THE EFFECTS OF DIFFERENT CONCENTRATIONS OF ACETYLSALICYLIC ACID ON PROLIFERATION AND VIABILITY OF LYMPHOCYTES IN CELL CULTURE

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Abstract

Numerous studies conducted on acetylsalicylic acid (ASA, aspirin) confirmed that ASA inhibits proliferation and induces apoptosis in various types of human cells. Therefore, it was of interest to examine possible effects of different concentrations of ASA on viability and proliferation of lymphocytes in the cell culture. After separation from blood, lymphocytes were suspended in RPMI 1640 medium and cultured at 37°C. Solution of ASA was added to cultures after 24h, in final concentrations of 1, 3 and 5 mmol/l. After 48h, proliferative response was evaluated by WST-1 assay. Significant difference in viability between controls and cell cultures treated with ASA in three different concentrations was observed ($p < 0,01$). Percents of viable cells in cultures after application of 1, 3 and 5 mmol/l ASA were 9,9%, 2,5% and 16,9% (compared to controls), respectively. To determine whether this cytotoxic effect was result of induction of apoptosis, DNA from cell cultures was isolated and subjected to agarose gel electrophoresis. Fragmentation of DNA was not detected, excluding apoptosis as possible cause of cytotoxic effects. Addition of ASA caused change of initial extracellular pH value for each treated culture. After addition of 1 mmol/l ASA, pH of culture was 7,19, after 3 mmol/L, 6,99 and after addition of 5 mmol/l solution, pH was 6,75. Decreased lymphocyte viability could be attributed to either the effects of the added substance or possible further acidification of cell cultures during three days of incubation.

KEY WORDS: acetylsalicylic acid (aspirin), lymphocyte cell culture

Introduction

Acetylsalicylic acid (aspirin, ASA) is a nonsteroid anti-inflammatory drug (NSAID), widely used for its antipyretic and analgesic properties. It acts by directly suppressing the cyclooxygenase enzyme (COX-1 and COX-2), the rate limiting enzyme catalyzing the biosynthesis

of prostaglandins, thereby blocking the production of proinflammatory prostaglandins. ASA is also known to induce gastrointestinal side effects, mainly in the form of gastric and duodenal ulcerations or erosions. Numerous *in vivo* and *in vitro* studies conducted on ASA, showed interesting potentials of this substance. Recent *in vitro* researches confirmed that ASA inhibits proliferation and induces apoptosis in human colorectal cancer cells (1, 2), inhibits the growth of endometrial cancer cells (3), induces apoptosis in OC2 cells, a human oral cancer cell line (4), induces apoptosis and activation of caspases in B-cell chronic lymphocytic leukemia cells (5). It has been shown that pretreatment by ASA augmented TRAIL-induced apoptotic death in human prostate adenocarcinoma LNCaP and human colorectal carcinoma CX-1 cells (6). Aspirin also sensitized human cervical carcinoma HeLa cells to TNF alpha-induced apoptosis and could be used to potentiate the effectiveness of TNF alpha-based therapeutic interventions in cancer treatment (7) as well as in chemoprevention for ovarian cancer (8). Culture of lymphocytes so far was rarely the subject of interest in studies related to ASA effects. In the present study, possible effects of different concentrations of ASA on viability of human peripheral blood lymphocytes were examined. It was of great interest to determine whether these effects were result of possible induced apoptosis of cells.

Materials and Methods

Aspirin (acetylsalicylic acid) was obtained from Panreac Quimica SA. Stock solution of aspirin in redistilled water was freshly prepared for each experiment. Lymphocyte populations for each test were obtained from blood sample of a consenting healthy volunteer. Samples were diluted with 0,9% NaCl solution (1:1) and after washing, suspended in RPMI 1640 medium containing 10% fetal bovine serum, L-glutamine, 35 µg/ml gentamicin and 2% phytohemaglutunine (PB KaryoMax, Gibco). The suspended cells were cultured in a 96-well microtiter plate in a final volume of 100 µl/well at 37°C in a humidified atmosphere containing 5% carbon dioxide (5x104 cells/well). Stock solution of acetylsalicylic acid (Panreac Quimica, SA) in redistilled water was sterilised and added to cultures after 24 hours, in final concentrations of 1, 3 and 5 mmol/l. After 48 hours, proliferative response was evaluated by Quick Cell Proliferation Assay Kit (K301-500, BioVision). The assay is based on the cleavage of the tetrazolium salt WST-1 to formazan. After addition of 10 µl WST-1 solution to each well, the cells were incubated for 2 hours in the same culture conditions. The formazan dye produced by viable cells was quantified by measuring its absorbance at 450 nm (reference wavelength was 630 nm) on microplate reader (Universal, ELX 800 UV, Bio-Tek Instruments) On the basis of these results, the percent of viable cells compared to controls was calculated based on the formula below:
% viability = (test cell OD / control cell OD) x 100

