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Aspirin and sodium salicylate inhibit proliferation and induce apoptosis in rheumatoid synovial cells

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Abstract

Aspirin has been reported to induce apoptosis in a variety of cell lines. In this study, we examined whether aspirin and sodium salicylate inhibit cell growth and induce apoptosis in rheumatoid synovial cells. Synovial cells were obtained from patients with rheumatoid arthritis, and the cells were treated with aspirin or sodium salicylate (0.1–10 mM) for 24 h. Cell proliferation and viability were assessed by 5-bromo-2′-deoxyuridine incorporation and by 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2*H*-5-tetrazolio]-1,3-benzene disulfonate (WST-1) assay, respectively. The apoptosis of synovial cells was identified by DNA fragmentation assay and terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) assay. Aspirin and sodium salicylate suppressed the proliferation (IC50 (concentration causing 50% inhibition of cell proliferation): 2.1 and 1.2 mM, respectively) and reduced the viability (IC50: 2.0 and 1.4 mM, respectively) of synovial cells in a concentration-dependent manner at 0.3–10 mM. Furthermore, they induced DNA fragmentation and increased the number of TUNEL-positive synovial cells. These results suggest that aspirin and sodium salicylate can inhibit the proliferation of rheumatoid synovial cells through induction of apoptosis.

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Introduction

Aspirin has been used in the treatment of rheumatoid arthritis for over 100 years because of its analgesic and anti-inflammatory activity. The major mechanism of action of aspirin is generally thought to be inhibition of cyclooxygenase (COX), the enzyme responsible for the biosynthesis of prostaglandins (Vane 1971). But aspirin reportedly has other mechanisms of action that contribute to its therapeutic effects in the treatment of inflammatory diseases (Tegeder et al 2001). For example, it blocks the activation of transcription factor nuclear factor-κB (NF-κB) (Kopp & Ghosh 1994; Yin et al 1998). Therefore, in addition to its effects on the biosynthesis of prostaglandins, aspirin may influence other cellular processes in rheumatoid arthritis.

In rheumatoid arthritis, an extensive tumour-like proliferation of synovial cells contributes to the hyperplasia of synovium and formation of pannus tissue that invades articular cartilage and surrounding tissues, resulting in joint destruction (Zvaifler & Firestein 1994). Agents that suppress the proliferation of rheumatoid synovial cells have therefore been the subject of much investigation (Matsubara et al 1988; Aono et al 1996; Hui et al 1997). Recently, evidence has emerged that aspirin has potent chemopreventive activity (Thun et al 1991; Giovannucci et al 1995). Aspirin also has been shown to have anti-proliferative and pro-apoptotic effects in a variety of cell lines such as colon cancer cells (Qiao et al 1998; Castaño et al 1999), endometrial cancer cells (Arango et al 2001), lymphocytic leukaemia cells (Bellosillo et al 1998) and myeloid leukaemia cells (Klampfer et al 1999), suggesting a mechanism for its chemopreventive activity. However, it remains unclear whether aspirin can suppress the proliferation of rheumatoid synovial cells.

In this study, we examined the effects of aspirin and sodium salicylate on the proliferation of rheumatoid synovial cells. In addition, we also analysed the induction of apoptosis as a possible mechanism of their action.

Materials and Methods

Materials

Aspirin, sodium salicylate and RPMI1640 were purchased from Sigma Chemical Co. (St Louis, MO). Fetal bovine serum (FBS) was purchased from Gibco (Grand Island, NY).

