GROWTH INHIBITION OF MESENCHYMAL STEM CELLS BY ASPIRIN: INVOLVEMENT OF THE WNT/β-CATENIN SIGNAL PATHWAY

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SUMMARY

1. Mesenchymal stem cell (MSC) therapy is drawing increasing attention in cardiology. However, the effect of aspirin, an assistant medication used extensively in the treatment of cardiovascular diseases, on MSC is not clear.

2. In the present study, we investigated the effect of aspirin on the growth of MSC in vitro and the underlying mechanism of its action.

3. The 3-(4,5-dimethyl-2-thiazoyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay revealed that 1, 5 and 10 mmol/L aspirin inhibited the growth of MSC by 18, 37 and 62%, respectively. DNA synthesis of MSC was inhibited by 25, 57 and 90% following treatment with 1, 5 and 10 mmol/L aspirin, respectively, as determined by the tritiated thymidine incorporation assay. No cytotoxicity was observed based on Trypan blue dye exclusion and cell morphological observations. Western blot analysis demonstrated that the protein level of phosphorylated β-catenin increased, whereas that of cyclin D1 decreased, after treatment of MSC with aspirin. Cell cycle analysis showed that aspirin failed to significantly alter the proportion of cells in different stages of the cell cycle.

4. These observations indicate that aspirin inhibits MSC proliferation and that the downregulation of the wnt/β-catenin signal pathway may be involved in the growth inhibition of MSC by aspirin.

Key words: aspirin, mesenchymal stem cells, wnt/β-catenin.

INTRODUCTION

Mesenchymal stem cells (MSC) have shown great promise in cardiology in recent years. Many studies have revealed that MSC transplant therapy can regenerate and repopulate the damaged myocardium, restoring its function owing to the ability of MSC to self-renew and differentiate into functional cell types, including cardiomyocytes or endothelial cells, in an appropriate environment. However, it is evident that transplanted stem cells do not survive well. For instance, over 99% of MSC injected into the left ventricle of CB17 SCID/beige adult mice died within 4 days of injection.

As shown in a previous study, in addition to low oxygen tension and serum deprivation, many other unidentified cellular events may jeopardize the survival of MSC in the ischaemic myocardium.

Aspirin is an effective and extensively used drug in cardiovascular diseases because of its remarkable ability to notably decrease mortality and reinfarction of patients who suffer from myocardial infarction (MI) and a wide range of other established cardiovascular diseases, such as arteriosclerosis, acute coronary disease and ischaemic cardiomyopathy. The clinical benefits of aspirin in cardiovascular diseases have been ascribed to its antiaggregation and anti-inflammation effects. However, in recent years, growth inhibition by aspirin has come to the fore. Many studies have indicated that aspirin is able to inhibit the growth of various cell types, including endothelial cells, vascular smooth muscle cells and, in particular, cancer cells. However, what effect aspirin may have on the growth regulation of MSC is unknown. In patients who are candidates for MSC transplant, there is usually a history of taking aspirin or the need for the further use of aspirin. Whether aspirin has any adverse effects on the survival of MSC in the myocardium needs to be determined.

Furthermore, the molecular mechanisms underlying the effect of aspirin are not well understood and are a matter of ongoing debate. In addition to the traditional accepted mechanism of reduced prostaglandin (PG) synthesis through the inhibition of cyclo-oxygenase (COX), recent studies have identified many other factors that are targets of aspirin, such as nuclear factor (NF)-κB, insulin-like growth factor I (IGF-I) and many other enzymes, as well as the disruption of wnt/β-catenin signal pathway, which has been demonstrated to be functional in MSC. Therefore, we are interested in unravelling the molecular mechanism involved in the effect of aspirin on MSC, especially the wnt/β-catenin signal pathway.

METHODS

Materials

Dulbecco’s modified Eagle’s medium (DMEM) was obtained from Hyclone (South Logan, UT, USA). Fetal bovine serum (FBS) was obtained from Gibco (Carlsbad, CA, USA). Aspirin, indomethacin, dexamethasone, ascorbate phosphate, β-glycerophosphate, insulin, trypsin, Alizarin red, Sudan III, Trypan blue and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyterazolium bromide (MTT) were from Sigma (St Louis, MO, USA). Tritiated thymidine (‘H-TdR) was obtained from China Institute of Atomic Energy (Beijing, China). The following mouse monoclonal antibodies were obtained from Becton Dickinson (San Jose, CA, USA): anti-CD29-polyethylene (PE), anti-CD34-PE, anti-CD44–fluorescein isothiocyanate (FITC) and anti-CD45–FITC. The homotype control (anti-IgG1–FITC and anti-IgG1–PE), along with anti-rat...
Trypan blue dye exclusion
Trypan blue dye exclusion was used to determine the cytotoxicity of aspirin to the cells. Living cells exclude the dye, whereas dead cells take up the blue dye. Cells were prepared and treated with or without aspirin (1, 5 and 10 mmol/L) for 24 h. Then, cells were harvested by trypsinization, washed with PBS, pelleted and resuspended in PBS. The cell suspension (0.5 mL) was mixed with an equal volume of 0.4% Trypan blue solution and incubated for 3 min at room temperature. The number of unstained cells and the total number of cells were counted on a haemocytometer under a microscope. The percentage of viable cells was determined by dividing the number of unstained cells by the total number of cells and multiplying by 100.

