Original Article

THE POTENTIAL ROLE OF NONSTEROIDAL ANTI-INFLAMMATORY DRUGS (NSAIDS) IN CHEMOPREVENTATION OF CANCER

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ABSTRACT:

Objective: To find out the probable effects of Nonsteroidal anti-inflammatory drugs (NSAIDs) in the preventation and treatment of cancer.

Design: The influence of NSAIDs on Fibrosarcoma cell line was investigated by using an in vitro cytotoxicity assay, Terminal deoxyribonucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay and gelatin zymography.

Setting: Department of Pathobiology, School of Public Health and Institute of Public Health Research, Tehran University of Medical Sciences.

Results: Cytotoxicity analysis of Diclofenac revealed much higher cell death than other examined agents. A potenet apoptotic influence of Diclofenac was also confirmed by data obtained from TUNEL assay. Dose-dependent inhibitory effects on Matrix metalloproteinases (MMPs) was seen in higher degree in Diclofenac compared with other examined NSAID(Piroxicam) and a synthetic glucocorticoid (Dexamethasone).

Conclusion: based on our data, the induction of apoptosis together with MMPs inhibition could be indicative of cancer treatment, thus Diclofenac might be considered as a chemopreventative agent in cancer.

KEYWORDS: Apoptosis, Cancer, Matrix metalloproteinases, NSAIDs

Abbrevaitions: MMPs: Matrix metalloproteinase, ECM: Extracellular matrix, SDS PAGE: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis, NSAIDs : Nonsteroidal anti-inflammatory drugs, TUNEL:Terminal deoxyribonucleotidyl transferase-mediated dUTP nick-end labeling.

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INTRODUCTION

Nonsteroidal anti-inflammatory drugs (NSAIDs) have been clinically used for their anti inflammatory, anti pyretic and analgesic properties. It is widely believed that their therapeutic actions are based on their ability to block the production of prostaglandins by their inhibitory effects against cyclooxygenase[1,2]. Cyclooxygenase has two isoforms (cyclooxygenase -1 and -2) which both be inhibited by NSAIDs[3-5], although there is some differences in their relative potency against the two forms of cyclooxygenase[6-7]. Cyclooxgygenase type 1 is constitutively expressed, and the prostaglandins produced by this isoform have thus been thought to play a "house keeping" function. In contrast,

Cyclooxgygenase type 2 is an inducible enzyme that is normally absent in intact cells but is expressed in response to growth factors, tumor promoters and cytokines[8-10]. Based on several observations, a regular intake of NSAIDs is associated with a decreased incidence of various types of cancers[11-15].

On the other hand, most investigators unanimously admitted that Matrix metalloproteinases (MMPs) are critical enzymes in tumor growth invasion, metastasis[16-18] and neovascularization[19,20]. MMPs are a family of highly homologous, zinc and calcium dependent endopeptidase that clear most, if not all, components of extracellular matrix (ECM). Thus, each component with potential inhibitory influence on MMPs expression is able to reduce the risk of cancer.

Here we study the probable antitumorigenic and MMPs expression blockage effects of NSAIDs for their ability to prevent and treatment of cancer by cytotoxicity, zymography and programmed cell death assay. In addition, their cytotoxicity effect has also been compared with synthetic corticosteroids.

MATERIALS AND METHODS

Cell culture: The Fibrosarcoma cell line (WEHI 164) was seeded at initial density of 2×10^4 cells/ well in 96-well tissue culture plates. Cells were maintained in RPMI-1640 medium supplemented with 5% fetal calf serum, penicillin at 100 units/ml, and streptomycin at 100 μg/ml, under 5% CO_2, 37°C and saturated humidity.

Dose-response analysis: Triplicate, two-fold dilutions of Dexamethasone and Diclofenac preparations were transferred to overnight cultured cells. Non-treated cells were used as control. Cells were cultured overnight and were then subjected to colorimetric assay. A sample of the media was used for zymoanalysis.

Colorimetric assay: After each experiment, the cells were washed three times with ice-cold PBS, followed by fixation in a 5% formaldehyde solution. Fixed cells were washed three times and stained with 1% crystal violet. Stained cells were washed, lysed and solubilised with 33.3% acetic acid solution. The density of developed purple color was read at 580 nm.

Zymoanalysis: This technique has been used for the detection of gelatinase (collagenase type IV or matrix metalloproteinase type 2, MMP-2) and MMP-9, in conditioned-media according to Heussen and Dowdle method (21) with some modifications. Briefly, aliquots of conditioned media were subjected to electrophoresis in (2mg/ml) gelatin containing polyacrylamide gels, in the presence of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under non-reducing conditions. The gels underwent electrophoresis for 3 hours at a constant voltage of 80 volts. After electrophoresis, the gels were washed and gently shaken in three consecutive washings in 2.5% Triton X100 solution to remove SDS. The gel slabs were then incubated at 37°C overnight in 0.1 M Tris HCl gelatinase activation buffer (pH 7.4) containing 10mM $CaCl_2$ and subsequently stained with 0.5% Coomassie Blue. After intensive destaining, proteolysis areas appeared as clear bands against a blue background. Using a UVI Pro gel documentation system (GDS_8000 System), quantitative evaluation of both surface and intensity of lysis bands, on the basis of grey levels, were compared relative to non-treated control wells and expressed as "Relative Expression" of gelatinolytic activity.

