown), but the expression of the Bax gene increased in the leukemic cells after the incubation with fluoride, particularly at the higher concentration (1.19 mM F). Figure 6 shows the expression of β-actin (as positive control) and Bax on mRNA level in the case of HL-60 cells exposed to NaF.

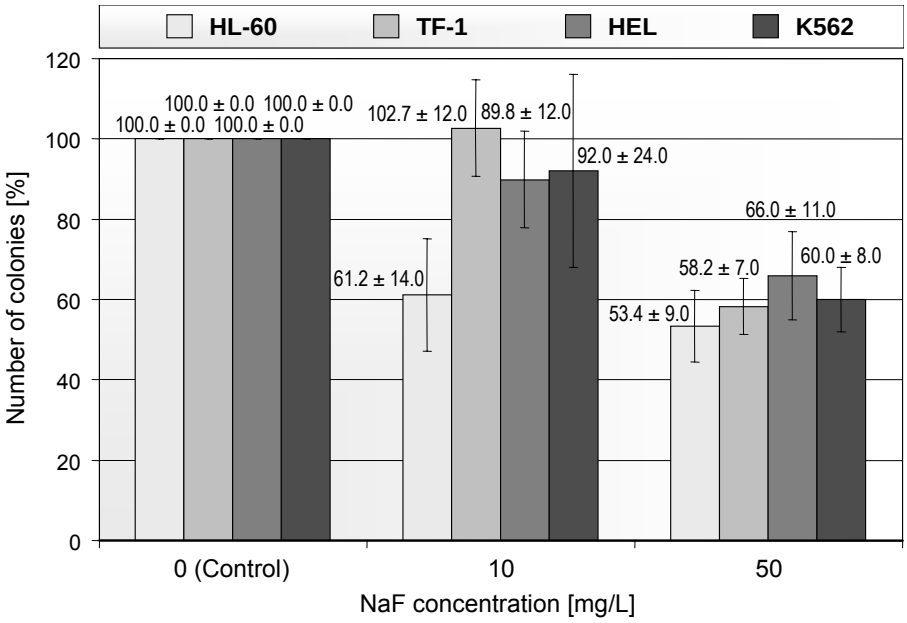

Figure 1. Influence of 24-hr NaF exposure on blast colony formation (human leukemic cells incubated at 37°C before plating).

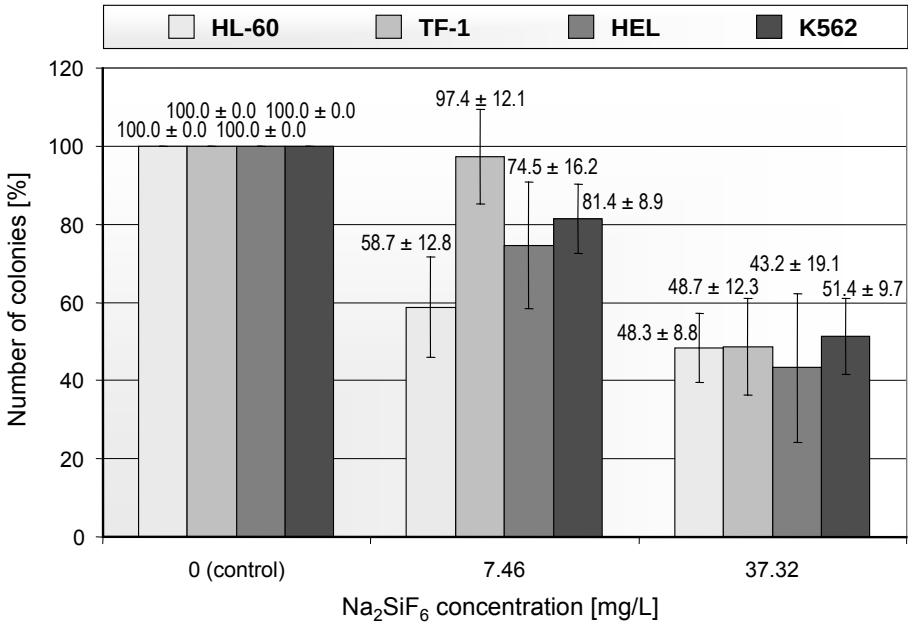

Figure 2. Influence of 24-hr Na_2SiF_6 exposure on blast colony formation (human leukemic cells incubated at 37°C before plating).

