

THE EFFECTS OF ASPIRIN ON APOPTOSIS OF NEUTROPHIL GRANULOCYTES

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Neutrophils are a part of the immune system, and they are involved in host defence against microorganisms. Neutrophil granulocytes have the shortest lifespan among leukocytes, which can be modulated by cytokines and pharmacological agents. The effect of aspirin on apoptosis of inflammatory granulocytes has not been studied in detail yet, and therefore was the chosen subject of this study. Inflammatory granulocytes have been isolated from polyvinyl sponges implanted under the skin of Albino Oxford (AO) rats. Inflammatory cells that were isolated 20 hours later were more than 95% neutrophil granulocytes. The cells were cultivated 24 h with different concentrations of aspirin ranging from 1 μ M to 10 mM. After the cultivation period, apoptosis of neutrophils was assessed by morphological criteria, as well as by flow cytometry (after staining the cells with propidium iodide). We found that at concentrations from 0,1 mM to 2,5 mM aspirin inhibited apoptosis of granulocytes, but at 10 mM aspirin induced apoptosis of these cells.

Key words: aspirin, neutrophil granulocytes, apoptosis

INTRODUCTION

Aspirin is a widely used antiinflammatory agent primarily because it is effective in the relief of pain and inflammation (Vane *et al.*, 1998; Awtry and Loscalzo, 2000; Hinz and Brune, 2002). Recently, aspirin and some other nonsteroidal antiinflammatory drugs (NSAIDs) received particular attention because of their protective effects against colon cancer (Thun, 1997; Porter *et al.*, 2000) and beneficial effects in cardiovascular diseases (Tegeger *et al.*, 2001).

Neutrophils are a part of the immune system, and they are involved in host defence against microorganisms. Neutrophils are the first leukocytes to migrate to the inflammation site, hours before other cells do so (Witko-Sarsat *et al.*, 2000; Girard, 2003). Neutrophil granulocytes have the shortest lifespan among leukocytes (12-48 h). Their lifespan can be modulated by cytokines, pharmacological agents and xenobiotics (Girard, 2003; Maianski *et al.*, 2004).

Apoptosis is a gene-directed mechanism for the elimination of unnecessary or unwanted cells from the organism. Apoptosis plays an important role in development, in tissue homeostasis and in the defence against viral infections and mutations. This process is regulated by complex molecular signaling systems (Kam and Ferch, 2000; Dlamini *et al.*, 2004; Guimaraes and Linden, 2004). Aspirin and other NSAID induce apoptosis in several cell types (human colorectal tumor cell lines, fibroblasts, B-cell chronic lymphocytic leukaemia cells, myeloid leukaemia cell lines and colon cancer cells *in vivo*) (Pique *et al.*, 2000). Apoptosis of neutrophil granulocytes is important for the resolution of inflammation (Kam and Ferch, 2000), since failure to undergo this form of cell death leads to increased tissue damage and exacerbation of the inflammatory response. It is of interest to prolong the lifespan of granulocytes in acute inflammation. Manipulation of the rate of apoptosis in inflammatory effector cells (neutrophils, eosinophils, monocytes and macrophages) could be of therapeutic benefit.

In the present study, we examined the effects of aspirin on the apoptosis of the rat inflammatory neutrophil granulocytes.

MATERIALS AND METHODS

Animals

Male 10 weeks old Albino Oxford (AO) rats, weighing 200-220 g, bred at the Institute for Medical Research, Military Medical Academy (MMA), Belgrade, were used in the experiment. The rats were fed standard laboratory chow (Veterinarski zavod Subotica) and tap water *ad libitum*. All experimental procedures on animals were conducted in adherence to the NIH guidelines for the use of experimental animals, with the approval of the MMA Ethical Committee.

Neutrophil collection

Effects of aspirin on the apoptosis of inflammatory neutrophil granulocytes was studied using a model of inflammation by implanting sterile polyvinyl sponges under the skin of rats (Middleton and Campebell, 1989). Rats were anaesthetized with ether (Merck) and their skin on the back was shaved and swabbed with 70% ethanol. Small incisions were made in the skin and pockets under the skin were made by using the tip of the scissors. Sterile polyvinyl sponge pieces (2 x 1,5 x 0,5 cm) were soaked in physiological saline, and two sponges per rat were implanted subcutaneously and pushed down under the skin. After 20 hours, the rats were sacrificed by inhalation anaesthesia and the sponges were retrieved aseptically. Infiltrating cells from the sponges were collected using RPMI 1640 medium containing 2% heat-inactivated fetal calf serum (FCS) (ICN Pharmaceuticals, Costa Mesa, USA), penicillin (50 IU/ml) and streptomycin (50 µg/ml), and RPMI 1640 medium with 0.04% EDTA. Cells were washed, resuspended, filtered through a nylon mesh, washed once more for 10 minutes and finally resuspended in complete RPMI 1640 medium containing 10% FCS. Cells were diluted in Trypan Blue and counted in a standard hemacytometer chamber. Isolated inflammatory cells were more than 95% neutrophil granulocytes. Viability of neutrophils was higher than 98%.

