SODIUM FLUORIDE ENHANCEMENT OF MONOCYTE DIFFERENTIATION VIA NUCLEAR FACTOR κB MECHANISM

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SUMMARY: Biologically, reactive oxygen species (ROS) may act as intracellular signal molecules participating in the process of growth, differentiation, cellular apoptosis, and gene expression. The objective of the present study was to determine the role of fluoride in differentiating monocytes to macrophages, and its potential for generating ROS in these cells. Adherent macrophages from human monocytic leukemia cells (THP-1) were incubated for 48 hr with 2.5, 62.5, and 125 µM NaF and used for quantitative measurement of ROS formation and estimation of apoptosis intensity. Additionally, peripheral blood mononuclear cells (PBMCs) of healthy volunteers were separated by density gradient. NaF at a concentration of 2.5 µM was added, and cells were then cultured for 48, 72, or 144 hr. Monocytes obtained from PBMCs were used for measurement of intracellular ROS formation, apoptosis, and monocyte differentiation by flow-cytometry. In these cells activation of transcription nuclear factor κB (NFκB) was measured using enzyme-linked immunoassay (ELISA). In monocytes isolated from PBMCs, NaF at a relatively low concentration (2.5 µM) was a stimulator of differentiation of monocytes to macrophages. Thus, because it can contribute to differentiation of monocytes and to more rapid activation of ROS generation, fluoride may be a potential activator of transcription factor κB. In macrophages obtained from THP-1 cells, ROS generation was most intense after 48 hr incubation with the lowest concentration of NaF (2.5 µM). Higher fluoride concentrations (65 and 125 µM) caused a limitation in ROS generation, which was associated with cell apoptosis.

Keywords: Apoptosis; CD 68 antigen; Cyclooxygenase-1; Cyclooxygenase-2; Human leukemia cells; Macrophage; Monocyte; Reactive oxygen species.

INTRODUCTION

The major physiological mechanism of cell removal from the body is apoptosis. Morphologically, in cells undergoing this change, there is disruption of the cytoskeletal architecture, condensation of plasma and nuclear membranes, and aggregation of nuclear chromatin (cell fragments) into “apoptotic bodies”. In apoptosis, cell remnants are phagocytosed by surrounding cells, i.e., by macrophages.1,2 Reactive oxygen species (ROS) such as superoxide radical anion (·O2), singlet oxygen (1O2), hydrogen peroxide (H2O2), and hydroxyl radical (·OH) are produced as by-products of oxidative metabolism in which energy activation is involved.3 They are produced in metabolic pathways of the xanthine oxidase system, the cyclooxygenase system, the mitochondrial respiratory chain, and the amyloid protein system.3,4 ROS may also act as intracellular signal molecules participating in the processes of growth, differentiation, cellular apoptosis, and gene expression.2,5,6 Therefore, their production is enhanced during inflammation, aging, radiation exposure, endotoxic shock, and ischemia-

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reperfusion of the heart, intestine, liver, kidney, and brain. They are therefore implicated in various cell dysfunctions.

Macrophages are one of the main sources of ROS in the human body. In these cells, the intracellular sources of ROS are certain enzymes: cyclooxygenases, respiratory chain enzymes, and NADPH-dependent oxidase. Cyclooxygenases are present in macrophages in two isoforms: cyclooxygenase1 (Cox-1) and cyclooxygenase2 (Cox-2). They catalyze the conversion of arachidonic acid to prostaglandins. Cox-1 is expressed constitutively, while Cox-2 is highly induced by many factors including cytokines, growth factors, and tumor promoters. In monocytes, only constitutive Cox-1 is present; in macrophages additionally inducible Cox-2 is expressed. Cox-2 expression is seen during monocyte-macrophage differentiation, e.g., as a result of contact with phorbol myristate acetate (PMA), or during long-term cultivation of monocytes in culture medium (Figure 1). The most popular, quantitative analytical method of monocyte differentiation is the cytometric analysis of expression of CD68—transmembrane glycoprotein highly expressed by tissue macrophages, or measurement of macrophages enzymes expression (or their product e.g., ROS).