Cell culture

Rheumatoid synovial cells were prepared from synovial tissues as described previously, with slight modification (Kawai et al 1998; Yamazaki et al 1999). The synovial tissues were obtained during surgery for total knee replacement from patients with rheumatoid arthritis who fulfilled the revised American Rheumatism Association criteria for the classification of rheumatoid arthritis (Arnett et al 1988). Experiments were carried out according to a protocol approved by the ethics committee of St Marianna University, and all patients gave written consent to the use of their tissues for this research. Synovial tissues were digested for 2 h with 0.2% (w/v) bacterial collagenase and for 2 h with 0.125% (w/v) trypsin, and then suspended in RPMI1640 with 10% (v/v) fetal bovine serum (FBS), 100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin (Gibco). The cells were incubated at 37°C in 5% CO_2 for several days, and non-adherent cells were removed. The fibroblast-like adherent cells were used as rheumatoid synovial cells within 2 passages. Among the adherent cells, T cells (CD3+) and macrophage/monocytes (CD14+) were not detected by two-colour immunofluorescence and flow cytometry.

Drug preparation

Test drugs were dissolved in dimethyl sulfoxide (DMSO) as ×1000 stock solutions and then diluted with RPMI1640 containing 1% FBS for cell culture experiments. The drug solutions were prepared on the day of testing. The final concentration of DMSO for all treatments, including the control culture, was maintained at 0.1%.

Cell proliferation assay

The proliferation of rheumatoid synovial cells was assessed by measuring the incorporation of 5-bromo-2′-deoxyuridine (BrdU). Rheumatoid synovial cells (1×10^4cells/well) on 96-well culture plates were treated with the test drugs in RPMI1640 containing 1% (v/v) FBS at 37°C in 5% CO_2. After 24 h, BrdU (10 μM) was added to the culture medium, and the cells incubated for another 16–18 h. The synovial cells were fixed and BrdU incorporation was determined with a cell proliferation enzyme-linked immunosorbent assay (ELISA) kit (Roche Diagnostics, Mannheim, Germany) using peroxidase-conjugated anti-BrdU Fab fragments according to the manufacturer's instructions. The results are presented as a percentage of the control value.

Cell viability assay

Rheumatoid synovial cells (2×10^4 cells/well) on 96-well culture plates were treated with the test drugs in RPMI1640 containing 1% (v/v) FBS at 37°C in 5% CO_2. After 24 h, cell viability was measured as mitochondrial NADH-dependent dehydrogenase activity with a cell counting kit (Dojindo, Kumamoto, Japan) using a sulfonated tetrazolium salt, 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2 *H*-5-tetrazolio]-1,3-benzene disulfonate (WST-1), according to the manufacturer's instructions (Ishiyama et al 1993). The results are presented as a percentage of the control value.

DNA fragmentation assay

Rheumatoid synovial cells (2×10^4 cells/well) on 96-well culture plates were treated with the test drugs in RPMI1640 containing 1% (v/v) FBS at 37°C in 5% CO_2 for 24 h. The level of fragmented DNA in the cells, which is characteristic of apoptosis, was measured by DNA Cell Death Detection ELISA^{PLUS} (Roche Diagnostics) using anti-histone mouse monoclonal antibody (clone H11-4) as primary antibody and anti-DNA mouse monoclonal antibody (clone MCA-33) as secondary antibody according to the manufacturer's instructions. The results are presented as fold-induction compared with the control culture.

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling assay

Rheumatoid synovial cells (6×10^4 cells/well) on 8-well chamber slides (IWAKI, Chiba, Japan) were treated with the test drugs in RPMI1640 containing 1% (v/v) FBS at 37°C in 5% CO_2. After 24 h, synovial cells were fixed with a 4% (w/v) formalin neutral buffer solution for 10 min at room temperature, and then apoptotic synovial cells were identified by terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) assay using an apoptosis in-situ detection kit (Wako, Osaka, Japan) according to the manufacturer's instructions. The synovial cells were also counter-stained using Methyl Green Solution (Wako).

Statistical analysis

Statistical analysis using Kruskal–Wallis test was done for comparison among different concentrations of aspirin or sodium salicylate in cell proliferation and viability assays. P values less than 0.05 were considered significant.