Cultivation of neutrophil granulocytes

Neutrophil granulocytes were plated in 96-well plates (5×10^5 cells/well, 200 μ l) along with increasing concentrations (0.01-10 mM) of aspirin (Sigma Chemical Co) and cultivated for 24 h. The control consisted of cells cultivated in complete RPMI 1640 medium. Culture supernatants were collected after 24 h and used for the measurement of nitric oxide (NO). The cells were used for the detection of apoptosis.

Measurement of apoptosis

For the measurement of apoptosis, two different methods were used. Morphological analysis using morphological criteria (chromatin condensation, nuclear pyknosis or nuclear fragmentation) was performed after fixation and staining of the cells with Türk reagent using light microscopy as previously originally described for lymphocytes (Čolić *et al.*, 2000). Apoptosis was also detected by staining hypodiploid nuclei using propidium iodide (PI) (Migita *et al.*, 1994; Vermes *et al.*, 2000). After incubation, neutrophils were washed once with phosphate buffered saline (PBS) and resuspended in 0,4 ml solution with 20 μ l of PI (1 μ g/ml). The analysis was performed on an EPICS XL-MLC cytofluorometer (Coulter, Germany).

Measurement of NO production

Production of NO, quantified by the accumulation of nitrite in the 24 h culture medium, was measured spectrophotometrically using the Griess reaction with sodium nitrite as a standard (Green *et al.*, 1982). Culture supernatants (50 μ l) were mixed with equal volumes of 1% sulphanilamide (Sigma Chemicals Co., St Louis, Mo., USA) in 5% H₃PO₄ and 0.1 % N-1-naphthylethylene diamine dihydrochloride (Sigma Chemicals Co., St Louis, Mo., USA) in distilled water, and after 10 minutes at room temperature the absorbance, was measured at 570 nm.

Statistical analysis

All data are expressed as mean \pm standard deviation. Each test was performed at least three times. Statistical analysis was performed by Student's t-test, using the PC Program. A p value less than 0.05 was considered significant.

RESULTS

Effect of aspirin on apoptosis of rat neutrophil granulocytes

After a 24-hour incubation of neutrophils isolated from sponges with different concentrations of aspirin at 37 °C, apoptosis was determined by the morphological method. It was found that aspirin, at concentrations from 100 μ M to 2,5 mM, significantly inhibited the spontaneous apoptosis of these cells. However, concentration of 10 mM of the drug promoted apoptosis of granulocytes (Figure 1). Similar results were confirmed by using flow cytometry after staining the cells with PI (Figure 2). Morphology of freshly isolated neutrophils is shown in Figure

3a. The appearance of viable and spontaneously apoptotic neutrophils in the culture is presented in Figure 3b. Figure 3c shows that most neutrophils incubated for 24 hours with 10 mM aspirin showed features of apoptosis such as condensed and/or fragmented nuclei.