Western blot analysis
Cells were rinsed twice with ice-cold PBS and then lysed with ice-cold lysis buffer (1% Triton X-100, 20 mmol/L HEPES (pH 7.5), 5 mmol/L MgCl2, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L diithiothreitol, 1 mmol/L phenylmethylsulphonyl fluoride and 1 mg/mL each of leupeptin, aprotinin and pepstatin) for 30 min. Cell lysates were centrifuged at 14,000 g for 10 min at 4°C. Equal amounts of protein (30 μg) were mixed with 5x sodium dodecyl sulphate (SDS) sample buffer, boiled for 5 min and then electro-phoresed on 10% SDS–polyacrylamide gel electrophoresis (PAGE) and transferred to nylon membranes using a standard protocol. The membrane was blocked in 5% skim milk for 2 h, rinsed and incubated overnight at 4°C with primary antibody in 5% fat-free milk. Excess antibody was then removed by washing the membrane in PBS/0.1% Tween 20 and membranes were incubated for 2 h with HRP-conjugated secondary antibodies. After being washed in PBS/0.1% Tween 20, bands were visualized by an enhanced chemiluminescence (ECL) system and exposed to radiography film.

Cell cycle analysis
Cell suspensions from aspirin-treated or -untreated cell cultures were prepared by trypsinizing the cells and washing them twice with cold PBS. Cells were resuspended at a density of 10^6 cells/mL in cold PBS and fixed with 75% ethanol overnight at −20°C. Ethanol-fixed cells were washed with cold PBS before being resuspended in 300 μL PBS containing 0.15% RNase A at 37°C for 30 min and stained with 50 μL/mL propidium iodide for 30 min. The DNA content was analysed using a FACScalibur Flow Cytometer (Becton Dickinson) and cell cycle data were analysed with Multicycle Software Autofit Version 2.50 (Phoenix Flow Systems, San Diego, CA, USA).

Data analysis
Data were analysed by F-test using Microsoft Excel 2003 software (Microsoft, Redmond, WA, USA). P < 0.05 was considered significant.

RESULTS
Characterization of MSC from bone marrow
The population of cells we purified from bone marrow was 98.42 ± 1.14% CD29+, 97.6 ± 1.85% CD44+ and negative for CD34 and CD45 (Fig. 1). After induction, Alizarin red and Sudan III staining showed the formation of calcium and oil-laden cells, respectively, indicating that the cells had the potential for osteoblastic and adipocytic differentiation (Fig. 2). Taking these data together, the cells were identified as MSC.

Growth inhibition of MSC by aspirin
The MTT assay demonstrated that aspirin significantly inhibited MSC proliferation in a concentration-dependent manner. The optical
density at 570 nm was decreased to 82, 63 and 38% of control by 1, 5 and 10 mmol/L aspirin, respectively. The effect of aspirin on the growth inhibition of MSC was confirmed by the [3H]-TdR incorporation assay, which demonstrated that DNA synthesis was inhibited by 25, 57 and 90%, respectively, in cells treated with 1, 5, 10 mmol/L aspirin for 24 h (Fig. 3).

Trypan blue dye exclusion was used to determine whether the observed growth inhibition of MSC by aspirin was caused by cytotoxicity. Viable cells as a percentage of cells treated with aspirin (1, 5 or 10 mmol/L) for 24 h did not differ from the percentage of viable cells in the control culture (data not shown). In addition, no morphological changes were observed, such as cell shape or detachment, for aspirin-treated cells, demonstrating that the growth inhibition of MSC by aspirin was not the result of cytotoxicity.

Involvement of the wnt/β-catenin signal pathway in growth inhibition of MSC by aspirin

As shown in Fig. 4, total β-catenin protein levels remained constant, whereas phosphorylated β-catenin (phospho-β-catenin) protein levels increased and showed a correlation with the concentration of aspirin after treatment of cells with 1, 5 and 10 mmol/L aspirin for 24 h. To study the time-course of the effect of aspirin on phospho-β-catenin protein level, cells were incubated with 5 mmol/L aspirin for 6, 12 and 24 h and it was seen that the phospho-β-catenin protein level increased with increasing incubation time (Fig. 4).

Cyclin D1 is one target protein modulated by the wnt/β-catenin signal pathway. In order to further examine the activity of the wnt/β-catenin signal pathway, we assessed the expression of cyclin D1.
Western blot analysis showed that cyclin D1 protein levels decreased with increasing time of incubation with aspirin (Fig. 5).