Results

Effects of aspirin and sodium salicylate on the proliferation and viability of rheumatoid synovial cells

We first examined the effects of aspirin and sodium salicylate on the proliferation (DNA synthesis) of rheumatoid synovial cells by measuring the cellular incorporation of

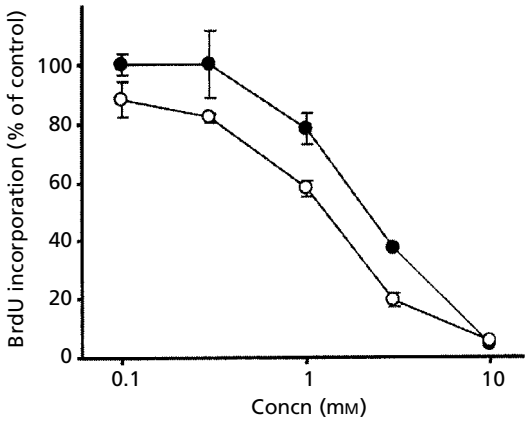

Figure 1 Effects of aspirin and sodium salicylate on the proliferation of rheumatoid synovial cells. Rheumatoid synovial cells were treated with aspirin (●) or sodium salicylate (○) for 24 h. Cell proliferation was estimated from the cellular incorporation of BrdU and presented as a percentage of the control value. Data are means±s.d. of triplicate cultures. Results are representative of three independent experiments. $P = 0.011$ for aspirin and $P = 0.006$ for sodium salicylate by Kruskal–Wallis test.

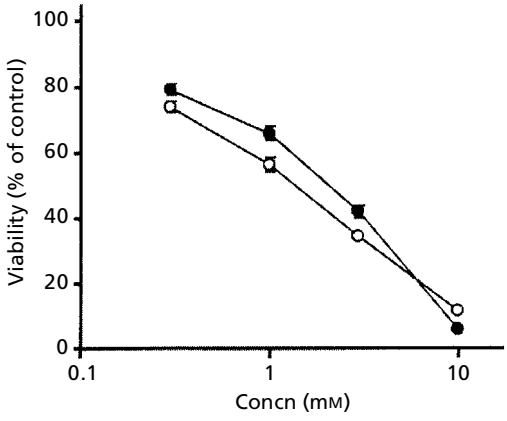

Figure 2 Effects of aspirin and sodium salicylate on the viability of rheumatoid synovial cells. Rheumatoid synovial cells were treated with aspirin (●) or sodium salicylate (○) for 24 h. Cell viability was measured by WST-1 assay and presented as a percentage of the control value. Data are means±s.d. of triplicate cultures. Results are representative of four independent experiments. $P = 0.009$ for aspirin and $P = 0.011$ for sodium salicylate by Kruskal–Wallis test.

BrdU (Figure 1). The cells were incubated with aspirin and sodium salicylate for 24 h. Both agents suppressed cell proliferation in a concentration-dependent manner over the range 0.3–10 mM, with IC50 values (concenctration causing 50% inhibition of cell proliferation) of 2.1 and 1.2 mM, respectively.

To explore whether cell death is involved in the suppression of cell proliferation caused by aspirin and sodium salicylate, the viability of rheumatoid synovial cells treated with these drugs was further examined by WST-1 assay (Ishiyama et al 1993). As shown in Figure 2, the viability

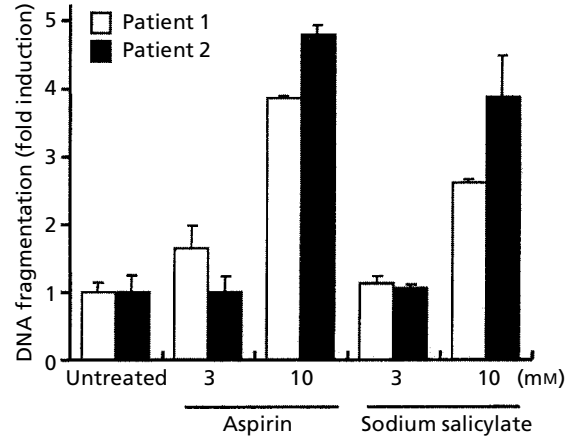

Figure 3 Effects of aspirin and sodium salicylate on DNA fragmentation in rheumatoid synovial cells. Synovial cells from two different patients with rheumatoid arthritis were treated with aspirin or sodium salicylate for 24 h. The level of fragmented DNA in the cytoplasm was measured by ELISA using anti-histone and anti-DNA mouse monoclonal antibodies. The fold-induction of DNA fragmentation is relative to untreated control cells.