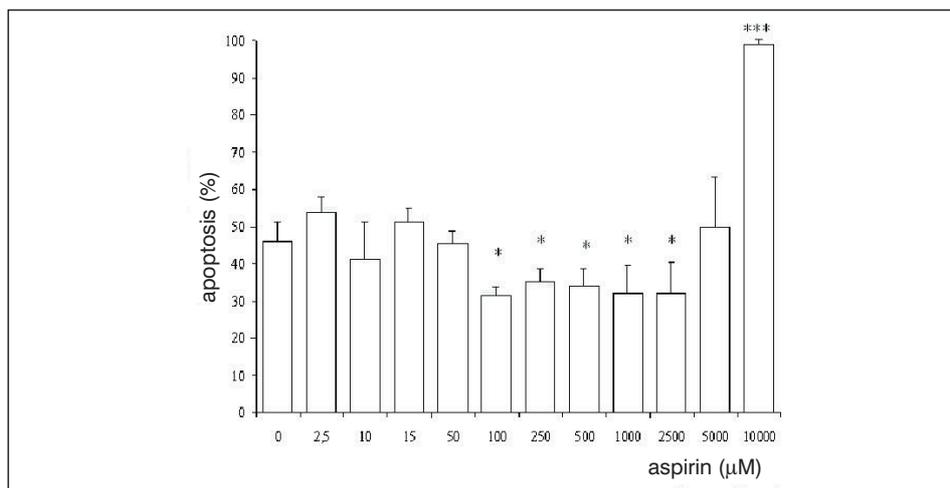


Figure 1. The effect of aspirin on apoptosis of neutrophil granulocytes in culture. Apoptosis was measured after staining the cells with Türk reagent and analysis by light microscopy, using morphological criteria as described in Materials and Methods. Values are given as mean \pm SD of triplicates from one representative experiment, out of several ones with similar results. Results of morphological analysis are given on the basis of 500 counted cells in each native preparation (* $p < 0.05$; *** $p < 0.001$ compared to control)

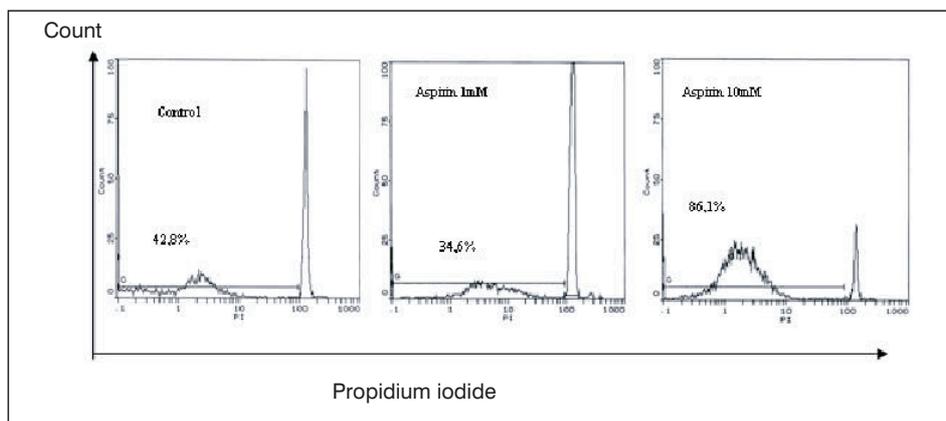


Figure 2. The effect of aspirin on apoptosis of neutrophil granulocytes in culture. Apoptosis was measured after staining the cells with propidium iodide using cytofluorometry as described in Materials and Methods. The number above the horizontal line shows the percentage of hypodiploid nuclei

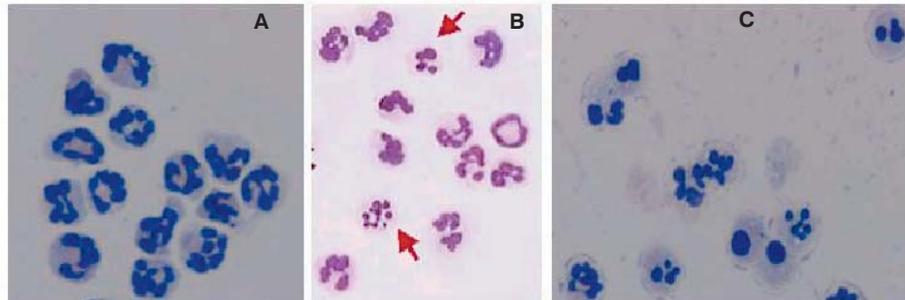


Figure 3. Cytospin preparations of neutrophil granulocytes, stained by May Grünwald-Giemsa. Freshly isolated neutrophils (A); After 24h incubation (B). Fig 3C shows the appearance of neutrophils incubated with 10 mM of aspirin for 24h

Effect of aspirin on NO production

It is known that NO, depending on the concentration, suppresses or promotes apoptosis. Therefore, in subsequent experiments, we investigated the effect of different concentrations of aspirin on NO production by inflammatory granulocytes. We found that aspirin at concentrations from 0,0025 to 5 mM, failed to produce any significant effect on the baseline production of NO (as assessed by the quantity of nitrite measured by the Griess reaction) by neutrophil granulocytes cultured for 24 hours. However, at the concentration of 10 mM, aspirin caused a statistically significant decrease in NO production by these cells (Figure 4).