The transcription nuclear factor κB (NFκB) has been shown to be important in cell proliferation. NFκB is a member of the protein family (Rel) that shares a 300 amino acid region, which mediates DNA binding, interaction with inhibitors, and nuclear translocation. NFκB typically is a heterodimer, consisting of two subunits, p65/RelA and p50/NFκB1, localized in the cytosol as a complex with the inhibitory protein IκB. Upon stimulation of the cell, IκB is degraded and free NFκB subunits are accumulated in the nucleus, where they can activate transcription of many genes, e.g., cyclooxygenase-2 gene.

A number of studies conducted to date have shown that fluoride may induce oxidative stress and enhance lipid peroxidation within the cell. The objective of the present study was to determine the role of fluoride in the process of monocyte differentiation into macrophages, apoptosis in monocyte/macrophage cells, and potential generation of ROS in these cells.

MATERIALS AND METHODS

Reagents and their sources were as follows: Human THP-1 monocytic leukemia cells from American Type Culture Collection (ATCC, Rockville, USA); RPMI (Roswell Park Memorial Institute) medium, glutamine, antibiotics (penicillin and
streptomycin), 2',7'-dichlorofluorescein diacetate (DCFH-DA), sodium fluoride (NaF) and phorbol myristate acetate (PMA) were from Sigma (Sigma–Aldrich, Poland), phosphate buffered saline (PBS) and foetal bovine serum (FBS) from Gibco (Gibco, UK), and Lymphozyten separations media from PAP Laboratories (PAP Laboratories, Austria). Nuclear Extract kit and TransAm NFκB p65/Rel A Transcription Factor Assay kits were from Active Motif (Rixensart, Belgium). The kit for apoptosis detection, Annexin V-fluorescein isothiocyanate (FITC) binding assay and the kit for CD68 expression analysis were from Becton Dickinson (Oxford, UK).

**Cell culture and treatment:** Human THP-1 monocytic leukemia cells were cultured in RPMI 1640 medium supplemented with 10% fatty acid free FBS, penicillin (100 U/mL) and streptomycin (100 mg/mL) at 37°C in 5% CO₂. THP-1 monocytes were treated with 100 nM PMA for 24 hr to facilitate differentiation into macrophages. Control THP-1 cells were cultured in a medium without PMA. After treatment, the adherent macrophages were washed three times with PBS and incubated with NaF at three concentrations: 2.5, 62.5, and 125 µM for 48hr at 37°C.

**Preparation of peripheral blood mononuclear cells (PBMCs):** Peripheral blood from young healthy volunteers was obtained in accordance with the principles outlined in the Declaration of Helsinki (Cardiovasc Research 1997;35:2-3). Mononuclear cells were separated by Lymphozyten separations media density gradient for 30 min, at 800×g and 20°C, as previously described. PBMCs (a mixture of monocytes and lymphocytes) were centrifuged, washed with ice-cold PBS, resuspended in PBS, and counted. Then, monocytes were isolated by selective adherence to the culture dishes for 2hr at 37°C, 5% CO₂. After 3 days, 2.5 µM NaF was added and monocytes were cultured for another 48, 72, or 144hours.

**Intracellular ROS formation:** The cells (monocytes from PBMCs or macrophages from THP-1) after incubation with NaF or immediately after NaF addition (considered as 0 hr in Table 1), were pre-loaded for 30 min at 37°C with 5 µM 2',7'-dichlorofluorescein diacetate (DCFH-DA), which became fluorescent on oxidation to DCF by hydrogen peroxide within the cell. When ROS synthesis increased, oxidation of DCFH-DA and fluorescent were automatically elevated. Cells were harvested and the number of cells exhibiting increased fluorescence of oxidized DCF was measured by flow cytometry (FACSCalibur, Becton Dickinson, USA) as previously described.