was concentration-dependently reduced by aspirin and sodium salicylate, with IC50 values of 2.0 and 1.4 mM, respectively. These doses corresponded to the doses that inhibited the cell proliferation as determined from the cellular incorporation of BrdU (Figure 1).

Induction of apoptosis by aspirin and sodium salicylate in rheumatoid synovial cells

To determine whether the inhibition of cell proliferation by aspirin and sodium salicylate was due to the induction of apoptosis, the effects of these agents on DNA fragmentation (a hallmark of apoptosis) in rheumatoid synovial cells were quantitatively analysed by fragmented DNA ELISA that specifically detects cytoplasmic histone-associated DNA fragments, mono- and oligonucleosomes. As shown in Figure 3, 3 or 10 mM of aspirin and sodium salicylate induced DNA fragmentation in synovial cells after a 24 h treatment. The inductive effect of aspirin was slightly more potent than that of sodium salicylate.

To confirm that apoptosis was induced by aspirin and sodium salicylate, apoptotic cells were detected by TUNEL assay after a 24-h treatment with these drugs. Untreated synovial cells were not stained by TUNEL assay (Figure 4). In contrast, treatment with aspirin (10 mM) and sodium salicylate (10 mM) resulted in a significant number of TUNEL-positive cells.

Discussion

In this study, we found that aspirin and sodium salicylate inhibited the proliferation of rheumatoid synovial cells in a concentration-dependent manner at 0.3–10 mM. The likely mechanism of the anti-proliferative effect of aspirin on

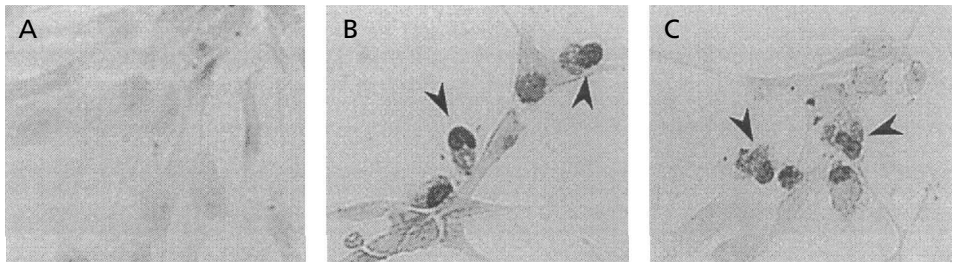

Figure 4 Detection of apoptotic rheumatoid synovial cells by TUNEL assay. Rheumatoid synovial cells were left untreated (A), or treated with 10 mM aspirin (B) or 10 mM sodium salicylate (C) for 24 h, and then apoptotic synovial cells were identified by TUNEL assay as described in Materials and Methods. The synovial cells were also counterstained with Methyl Green. Arrows indicate representative TUNEL-positive cells (apoptotic cells) (original magnification ×60).