Cell apoptosis assay: The cells were treated with the different agents for 24 hour and then fixed in 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. Terminal deoxyribonucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay for detecting DNA fragmentation was performed by flocytometric analyze as indicated by kit instructions. (APO-BRDU, Roche, CA, USA). The cell nuclei were stained with Fluorescein and Propidium iodide and apoptotic and total cells were counted by flocytometry instrument

(FACSCalibar Becton Dickinson, USA). The results were expressed as percentage of apoptotic cells.

Statistical analyses: The differences in cell proliferation and gelatinase activity were compared using the Student's *t* test. *P* values <0.05 were considered significant.

RESULTS

Cell cytoxicity of Diclofenac comparing to Piroxicam and Dexamethasone is illustrated in Figure-1. The cytotoxic effects of Diclofenac caused 40% and 77.5% cell death at 10 and 20 ìg/mL respectively. Besides, the majority of cells seem to be dead up to 40 ìg/mL. Cytotoxicity analysis of Piroxicam and Dexamethasone were almost similar to each other and revealed considerable decreases in cell number up to 40 ìg/mL. Data obtained from TUNEL assay indicat that Diclofenac with concentrations 20 ìg/mL, 40 ìg/mL and 80 ìg/mL caused 19.4%, 60.7% and 78% cell apoptosis respectively. Diclofenac in comparison with Piroxicam and Dexamethasone induced 3 to 4 fold increase in total apoptotic cells, approximately (Figure 2).

The Relative expression of MMPs activity of Diclofenac, Piroxicam and Dexamethasone is presented in figure-3. Higher inhibitory activities have been seen in Diclofenac rather than examined agents at all concentrations.
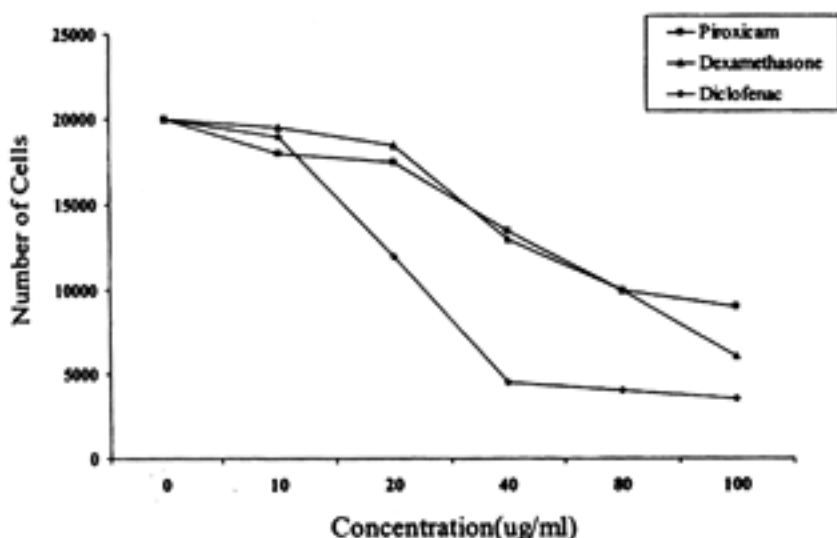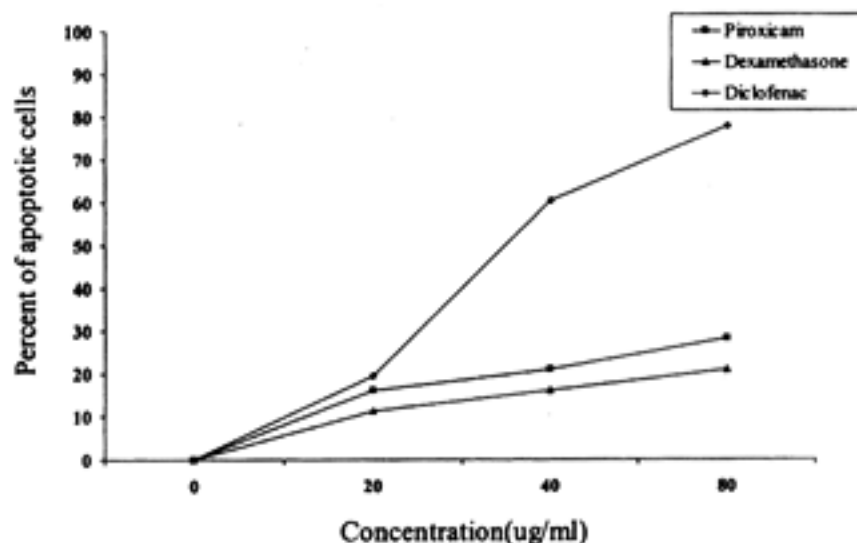

