IN VIVO EFFECTS OF SODIUM FLUORIDE ON BONE MARROW TRANSPLANTATION IN LETHALLY IRRADIATED MICE

Anna Machalinska, a Jaroslaw Nowak, b Alina Jarema, c Barbara Wiszniewska, d Boguslaw Machaliński a
Szczecin, Poland

SUMMARY: In our previous studies we found that sodium fluoride (NaF) might be toxic to early human hematopoietic cells. One of the mechanisms for such an effect was the induction of apoptosis in affected cells. Our new experiments were designed to evaluate whether hematopoietic cells exposed to sodium fluoride in ex vivo conditions are able to engraft into lethally irradiated mice and successfully reconstitute hematopoiesis. Untreated cells as well as cells exposed to a moderate (10 mg/L) dose of NaF were able to establish a significant number of new hematopoietic islets on the spleen surface. In contrast, fewer hematopoietic islets were found when the cells were exposed to a higher dose of sodium fluoride (50 mg/L). Morphological parameter values of peripheral blood derived from the mice confirmed these effects. From the results we conclude that a relatively high dose of fluoride may significantly influence hematopoiesis.

Keywords: Bone marrow cells, Hematopoietic cell transplantation, Mice irradiation, Sodium fluoride (NaF), Spleen surface.

INTRODUCTION

Blood cell production is a continuous process, which results from the proliferation and differentiation of hematopoietic stem cells (HSCs) and hematopoietic progenitor cells (HPCs). Biologically, HSCs are characterized by their ability to self-renew and to differentiate into HPCs of all lineages – both myeloid and lymphoid.1, 2 Each time an HSC divides, two functionally distinct daughter cells are produced. One remains “stem-like” and replenishes the HSC pool. The other enters a differentiation pathway that gives rise to an HPC and undergoes further divisions. Thanks to them the hematopoietic system is constantly renewed. Because HSCs and HPCs exist as a developmental continuum, it is technically extremely difficult to isolate a pure fraction of HSCs.1 The functional proof that HSCs exist in a cell suspension is the full reconstitution of hematopoiesis in patients undergoing allogeneic bone marrow transplantation.

HSCs are influenced by a wide range of stimulatory and inhibitory signals delivered in the form of cytokines and growth factors. Certain biological microelements can influence or disturb normal hematopoiesis.1, 3 Among them fluorine is one of the most reactive.4

aFor Correspondence: Boguslaw Machaliński MD PhD, Dept. of General Pathology, Pomeranian Academy of Medicine (PAM), Al. Powstancow Wilk. 72, 70-111 Szczecin, Poland; bProvincial Blood Bank, ul. Wojska Polskiego 80/82, 70-482 Szczecin, Poland; cClinic of Radiotherapy, PAM, ul. Strzalowska 22, 71-730 Szczecin, Poland; dDept. of Histology and Embryology, PAM, Al. Powstancow Wilk. 72, 70-111 Szczecin, Poland. E-mail: machalin@sci.pam.szczecin.pl
Previously, we found that fluorides could influence clonogenic growth and also induce apoptosis in human hematopoietic progenitor cells.\textsuperscript{5,6,7} Extending that research, we have now performed additional experiments designed to evaluate the influence of sodium fluoride (NaF) on hematopoietic stem cells using an animal model. In this study, we investigated the hematopoiesis reconstituting ability of such cells exposed to different doses of NaF and subsequently engrafted to lethally irradiated Balb C mice.