DNA from treated cell cultures was extracted by Miller's protocol. Cell culture (10 ml) was lysed for 20 minutes at +4°C in lysis buffer containing 0.155 NH_4Cl, 10 mmol/l $KHCO_3$ and 1 mmol/l EDTA (pH 7,48). After centrifugation at 1500 g for 10 minutes, pellet was washed in phosphate buffered saline (PBS), dissolved in Kern-lysis buffer (digestion or extraction buffer containing 10 mmol/l Tris, 400 mmol/l NaCl and 2 mmol/l EDTA) and then treated with protease (Quigen) and 20% sodium dodecyl sulfate (SDS) at 37°C overnight. After incubation, 6 mmol/l NaCl was added to precipitate proteins. Samples were centrifugated at 2500 g for 15 minutes. Supernatants were transferred and DNA was precipitated with 2 volumes of cooled absolute ethanol. DNA precipitates were washed in 70% ethanol. After centrifugation at 13 000 g for 10 minutes, DNA pellet was dried and dissolved in TE buffer (containing 10 mmol/l Tris-HCl, pH 7,4 and 0,1 mmol/l EDTA). Electrophoresis of DNA preparations on 1% agarose gel was run under following conditions: a) 1 hour at 90 V and b) 3 hours at 60 V. Gels were stained with ethidium bromide and visualized under UV light.

Levels of significance between samples were determined using the t-test for non-paired samples.

Results

Effects of aspirin on lymphocyte viability.

The results of cell viability assay are presented in table 1. As it can be seen from the table, detected absorbances of samples treated with ASA in three different concentrations were significantly lower than those obtained for controls – $p < 0,01$.

	X ± SEM	p
Controls	0,283 ± 0,008	
A_1 (c = 1 mmol/l)	0,028 ± 0,012	< 0,01
A_3 (c = 3 mmol/l)	0,007 ± 0,002	< 0,01
A_5 (c = 5 mmol/l)	0,048 ± 0,008	< 0,01

TABLE 1. Mean values of absorbances (X) obtained for controls and samples treated with 1, 3 and 5 mmol/l ASA (A_1, A_3 and A_5)

The same results are shown in Figure 1 - applied concentrations of ASA (compared to controls) obviously caused significant decrease in number of viable cells .

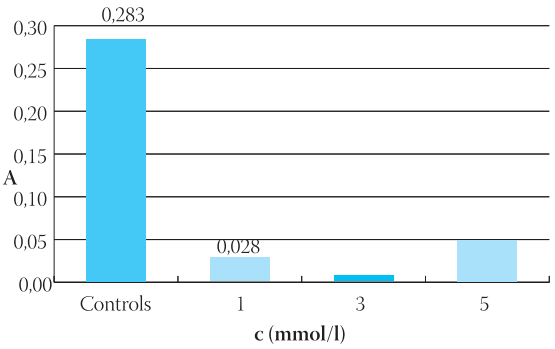

FIGURE 1. Mean values of absorbances (A) of controls and samples treated with 1, 3 and 5 mmol/l ASA

As shown in Figure 2, the percent of viable cells drastically decreased after addition of ASA.

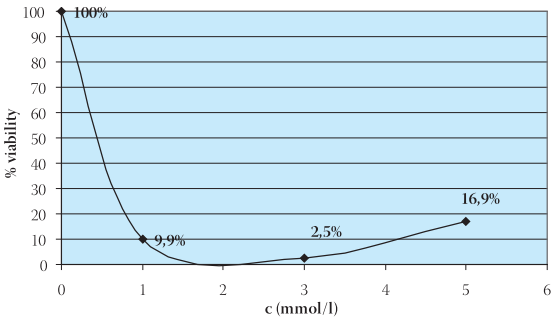

FIGURE 2. MThe percent of viable cells in samples treated with different concentrations of ASA with respect to controls (100%)

DNA from lymphocyte cell culture.

To determine whether this obvious cytotoxic effect was result of induction of apoptosis, it was of interest to analyze whether incubation with aspirin, induced DNA fragmentation in cells. In these experiments, cells were treated with the same doses of aspirin (1, 3 and 5 mmol/l) used in previous experiments. DNA was extracted from samples and subjected to agarose gel electrophoresis for 1 hour at 90V and for 3 hours at 60V. As shown in Figures 3 and 4, fragmentation of DNA was not detected under these experimental conditions.