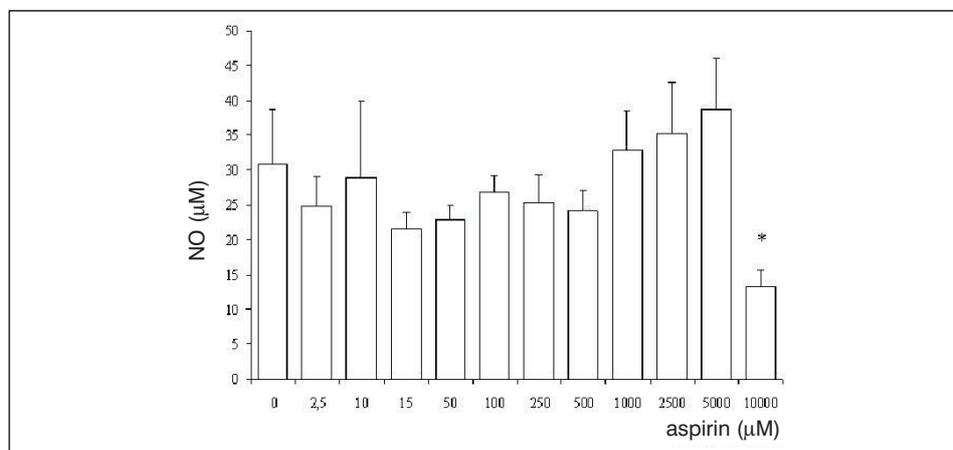


Figure 4. The effect of aspirin on the production of nitric oxide by neutrophil granulocytes in 24h culture. Production of nitric oxide was measured spectrophotometrically using the Griess method as described in Materials and Methods. Values are given as mean \pm SD of triplicates from one representative experiment, out of several ones with similar results (* $p < 0.05$ compared to control)

DISCUSSION

In our experiment, we found that aspirin, in concentrations from 2.5 to 50 μM , did not affect, with any statistical significance, the degree of spontaneous apoptosis of rat neutrophil granulocytes after 24 hours of incubation at 37 °C. When incubated with neutrophils in concentrations ranging from 100 to 2500 μM , aspirin significantly inhibited the spontaneous apoptosis of these cells. The relative numbers of apoptotic cells have fallen from 45 %, which has been the level of spontaneous apoptosis, to 37.9 and 30.0% with aspirin. However, when incubated with a high aspirin concentration of 10 mM, neutrophils were induced towards apoptosis. Namely, their spontaneous apoptosis of 45% increased to 85-100%.

Similar results have not yet been published. There are very few works about the effect of aspirin on the apoptosis of neutrophil granulocytes. Garcia-Martinez *et al.* (2003) have found that, after an incubation of 24 hours, aspirin, in the concentration of 10 mM, induces apoptosis of human neutrophils (in peripheral circulation), while in the concentration of 75 μM it does not modulate this process. Galichs *et al.* (2004) have determined that the apoptosis of neutrophils from peripheral blood of patients taking aspirin as their antiaggregation therapy, after 24 hours in culture, equals that of the controls. However, in patients suffering from acute coronary syndrome, neutrophil apoptosis was reduced. Sera of these patients inhibited apoptosis of neutrophils from healthy donors, due to the presence of $\text{TNF}\alpha$, GM-CSF and $\text{IL-1}\beta$.

There are numerous publications regarding the pro- and anti-apoptotic effect of aspirin on different cell types. When Ricci *et al.*, (2003) incubated caco-2 cells (a human colon adenocarcinoma cell line) with 2 and 5 mM of aspirin, they found that it caused apoptosis. Also, aspirin, in concentrations of 1 and 2,5 mM, induces a statistically significant degree of apoptosis in three different cell lines of human colon cancer, after an incubation lasting 48 or 72 hours (Goel *et al.*, 2003). In the concentration of 10 mM, aspirin induces apoptosis after 12 hours of incubation in Jurkat and Molt-4 cells (acute T cell leukaemia lines), as well as Raji cells (a Burkitt B cell lymphoma line) (Pique *et al.*, 2000).

The finding that aspirin, in concentrations up to 100 μM , does not affect neutrophil apoptosis, while at 10 mM it induces apoptosis, is in concurrence with other studies describing the effects of aspirin on neutrophils. Our data agree well with the pro- and anti-apoptotic effects of aspirin on other cells.