rheumatoid synovial cells is apoptosis. Studies have showed that aspirin induces apoptosis in a variety of cell lines (Bellosillo et al 1998; Qiao et al 1998; Castaño et al 1999; Klampfer et al 1999; Arango et al 2001). However, there is also evidence that aspirin cannot induce apoptosis, although it reduces cell proliferation (Shiff et al 1996; Subbegowda & Frommel 1998; Smith et al 2000). Therefore, it is inconclusive whether the anti-proliferative effect of aspirin is associated with induction of apoptosis. However, in this study, aspirin and sodium salicylate certainly reduced cell viability, induced DNA fragmentation and increased TUNEL-positive cell numbers in rheumatoid synovial cells. The effective dose range of aspirin and sodium salicylate to induce apoptotic cell death in synovial cells was the same as that needed to induce apoptosis in colon cancer cells (Qiao et al 1998; Castaño et al 1999), endometrial cancer cells (Arango et al 2001), lymphocytic leukaemia cells (Bellosillo et al 1998) and myeloid leukaemia cells (Klampfer et al 1999). The evidence suggests that aspirin and sodium salicylate act by inducing apoptosis. Since the inductive effect of aspirin on DNA fragmentation was slightly more potent than that of sodium salicylate, the apoptotic effect of aspirin was not mediated by conversion to salicylic acid in the culture medium. To our knowledge, this is the first report to document that aspirin and sodium salicylate have anti-proliferative and pro-apoptotic effects in synovial cells from a patient with chronic inflammation.

Although COX is the molecular target of most NSAIDs, not only a COX-dependent (Souza et al 2000; Li et al 2001a, b) but also a COX-independent (Hanif et al 1996; Elder et al 1997; Smith et al 2000) mechanism for the apoptotic action of NSAIDs has been reported. In our study, aspirin had no effect on the proliferation or viability of synovial cells at 100 μM, a concentration that completely suppresses PGE_2 production in rheumatoid synovial cells (Kawai et al 1998). In addition, aspirin and sodium salicylate exerted anti-proliferative and pro-apoptotic effects over the same dose range, although aspirin is a much more potent inhibitor of COX than sodium salicylate (Mitchell et al 1994). Therefore, it is possible that the mechanism by which aspirin and sodium salicylate cause apoptosis in rheumatoid synovial cells is COX-independent. Several cellular transcription factors have been shown to be targets of NSAIDs, which may mediate not only the anti-inflammatory action of NSAIDs but also NSAID-induced apoptosis (Tegeder et al 2001). For example, aspirin and sodium salicylate are potent inhibitors of transcription factor NF-κB (Kopp & Ghosh 1994; Yin et al 1998). It has been shown that inhibition of the NF-κB pathway by pyrrolidinedithiocarbamate and *N*-acetylcysteine is linked to the induction of apoptosis in a variety of cells (Tsai et al 1996; Wu et al 1996; Ozaki et al 1997). However, in our additional study, pyrrolidinedithiocarbamate and *N*-acetylcysteine did not cause apoptosis in rheumatoid synovial cells (data not shown). Therefore, the NF-κB pathway seems not to contribute to the inhibition of proliferation and the induction of apoptotic cell death by aspirin and sodium salicylate in rheumatoid synovial cells. Elucidation of the mechanisms involved in the pro-apoptotic action of aspirin and sodium salicylate in rheumatoid synovial cells needs further investigation.

It remains to be seen whether the results presented here, obtained in-vitro, can be extrapolated to man. In fact, much higher doses of aspirin were required to achieve the induction of apoptosis than to inhibit PGE_2 production in rheumatoid synovial cells (Kawai et al 1998). However, it is possible that the concentrations of aspirin and sodium salicylate used here are attainable in plasma. For example, it was reported that the usual range of plasma salicylate concentrations for anti-inflammatory effects in patients with rheumatoid arthritis was 120–350 μg mL^{-1} (0.9–2.5 mM) by daily ingestion of 4 or 5 g of aspirin (Insel 1990). Therefore, it is conceivable that the cellular effects we observed in-vitro might occur in man.

Conclusions

We demonstrated that aspirin and sodium salicylate inhibited the proliferation of rheumatoid synovial cells. In addition, at similar concentrations, these compounds induced apoptosis in synovial cells. The results suggest that aspirin and sodium salicylate inhibit the growth of the cell population through the induction of apoptosis. Although the relevance of these observations to the therapeutic effects of aspirin and salicylate in the treatment of rheumatoid arthritis as yet remains to be elucidated, this study provides important information for the design of NSAIDs to treat rheumatoid arthritis more effectively.

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