**MATERIALS AND METHODS**

**Cells:** Bone marrow cells were obtained from 12 freshly decapitated 6-week old mature, healthy, female inbred Balb C mice (Polish Academy of Sciences, Wroclaw, Poland). A fraction of bone marrow mononuclear cells (BM MNCs) was isolated after centrifugation over Gradiol L (Polfa Kutno, Poland) gradient and depleted of adherent cells after overnight incubation in plastic Petri dishes as described.\textsuperscript{5,9} Cell viability was assessed by the 0.5% trypan blue exclusion test. Sodium fluoride (Sigma, USA) was diluted in a phosphate-buffered saline (PBS) at a concentration of 1 g/kg and was stored at 4°C. BM MNCs ($5 \times 10^7$) were resuspended in 1 mL of PBS + 5% BCS (bovine calf serum) and then exposed to NaF at different concentrations: 0 mg/L (group 1), 10 mg/L (0.24 mM; group 2), and 50 mg/L (1.19 mM; group 3) for 12 hr at 37°C. After incubation the cells were washed twice in IMDM (Iscove’s Modified Dulbecco’s Medium) and subsequently used for transplantation studies.

**Mice:** The transplantation experiment was performed on pathogen-free, 6-week old mature female inbred Balb C mice (Polish Academy of Sciences, Wroclaw, Poland). The animals were randomly divided into three experimental groups (group 1 – irradiated mice injected with cells unexposed to NaF; group 2 – irradiated mice injected with cells exposed to NaF at a concentration of 10 mg/L; group 3 – irradiated mice injected with cells exposed to NaF at a concentration 50 mg/L), 1 control group (mice not irradiated) and 1 blind group (irradiated mice injected with saline instead of cells). Groups 1, 2, and 3 contained 6 animals each; the control and blind groups consisted of 3 animals each. The animals were maintained under standard laboratory conditions in a 12hr/12hr light-dark cycle, at 21°C. The mice (except for the control group) were exposed to 800 cGy from a gamma irradiation source 24 hr prior to transplantation.

**Hematopoietic cell transplantation:** The day after irradiation, mice from groups 1, 2, and 3 were given a transplant of $5 \times 10^7$ BM MNCs/animal via tail vein injection. Mice from the blind group were injected with saline instead of cells. After transplantation, hematological parameters of the animals were monitored by blood sampling from the retroorbital plexus. Ten and 14 days after the transplantation, mice (half of the animals from each group)
were sacrificed, and the presence of hematopoietic islets on the surface of spleens was examined under the light microscopy, and the livers and spleens were collected for microscopic analysis.

\textit{Hematological parameters:} Hematological parameters of the peripheral blood samples (n = 5) were evaluated by employing an automatic blood-analyzer (Cell-DYN 1700, Abbott, USA) according to the manufacturer’s protocol.

\textit{Microscopic analyses:} Hematopoietic stem cell recovery was evaluated using the endogenous spleen colony-forming unit (E-CFU) assay. The number of hematopoietic islets on spleen surfaces were evaluated under a stereoscopic microscope immediately after obtaining the organ. For morphological studies the tissues (livers and spleens) were fixed in Carnoy’s solution and stained with H-E (Hematoxylin-Eosin) and PAS (Periodic-Acid-Schiff) methods. Microscopic slides from the livers and spleens of the sacrificed mice were evaluated under a light microscope (Olympus, Japan) and subsequently photographed.

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

\textbf{RESULTS}

\textit{Ability of cells exposed to NaF to engraft in vivo}

Some of the mice injected with cells exposed to the higher dose of NaF (group 3) as well as all animals from the blind group (injected with saline instead of cells), became sick within 24 hr after the injection. On the 5th day two animals from group 3, and all from the blind group died. Three mice from the control, and groups 1 and 2, along with two from group 3 were sacrificed on the 10th day. The rest of the mice were sacrificed on the 14th day of the experiment.