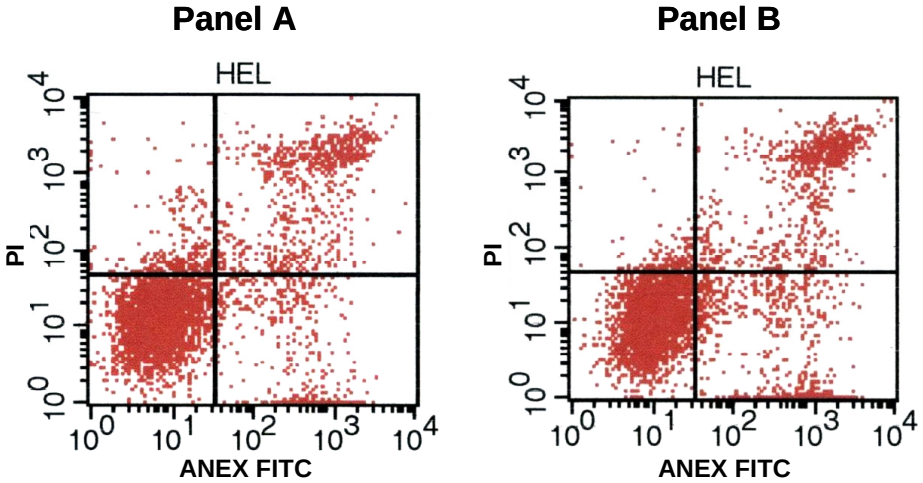

Figure 3. A representative study for detection of the number of apoptotic (x axis – Annexin V-Fluorescein Isothiocyanate [ANEX FITC]) and death HEL cells (y axis – propidium iodide [PI]) in the leukemic cell samples of the control group (panel A) and the group exposed to NaF at the concentration 50 mg/L (panel B).

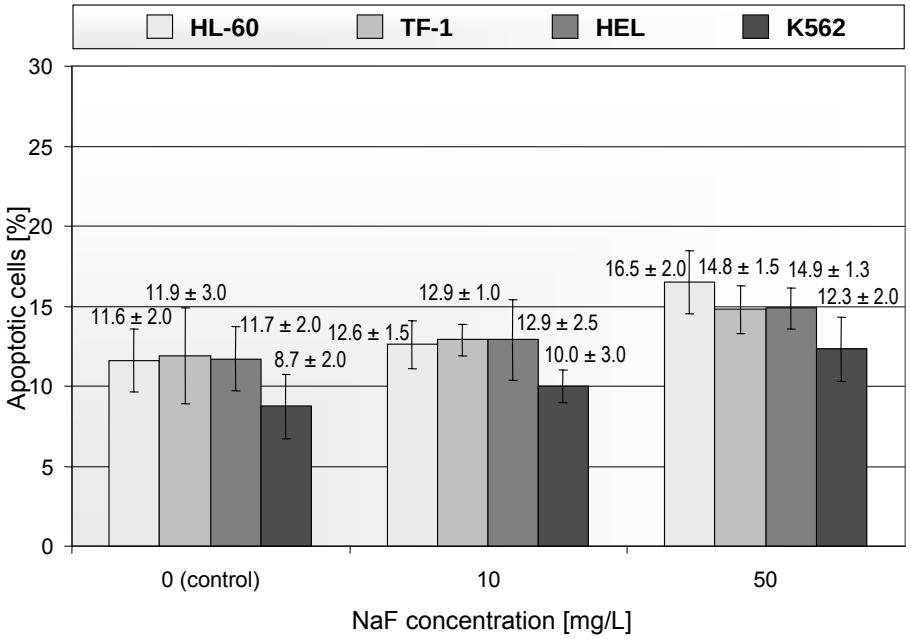

Figure 4. Percent of apoptotic human leukemic cells, after 24-hr incubation with increasing concentrations of NaF, evaluated by flow cytometry.