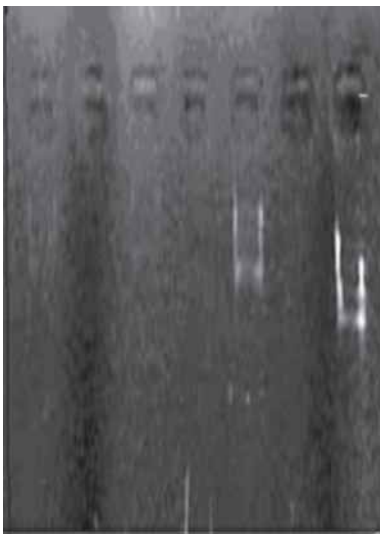

FIGURE 3. DNA preparations extracted from control (C) and treated lymphocyte cell cultures after agarose gel electrophoresis for 1 hour at 90 V

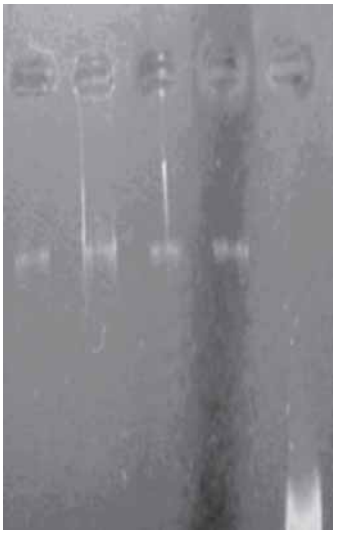

FIGURE 4. DNA preparations extracted from control (C) and treated lymphocyte cell cultures after agarose gel electrophoresis for 3 hours at 60 V

Discussion

As it can be seen from the results, aspirin applied in 1, 3 and 5 mmol/l concentrations had obvious cytotoxic effect on cultured human normal lymphocytes. Viability of cells was decreased significantly. In the study conducted by Bellosillo et al. (5), incubation of normal peripheral blood mononuclear cells with aspirin produced no significant cytotoxic effect with doses ranging up to 7,5 mmol/l. This concentration was the lowest one being able to induce detectable DNA fragmentation in the same type of cells (5). We assumed that cytotoxic effect observed with lower concentrations of ASA in our study, was due to changes in pH of culture medium. Namely, after addition of 1 mmol/l ASA, measured pH in culture was 7,19, after addition of 3 mmol/l ASA, it was 6,99 and after addition of 5 mmol/l ASA, measured pH was 6,75.

Numerous studies have shown that decrease of extracellular pH ranging from pH 6,5 to 6,8 can induce apoptosis of various cell cultures (9, 10, 11, 12, 13). However, fragmentation of DNA (a hallmark of apoptosis) in lymphocyte cell cultures treated with 1, 3 and 5 mmol/l concentration of ASA, was not detected in our study, thus excluding apoptosis as possible cause of these effects. Available literature data stress the role of extracellular pH as a fundamental parameter that has many different effects on cultured cells. In the study of Carswell and Papoutsakis (14), peripheral blood mononuclear cells, when stimulated with phytohemagglutinin and cultured at pH values of 7,0; 7,2 and 7,4, showed increased proliferation capacity and lower portion of apoptotic cells in cultures with pH of 7,0 compared to cultures with pH of 7,2 and 7,4. Bental and Deutsch studied intracellular pH of purified human T lymphocytes using NMR spectroscopy under physiological conditions (15). They found that proliferation of T cells was optimal at initial external pH values between 6,8 and 7,6 after phytohemagglutinin stimulation (intracellular pH remained 7,25 during the study). Proliferation was completely abolished when the initial extracellular pH was <6,5 or >7,8 (15). In our study, initial extracellular pH varied between 6,75 and 7,19. Having in mind results mentioned above (14, 15), suppressive effect of applied ASA on lymphocyte proliferation cannot be explained simply by change of extracellular pH measured in culture. Bental and Deutsch study cites that in vitro cultures typically acidify during 3 days of stimulation and suggest that decrease in medium pH below pH value of 7,05 is being accompanied by significant changes in intracellular pH of cells (15). This can be possible cause for the observed decrease in viability of cells in our study, however, this has to be at the level of speculation since pH of cell cultures at the end of incubation was not measured as well as intracellular pH. Other possible cause of decreased viability of cells could be attributed to the effects of substance itself.

Conclusion

Our study has shown that aspirin applied in 1, 3 and 5 mmol/l concentrations, significantly decreased viability of cultured human lymphocytes. DNA fragmentation was not detected in these studies, excluding apoptosis as possible cause of these effects. Decreased lymphocyte viability could be attributed to either the effects of the added substance itself or possible further acidification of cell cultures during three days of incubation. Having in mind the fact that pH of cell cultures at the end of incubation period was not measured as well as intracellular pH, further studies are needed to in order to substantiate these findings.

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