**Apoptosis measurements:** Annexin V-fluorescein isothiocyanate (FITC) binding assay was performed to determine the apoptotic cell death. After treatment, cells (monocytes from PBMCs or macrophages from THP-1) were counted using a Bürker chemocytometer and then, 5×10⁵ cells were collected and washed twice with PBS. Next, the cells were suspended in a binding buffer and stained with 1 ng/mL Annexin V-FITC and 5 ng/mL propidium iodide for 30 min in the dark. The stained cells were analysed directly by flow cytometry using the Cell Quest program (Becton Dickinson, USA).

**Flow cytometry:** The percentage of CD68 monocytes isolated from PBMCs was assessed by flow cytometry (FACScan) using CellQuest software. CD68 cells
were washed with ice-cold PBS, fixed with 4% paraformaldehyde and permeabilized using 0.5% Triton X-100, then incubated with fluorochrome-conjugated monoclonal antibody CD68 for 30 min. Mouse IgG2b,κ was used as isotype control antibody.15

**NFκB activation measurement:** To measure NFκB activation, monocytes isolated from PBMCs were incubated with 2.5 µM NaF for 144 hr. NFκB activation was measured in nuclear extract using an enzyme-linked immunoassay (ELISA)-based kit. In this method, the primary antibodies used to detect NFκB recognize one of NFκB subunits named p65/Rel A, an epitope which is only accessible when NFκB is activated and bound to its target DNA.

Briefly, NFκB was isolated using complete lysis buffer containing dithiothreitol and protease inhibitor cocktail according to instructions for the Nuclear Extract Isolation Kit (Active Motif). For measurement of protein concentration in nuclear extract, the Bradford protein measurement kit was used (Sigma-Aldrich, Poland), and then the nuclear extract was dissolved in suspensions containing 20 µg protein/probe. To determine NFκB activation with the ELISA-based kit, an oligonucleotide containing the NFκB consensus binding site (5’-GGGACTTTCC-3’) specific for the active form of NFκB was immobilised on a 96-well plate and the well was filled with uniform volumes of solutions containing 3.3 µg protein of the nuclear extract/well. After incubation, the primary antibody (against the active form of NFκB) and then the secondary antibody conjugated to horseradish peroxidase were added to achieve a reliable result by spectrophotometry at 450 nm. The experiments were performed in duplicate and the results were expressed as OD_{450nm}.

**Statistics:** As the distribution in most cases deviated from normal (Shapiro-Wilk test), non-parametric tests were used. Significance was first checked with Friedman’s ANOVA; significant results were the subjected to the Wilcoxon matched-pairs test.

### RESULTS

Table 1 presents the effect of a relatively small dose of fluoride (2.5 µM) on the synthesis of ROS in monocytes isolated from PBMCs depending on exposure time. An increase in ROS generation versus control cells was observed. ROS were measured by flow cytometry as described in the Materials and Methods. The highest quantitative increase in ROS synthesis was seen in both groups (experimental and control) after 48 hr of incubation.

**Table 1.** Fluoride activation (expressed as mean fluorescence intensity in relation to time of incubation) of systems responsible for synthesis of ROS in monocytes.

<table>
<thead>
<tr>
<th>Synthesis of ROS Monocytes</th>
<th>Time of incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 hr</td>
</tr>
<tr>
<td>Control without NaF</td>
<td>55.1±0.89</td>
</tr>
<tr>
<td>2.5 µM NaF</td>
<td>69±8.74</td>
</tr>
</tbody>
</table>

*p<0.05 (Wilcoxon’s test) in comparison with 0 hr value.
The effect of sodium fluoride (2.5 µM) on monocyte differentiation into macrophages is presented in Table 2. It was found that NaF added to the culture medium increased the expression of CD68 antigen compared to control (Table 2, Figure 2A and 2B). Monocytes were isolated from PBMCs.

Table 2. CD68 expression (measured as percentage of gated cells) in monocytes obtained from PBMCs and cultured with 2.5 µM NaF or without fluoride (control), n=4.

<table>
<thead>
<tr>
<th>Induction of CD68</th>
<th>Time of incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Gated events</td>
<td>48 hr</td>
</tr>
<tr>
<td>Control without NaF</td>
<td>1</td>
</tr>
<tr>
<td>2.5 µM NaF</td>
<td>1</td>
</tr>
</tbody>
</table>

*p<0.02 (Wilcoxon’s test) between groups at indicated times.