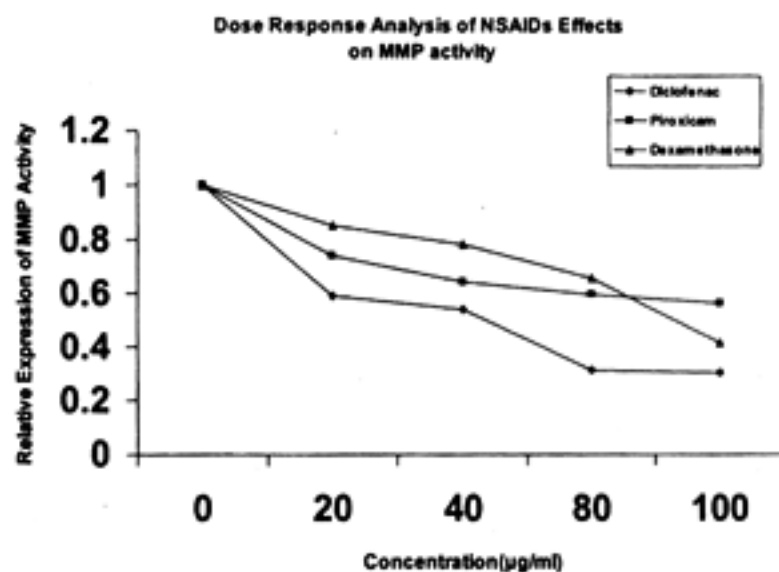Fig 2: Apoptotic assay of Diclofenac compared to Dexamethasone and Piroxicam

Fig 3: Fibrosarcoma cell lines (2x10⁴ cell/well) were incubated for overnight with increasing dose of Diclofenac as described in *Materials and Methods*. dexamethasone and piroxicam treated cells were used as controls. Analyses were performed using UVI Pro Gel Documentation System. Surface and intensity of lysis bands on the basis of grey levels were analysed. All treated and non-treated cells were investigated in triplicate.

Fig 1: Cytotoxic Analysis of Diclofenac compared to Dexamethasone and Piroxicam

DISCUSSION

In the present study, the effect of NSAIDs on the apoptotic DNA fragmentation was examined in cultured fibrosarcoma cells. DNA fragmentation, a typical feature observable in cells undergoing apoptotic cell death[22,23], was significantly induced in WEHI cells by treatment with Diclofenac and Piroxicam which are potent and non-specific inhibitors of cyclooxygenase. The same effect was also seen by treatment with a synthetic glucocortocoid, Dexamethasone.

Cytotoxicity analysis test for Diclofenac treated cells revealed that this substance showed significant cytotoxic effect up to 10 ìg/mL. By contrast, our data showed that non-steroidal anti-infalammatory agent i.e., Piroxicam eliceted less cytotoxic characteristics as compared to Diclofenac at low concentrations (< 20 ìg/mL). Among steroidal drugs, Dexamethasone was examined and its cytotoxic pattern was similar to piroxicam. Moreover, our findings showed that these drugs were all cytotoxic, especially in high dose treatments (Figure 1).

In addition, in this study the induction of apoptotic DNA fragmentation by NSAIDs was demonstrated in cultured cells. Concerning data obtained from TUNEL assay, induction of cell apoptosis by Diclofenac was similar to Piroxicam and Dexamethasone at low concentration. In contrast, Diclofenac caused significantly higher cell death at elevated doses. Our data are in agreement with experiment by Elder and Smith who demonstrated that NSAIDs are able to induce programmed cell death in other cancer cell lines[24-26]. Evidence to date indicates that the majority of NSAIDs are mediating their anti-proliferative effects by cyclooxygenase (COX) inhibiting activity[27,28]. Furthermore, recent investigations demonstrate that specific inhibitors of COX-2 are able to reduce recurrence of malignancy[29-32]. Concerning potential less toxicity of COX-2 inhibitors, as compared to traditional NSAIDs, their application for cancer chemoprevention seems to be more rational. However, studies on the definite molecular mechanisms by which NSAIDs induce apoptosis are thus still called for.

Besides, as one of the critical steps for tumor invasion and metastasis is the destruction of ECM that is catalyzed mainly by the MMPs[33], inhibition of MMPs could be beneficial in preventing tumor metastasis. Only limited information is available on the effects of non-steroidal anti-infalammatory drugs and glucocorticoides in regulating MMPs activity and/or biosynthesis. Our zymogeraphy analysis test of non-steroidal and steroidal anti-inflammatory drugs showed similar results with previous studies[34-36]; reduction of expression of MMPs, are associated with increasing concentration of these drugs. Indeed, reduction of MMPs expression might be indicative of the cell death processes. Moreover, all these agents inhibited MMPs-activity in a dose-response fashion (Fig 3).

With this regard, potential ability of these agents to inhibit MMPs could be an extra characteristic to prevent tumor invasion and metastasis. Collectively, Diclofenac might be assumed as a component, which has the potential to be applied to chemoprevention of cancer.

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