Figure 1 shows the relative numbers of hematopoietic islets on the surface of spleens obtained from the examined mice. A moderate concentration of NaF (10 mg/L) used during preincubation of the injected cells caused little inhibitory effect on the growth of murine early hematopoietic cells 10 days after transplantation, compared to the irradiated mice that were treated with unexposed cells. In contrast, a higher concentration of NaF (50 mg/L) damaged part of the stem and progenitor cells and significantly decreased the number of these islets. Fourteen days after transplantation, renewal of hematopoiesis was not inhibited by sodium fluoride used at a moderate concentration (10 mg/L). At the same time, inhibition of hematopoietic islets formation by the high dose of NaF was similar to that found on the 10th day of the experiment.
In these studies we also evaluated several standard hematological parameters of the peripheral blood (Table). We found that all parameters – white blood cells (WBC), red blood cells (RBC), hemoglobin (HGB), hematocrit (HCT), and platelets (PLT) - in the samples collected from mice injected with the cells exposed to 50 mg NaF/L were significantly lower (p < 0.05) compared to those in the samples collected from the other groups of mice. On the other hand, no significant differences in recovery of hemopoiesis were found between peripheral blood samples aspirated from mice injected with cells not exposed to NaF and from mice injected with cells exposed to 10 mg NaF/L (Table).

**Morphological changes**

**Livers:** Cytoplasm of the hepatocytes stained with H-E in all the irradiated groups of mice had regular structure and the chromatin in the nuclei formed nodules located near the nuclear membrane. In every instance, regular distribution of glycogen, which was located mostly around the central vein, was found in the hepatocytes stained with PAS.

*Figure 1.* The influence of NaF on hematopoietic islets formed on spleen surface by cells transplanted into mice. The hematopoietic cells before transplantation were exposed to NaF at doses of 10 and 50 mg/L for 12 hr at 37°C. The numbers of islets are shown as a percent of the islets formed by cells that were not exposed to NaF.
**Table.** Hematological parameters (mean, SD) of peripheral blood obtained from mice after 10 and 14 days since transplantation (n = 5). NI – mice not irradiated; group 1 – mice injected with unexposed cells; group 2 – mice injected with cells exposed to 10 mg NaF/L; group 3 – mice injected with cells exposed to 50 mg NaF/L. WBC – white blood cells; RBC – red blood cells; HCT – hematocrit; HGB – hemoglobin; PLT – platelets. G/L – 10^9/L; T/L – 10^12/L

<table>
<thead>
<tr>
<th>Group</th>
<th>Time</th>
<th>WBC  G/L</th>
<th>RBC T/L</th>
<th>HCT %</th>
<th>HGB mM</th>
<th>PLT G/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>NI</td>
<td>10 days</td>
<td>9.2 (1.0)</td>
<td>6.12 (0.9)</td>
<td>0.28 (0.05)</td>
<td>6.8 (0.5)</td>
<td>403 (79)</td>
</tr>
<tr>
<td>Group 1</td>
<td>10 days</td>
<td>3.3 (0.8)</td>
<td>5.17 (1.0)</td>
<td>0.23 (0.03)</td>
<td>6.4 (0.3)</td>
<td>243 (51)</td>
</tr>
<tr>
<td></td>
<td>14 days</td>
<td>8.1 (1.1)</td>
<td>5.91 (0.8)</td>
<td>0.29 (0.05)</td>
<td>6.9 (0.4)</td>
<td>367 (88)</td>
</tr>
<tr>
<td>Group 2</td>
<td>10 days</td>
<td>2.5 (0.7)</td>
<td>5.44 (1.7)</td>
<td>0.24 (0.05)</td>
<td>6.0 (0.5)</td>
<td>272 (92)</td>
</tr>
<tr>
<td></td>
<td>14 days</td>
<td>8.0 (0.8)</td>
<td>6.0 (1.3)</td>
<td>0.28 (0.03)</td>
<td>6.2 (0.6)</td>
<td>398 (78)</td>
</tr>
<tr>
<td>Group 3</td>
<td>10 days</td>
<td>2.1 (0.5)</td>
<td>3.7 (1.2)</td>
<td>0.17 (0.02)</td>
<td>4.6 (1.0)</td>
<td>210 (37)</td>
</tr>
<tr>
<td></td>
<td>14 days</td>
<td>2.0 (0.4)</td>
<td>4.3 (1.3)</td>
<td>0.20 (0.03)</td>
<td>4.7 (0.9)</td>
<td>322 (45)</td>
</tr>
</tbody>
</table>

**Spleens:** Figure 2 shows spleen morphology of a control, non-irradiated mouse. Ten days after injection of murine cells unexposed to NaF into mice of group 1, large amounts of lymphatic nodules with germinal centers and numerous early cells were found (Figure 3). A small number of megakaryocytes was also visible. After 14 days, expanded white pulp in the spleen sections was noted. Numerous early lymphocytes with normal nuclei as well as megakaryocytes were likewise present.