Neutrophils are highly specialized white blood cells that participate in the host defense against infection (Simon, 2003). These cells are the first line of defense of the innate immune system. Neutrophils are especially important when fighting microorganisms (bacteria and certain fungi) by phagocytosis and releasing toxic mediators. They have a short half-life if they are not recruited to the site of inflammation. Inflammatory mediators, cytokines, growth factors and chemical mediators mediate duration of their life-span (Ward *et al.*, 1999, Simon, 2003). However, neutrophils are terminally differentiated cells and, therefore, because of their short life-span it is difficult to study neutrophils from the blood *in vitro* (Simon, 2003). Nevertheless, neutrophils life span changes as they

transmigrate from blood to inflammatory tissue. These changes can be examined *ex vivo* and *in vitro*, where these inflammatory cells live longer. It is easy to obtain such a cell population from the blood, as well as from subcutaneously implanted sponges.

The tight regulation that governs neutrophils behavior is necessary because the content of neutrophil granules can be noxious to the host tissue (Ćupić, 1997). In the resolution of inflammation, the accumulated neutrophils need to be safely removed. Apoptosis plays an important role in eliminating such neutrophils from inflamed tissues. Most studies have been performed on purified blood neutrophils aiming to understand the molecular events that control apoptosis in these cells (Simon, 2003). Apoptosis is the physiologic cell death of neutrophils. The number of neutrophils is regulated *in vivo* by their production in the bone marrow and by apoptosis. Apoptosis at the inflammatory site appears to be delayed when survival factors are generated by pro-inflammatory cytokines (Akgul *et al.*, 2001).

Nitric oxide (NO) is the key mediator in a great number of physiological and pathophysiological processes. It can regulate the rate of apoptosis in many cell types including human inflammatory cells. NO has both pro and antiapoptotic properties depending largely on the concentration and flux of NO and cell type. Low concentrations of NO (less than 100 μM) (Bogdan *et al.*, 2000) usually have protective effects on cells, whereas higher concentrations derived from inducible isoform of NO synthase drive the cell to death (Taylor *et al.*, 2003). NO is able both to promote and delay inflammatory cell apoptosis and the mechanisms are still not fully elucidated. Because of the importance of NO in apoptosis of neutrophils we examined its concentration in culture. In our experiments, aspirin decreased the quantity of NO in neutrophil culture supernatants, when incubated with these cells in a concentration of 10 mM, only. It is possible that this observation is related to the induction of apoptosis by aspirin in this concentration.

Based on the results of our work, the following may be concluded: aspirin has a dichotomal effect on the apoptosis of rat inflammatory neutrophils. Namely, in concentrations of 100-2500 μM , aspirin inhibits apoptosis, but in the concentration of 10 mM, it has the opposite effect, i.e. induces apoptosis. The fact that aspirin, in concentrations that lie within the range of therapeutic plasma levels, causes inhibition of neutrophil apoptosis is a very important finding that may contribute to our knowledge of mechanisms of aspirin-induced damage to the mucosa of the stomach and intestine.

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EFEKTI ASPIRINA NA APOPTOZU NEUTROFILNIH GRANULOCITA

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SADRŽAJ

Neutrofilni granulociti su deo imunog sistema, uključenog u odbranu od mikroorganizama. Oni imaju najkraći životni vek među leukocitima, koji se može modulirati citokinima i farmakološkim agensima, a do sada nije ispitan efekat aspirina na apoptozu inflamatornih granulocita. Zbog toga je u ovoj studiji ispitan efekat aspirina na apoptozu inflamatornih neutrofilnih granulocita pacova. Inflamatorni granulociti su izolovani iz polivinilskih sunđerica, potkožno implantiranih, pacovima Albino Oxford (AO) soja. Inflamatorne ćelije, izolovane 20 sati kasnije, najvećim delom (više od 95 %) predstavljaju neutrofilne granulocite. Ove ćelije su kultivisane 24 sata sa aspirinom u koncentracijama od 1 μ M do 10 mM. Posle ovog perioda, supernatanti su sakupljeni i korišćeni za merenje koncentracije NO. Ćelije su bojene propidijum jodidom i apoptoza je analizirana na protočnom cito-fluorimetru, kao i pomoću morfoloških kriterijuma. Ustanovljeno je da u koncentracijama od 0,1 do 2,5 mM aspirin inhibira, a samo u visokim koncentracijama (10 mM) indukuje apoptozu ovih ćelija. Aspirinom indukovana apoptoza je bila u pozitivnoj korelaciji sa smanjenom produkcijom NO.