In parallel with the measurement of ROS production intensity and CD68 antigen expression, the intensity of apoptosis in monocyte cells (from PBMCs) incubated for 48 hr, 72 hr, or 144 hr in a medium containing 2.5 µM NaF or in NaF-free medium was measured. The presence of 2.5 µM NaF in the monocyte culture slightly stimulated apoptosis compared to control monocytes (Table 3, Figure 3A and 3B).
Fluoride enhancement of monocyte differentiation

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November 2005

**Table 3.** Apoptosis intensity expressed as percentage of viable (non-apoptotic) monocyte cells from PBMCs cultivated in a medium without NaF (control) and with 2.5 µM NaF, n = 4.

<table>
<thead>
<tr>
<th>Apoptosis Non-apoptotic cells</th>
<th>Time of incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>48 hr</td>
</tr>
<tr>
<td>Control cells without NaF</td>
<td>91±2</td>
</tr>
<tr>
<td>2.5 µM NaF</td>
<td>94±2</td>
</tr>
</tbody>
</table>

**Figure 3A, B.** Intensity of apoptosis expressed as percentage of viable in monocyte cells from PBMCs incubated for 72 hr in medium without NaF (A) or with 2.5 µM NaF

Similar measurements were performed in a culture of macrophages obtained from the THP-1 cell line. Increased ROS generation was seen in macrophages cultured at low fluoride concentration (2.5 µM) after 48 hr incubation. After that time, ROS generation in the cell gradually declined (Table 4).

**Table 4.** ROS production (expressed as mean fluorescence intensity) in macrophages obtained from THP-1 cells by addition of PMA (100 nM/24 hr) to the culture medium.

<table>
<thead>
<tr>
<th>Synthesis of ROS Macrophage</th>
<th>Time of incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 hr</td>
</tr>
<tr>
<td>Control without NaF</td>
<td>63.33±5.13</td>
</tr>
<tr>
<td>2.5 µM NaF</td>
<td>218.2±15.69</td>
</tr>
</tbody>
</table>

*P<0.05 (Wilcoxon’s test) in comparison with 0 hr value.

When THP-1 macrophages were cultured for 48 hr in a high-fluoride environment (65 µM and 125 µ5M), a reduction in ROS synthesis proportional to NaF concentrations in the medium was observed (Figure 4).
Figure 4. DCF fluorescence measurement of ROS in macrophages obtained from THP-1 cells after addition of PMA (100 nM), followed by cultivation for 48 hr in medium containing 65 µM and 125 µM NaF.

Figure 5 presents the percentage of apoptotic cells measured after 48 hr incubation. A significant number of macrophages cultured for 48 hr in a medium containing 65 µM NaF were found to be in the apoptotic state. Total cell apoptosis was obtained when 125 µM NaF was added to the macrophage culture and the macrophages were cultured for 48 hr. Under the same conditions, control macrophages still retained their activity, and the ROS-generating systems in the cells remained active.

![Apoptosis Graph](image)

Figure 5. Apoptosis (expressed as percentage of viable cells) in macrophages cultivated for 48 hr without NaF (control) and at high concentrations of NaF.

Activation of transcription nuclear factor NFκB in monocytes isolated from PBMCs and cultured with fluoride ion was observed. Cells were treated with NaF at a concentration of 2.5 µM or without F⁻ for 144 hr, and then activation of NFκB was measured by ELISA kit (Figure 6).