After 10 days, in the case of injection of cells exposed to a moderate dose of NaF (10 mg/L; group 2), large lymphatic nodules were found in which few lymphatic cells with low levels of condensed chromatin were visible (Figure 4). Also few megakaryocytes were present. After 14 days from the injection, many more early forms of lymphatic cells were visible in large lymphatic nodules. The number of megakaryocytes in the spleens was higher.

In group 3, 10 days after injection of the cells exposed to the high dose of NaF (50 mg/L), lymphatic nodules containing early forms of lymphocytes were found (Figure 5). Singular megakaryocytes were present. After 14 days the spleen sections were very similar to those in group 2 after 14 days.
Figure 2. Spleen morphology of a control, non-irradiated mouse after 10 days. H-Ex160.

Figure 3. Spleen morphology of an irradiated mouse 10 days after injection with bone marrow mononuclear cells not exposed to NaF. Lymphatic nodules with germinal centers and numerous early cells are visible. H-Ex160.
Figure 4. Spleen morphology of an irradiated mouse 10 days after injection with bone marrow mononuclear cells exposed to 10 mg NaF/L. Large lymphatic nodules in which few lymphatic cells with low levels of condensed chromatin are visible. H-Ex160.

Figure 5. Spleen morphology of the mouse 10 days after injection with bone marrow mononuclear cells exposed to 50 mg NaF/L. Lymphatic nodules containing early forms of lymphocytes are visible. H-Ex330.
DISCUSSION

Our earlier studies indicated that sodium fluoride affected human hematopoietic progenitor cells (HPCs) and significantly decreased their clonogenic growth.5,7 This phenomenon occurred at NaF concentrations of 50 mg/L and higher. Moreover, exposure to the same concentration of sodium fluoride induced significant apoptosis in human HPCs.6

Following these reports, we performed additional experiments designed to evaluate the potential influence of sodium fluoride in increasing doses on hematopoietic stem cell populations. So far, it has not been clear whether murine hematopoietic stem and early progenitor cells, obtained from healthy inbred Balb C mice and subsequently exposed to sodium fluoride in ex vivo conditions, would be able to engraft into lethally irradiated animals (taken from the same breeding) and successfully reconstitute hematopoiesis.

Hematopoietic stem and early progenitor cells, which are responsible for hematopoiesis renewal, are very primitive, non-cycling and metabolically quiescent.1,2 More importantly, they are relatively resistant to different toxic agents.1 Our present results, which involve numeration of early hematopoietic islets visible on the spleen surface, morphologic examination of spleen and liver and analysis of standard hematological parameters of peripheral blood, suggest that a relatively high concentration of NaF (50 mg/L) disturbed and significantly delayed the reconstitution of hematopoiesis in lethally irradiated mice.

Our findings therefore revealed that stem and early progenitor cells can be influenced and damaged by sodium fluoride. Because of the potential toxic effect on the hematopoietic system, we suggest introduction of a professional screening program in regions affected by endemic fluorosis or intensive industrial pollution. Such examination should focus not only on hard tissues but also on hematopoiesis, particularly in children and young adults.

ACKNOWLEDGMENT

This work was presented at the XXIIIrd Conference of the International Society for Fluoride Research in Szczecin, Poland, June 11-14, 2000 (Program abstract P-28).

REFERENCES


Fluoride 35 (2) 2002