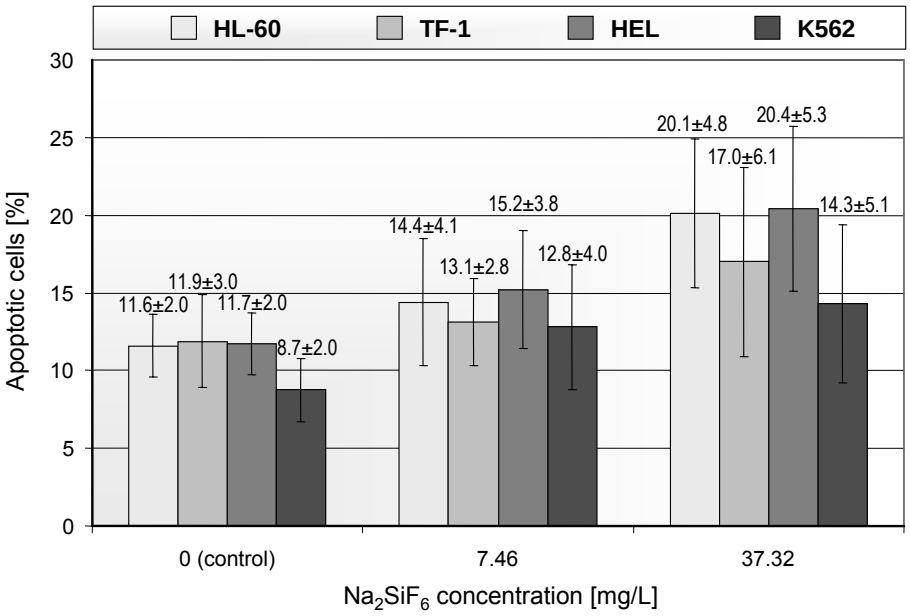

Figure 5. Percent of apoptotic human leukemic cells, after 24-hr incubation with increasing concentrations of Na_2SiF_6, evaluated by flow cytometry.