![Fluoride Activation ELISA](image)

Figure 6. Fluoride activation measure by ELISA kit of NFkB in nuclear extract of cells from PBMCs without NaF (control) and with NaF (2.5 µM) for 144 hr.
DISCUSSION

During an infective process in the body, circulating blood monocytes migrate from the vasculature into the extravascular compartment. In the tissues, monocytes differentiate into macrophages; they lose their ability to replicate but enhance their properties that allow them to participate in the inflammatory and immune responses. From the results obtained here, we conclude that fluoride at the relatively low concentration of 2.5 µM is a stimulator of the process of monocyte differentiation into macrophages. This is evidenced by increased expression of CD68 antigen (Table 2) and increased ROS generation in monocytes cultured with sodium fluoride (Table 1). The activation of ROS generation is associated with the effect on the expression of enzymatic systems specific for mature macrophages. A main source of ROS in macrophages is cyclooxygenase-2, which is expressed during the monocyte differentiation process.

Expression measurement of the CD68 protein is a quantitative method for determining the degree of monocytes differentiation into macrophages. As shown here, the number of cells containing the CD68 antigen increased (in comparison with control) in monocytes cultured with 2.5 µM NaF in the medium (Table 2). Therefore, monocytes cultured with sodium fluoride seem to differentiate into macrophages more rapidly than cells cultured in similar conditions without fluoride (Table 2). However, what is also important is that 2.5 µM fluoride did not reduce monocyte viability with respect to control cells (Table 3, Figure 3A and 3B).

How can the role of fluoride in the process of activation of monocyte differentiation into macrophages be explained?

During monocyte differentiation, cyclooxygenase-2 (Cox-2) induction takes place. Cox-2 expression is associated with activation of the NFκB pathway; the NFκB factor has been shown to control the induced transcription of Cox-2 gene. Fluoride seems to act as a ligand of the transcriptional factor NFκB. We propose that fluoride may be a potential activator of NFκB because it contributes to more rapid differentiation of monocytes, reflected by CD68 expression, and to activation of ROS generation as a result of Cox-2 expression (Table 1, Table 2, and Figure 6). The role of fluoride as a possible activator in the monocyte differentiation process also seems to be confirmed by the results of in vivo studies. In these experiments, macrophages were isolated from rats exposed to fluoride administered by inhalation. It was also noted that fluoride caused polymorphonuclear leukocyte (PMN) infiltration in the lungs, likewise supporting the role of fluoride in the differentiation process. In addition, fluoride acts as an activator of alveolar macrophages enhancing the production of chemokines and pro-inflammatory cytokines (pro-inflammatory activity).

In contrast to monocytes, macrophages have a fully mature enzymatic system; both cyclooxygenase isoforms are present in these cells. Therefore, ROS generation is greater in macrophages than in monocyte cells (Table 1, Table 4). In macrophages ROS synthesis was most intense after 48 hr incubation with 2.5 µM NaF (Table 4), the same as in the monocytes (Table 1).
Interesting information is provided by the observation of metabolism of macrophages cultured in media with the addition of relatively higher sodium fluoride concentrations (65 µM, 125 µM) (Figure 4). Proportionally to NaF concentration in the medium, the increased fluoride concentration caused a limitation in ROS generation (Figure 4). This process was strictly associated with pro-apoptotic properties of high fluoride concentrations (Figure 5). ROS generated as a result of Cox-2 activation by fluoride seem to trigger the whole chain of events characteristic of the apoptotic process: damage of cellular structures, DNA damage, and cell disintegration. The pro-apoptotic properties of fluoride and the mechanism of apoptosis induction by the addition of fluoride to the cells have been well described. In the study of Thrane et al., apoptosis in epithelial lung cells from rats and humans induced by high doses of sodium fluoride (1–5 mM) was assessed. These authors attributed to fluoride the role of activator of kinases critical for apoptosis induction. Additionally, epithelial lung cell exposure to fluoride has been shown to release increased amounts of inflammatory cytokines. At high concentrations, fluoride has also been found to inhibit protein synthesis and cell cycle progression. It has also been reported that epithelial lung cells and alveolar macrophages undergo apoptosis after fluoride exposure. Also, in human leukemia cells (HL-60) NaF induced apoptosis in a dose and time-dependent manner. Fluoride at a high doses (2–5 mM) led to activation of caspase-3. It is suggested that fluoride causes cell death in human leukemia cells by the activation of caspase-3 which leads to DNA damage and cell death.

REFERENCES