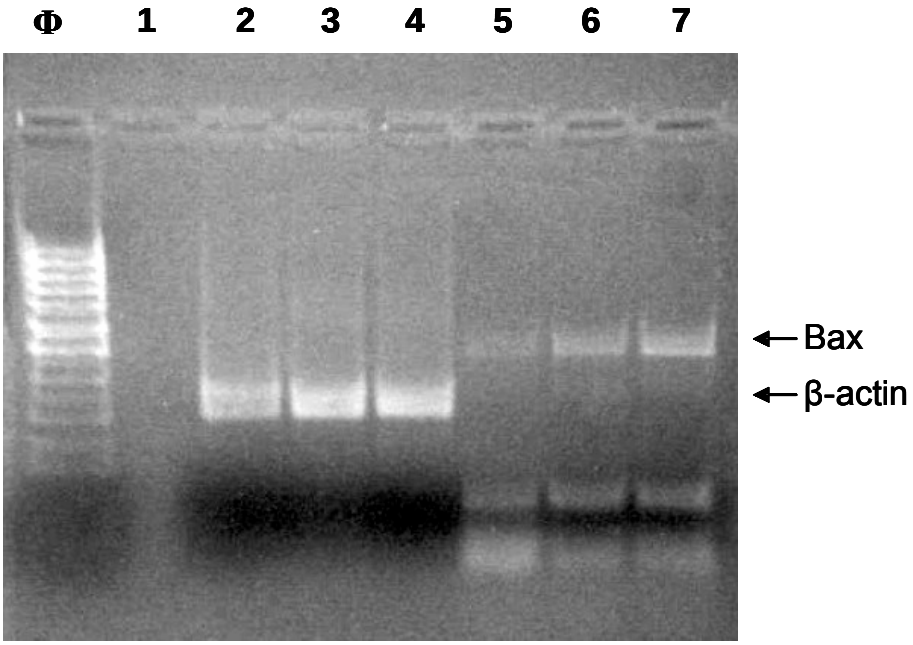

Figure 6. mRNA expression of Bax gene in HL-60 cell line. Φ – marker; negative control (H_2O) (lane 1); positive control (β-actin) (lanes 2-4); cells not exposed to NaF (lane 5); cells exposed to 10 mg NaF/L (lane 6); cells exposed to 50 mg NaF/L (lane 7).

DISCUSSION

In this report, different human leukemic cells were found to differ in their resistance to the influence of fluoride. The most sensitive were promyelocytic HL-60 cells. Our findings are in good agreement with those of Kawase *et al*, who reported that NaF at 0.5 mM inhibited proliferation of HL-60 cells.[6]

Other cells tested in our work were more resistant, and significant inhibition of their clonogenic growth was observed only after exposure to the higher concentration of NaF as well as Na_2SiF_6 (1.19 mM F). Strong expression of the multidrug resistance gene (mdr-1) in leukemic blasts seems to be important in the explanation of these results.[12,13]

Apoptosis was detectable in similar proportions after exposure of the cells to both NaF and Na_2SiF_6. Higher concentrations of fluorine compounds in-

duced a larger number of the cells to enter the early phase of apoptotis measured by flow cytometry.

In dilute aqueous solution, essentially 100% of NaF dissociates to sodium ions and fluoride ions. By contrast, Na_2SiF_6 readily dissociates only to sodium ions and hexafluorosilicate ions. In theory, if 100% of Na_2SiF_6 dissociated to fluoride ions and hydrated silica, as is claimed to occur at the pH of drinking water and at the concentration usually used for fluoridation (1 mg F^-/L),[14] the level of F^- derived from sodium hexafluorosilicate would be the same as from NaF in the respective concentrations used here. Nevertheless, according to another report,[15] fluoride does not dissociate completely from $[SiF_6]^{2-}$ at pH 7.4 and 37°C.

Our finding of a significantly higher inhibition of clonogenic growth of HEL cells as well as a higher percentage of apoptotic HL-60 and HEL cells exposed to Na_2SiF_6 (at 1.19 mM F), compared to the same concentration of NaF, thus supports a report that the silicofluoride complex (SiF) has biological effects that are even more potent than those of simple fluoride released by sodium fluoride.[15]

Apoptosis is characterized by a loss of membrane phospholipid asymmetry. Phosphatidylserine (PS), which is located in an inner leaflet of the plasma membrane, translocates to the surface of the cell, thereby playing an important role in the removal of the apoptotic body by the surrounding cells.[16] Since Annexin V has an affinity to PS, staining the cells with fluorescein isothiocyanate-labeled Annexin V enables the detection of apoptotic cells by flow cytometry.[17] Changes in the structure of the cell membrane are considered characteristic for an early stage of apoptosis. Nevertheless, this simple and relatively inexpensive method is not very specific and requires additional verification by other tests.[18]

We confirmed our results by RT-PCR (reverse transcription followed by polymerase chain reaction) estimation of the Bax and Bcl-X_L gene expression for a better understanding of the mechanism of apoptosis.[16] Semiquantitative analysis of our results showed that NaF and Na_2SiF_6 used in higher concentrations (1.19 mM F) caused induction of programmed cell death in leukemic blasts. In this regard, our study extends the findings of Anuradha *et al*, in which 24-hr exposure to 2 mM NaF induced apoptosis in HL-60 cells by downregulation of Bcl-2.[7] Unfortunately, these authors did not examine Bax protein as well as the other cell lines we evaluated here.

In conclusion, our findings revealed that human leukemic cells can be influenced and damaged by different forms of fluorine compounds. A substantially more evident effect was caused by silicofluoride complex (SiF) compared to simple fluoride ion released by sodium fluoride.

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