Cell cycle not significantly altered by aspirin

Cell cycle analysis revealed that the proportion of untreated MSC in the G0/G1 phase, S phase and G2/M phase was 0.73 ± 0.03, 0.16 ± 0.03 and 0.11 ± 0.01, respectively. Aspirin treatment (5 mmol/L) for 6, 12 and 24 h failed to significantly alter the proportion of cells in the different stages of the cell cycle. A trend towards a decreased proportion of cells in the S phase, with relative increases in the proportion of cells in the G0/G1 phase was observed, but this failed to reach statistical significance (Table 1).

DISCUSSION

Aspirin has been used extensively in the primary and secondary prevention of cardiovascular diseases owing to its anti-inflammatory and platelet inhibitory effects.9,10,33 In the present study, we demonstrated that aspirin inhibited the growth of MSC in vitro, which may indicate that in the case of terminal cardiovascular patients in whom MSC transplants are being considered, the use of aspirin has to be taken into careful consideration. The present data showed that 1–10 mmol/L aspirin was effective in inhibiting the growth of MSC, an effect that appears to be mediated through the inhibition of proliferation, with no accompanying increase in cell death. To our knowledge, this is the first time that the effect of aspirin on MSC has been investigated. The present results are consistent with those of other studies demonstrating that aspirin is able to inhibit the proliferation of a variety of cell types12,13,17 and the concentrations of aspirin used in the present study were consistent with those used previously as well. Both aspirin and MSC are important medications for cardiovascular disease, but our experiments indicate that aspirin may be disadvantageous at the initial stage of MSC transplantation because of the well-known fact that proliferation is an essential process in MSC therapy. In order to effect tissue repair, engrafted cells must proliferate to provide adequate new tissue before differentiating into...
Because there is growing conviction that stem cells share much in common with nonstem cells,35 and there are many studies demonstrating that aspirin can inhibit the growth of cancer cells,14–19,36,37 suggesting that aspirin may be favored in the later stage of MSC transplantation owing to its ability to prevent neoplasia from the transplanted cells in addition to its anti-inflammatory effects.

There are several explanations regarding the molecular mechanisms by which aspirin inhibits cell proliferation.20–26 Recently, the Wnt/β-catenin signal pathway has been linked to the mechanism of action of aspirin. Wnts bind to frizzled receptors and subsequent signaling inhibits glycogen synthase kinase-3β (GSK-3β), causing the accumulation of β-catenin in the cytoplasm and translocation into the nucleus to induce target gene expression.38 Etheridge et al.28 have identified the expression profile of Wnt signaling molecules in MSC and have provided evidence that an endogenous Wnt/β-catenin pathway functions in these cells. Boland et al.39 have demonstrated that Wnt/β-catenin signaling functioned in maintaining an undifferentiated, proliferating progenitor MSC population. β-Catenin is a key molecule of the Wnt/β-catenin signal pathway. Under unstimulated conditions, β-catenin is continuously targeted for ubiquitin-dependent degradation, which depends on its NH2-terminal phosphorylation by GSK-3β in association with a multiprotein complex. However, when the Wnt signal is transduced, phosphorylation of β-catenin by GSK-3β is inhibited, causing β-catenin stabilization and accumulation before translocation to the nucleus, where it binds with members of the T cell factor (TCF) and lymphoid enhancer factor (LEF) transcription factor family to induce expression of target genes.39 Thus, there are two different cytosolic β-catenin pools: phosphorylated β-catenin for degeneration and monomer β-catenin for translocation into the nucleus. The present study revealed that, in aspirin-treated MSC, the total β-catenin protein levels remained constant, but the levels of phospho-β-catenin protein increased and were correlated with the concentration of aspirin and the duration of incubation. Thus, we can deduce that the cytosolic monomer β-catenin levels decreased, which may be the result of enhanced phosphorylation by GSK-3β and, finally, led to the arrest of signal transduction into the nucleus. Investigations of colorectal cancer cells by Dihlmann et al.40,41 demonstrated that aspirin inhibited the growth of colorectal cancer cells by downregulating Wnt/β-catenin signalling and that the reduced signalling activity of β-catenin in response to aspirin was the result of its enhanced phosphorylation. It could be reasoned that aspirin works on MSC and cancer cells through a similar mechanism because there is growing conviction that stem cells share much in common with cancer cells.41–43 Furthermore, after treatment of cells with aspirin, we also detected a decrease in cyclin D1, the target protein that is regulated by the Wnt/β-catenin signal pathway.44,45 Taken together, these results could infer that the Wnt/β-catenin signal pathway may be involved and is downregulated in the growth inhibition of MSC by aspirin. The antiproliferative effect of aspirin in a variety of cell types is associated with the induction of apoptosis. However, there is conflicting evidence that aspirin induces apoptosis in colorectal cancer cell lines. Shiff et al.46 were not able to demonstrate an effect of aspirin. In contrast, Elder et al.47 demonstrated a convincing, dose-dependent apoptotic response using salicylate. Further studies are required to determine whether aspirin causes an apoptotic response in MSC.

In the present study, flow cytometry analysis failed to identify significant differences in the proportion of cells in the different stages of the cell cycle following treatment with aspirin, despite a decrease in cyclin D1. However, a trend towards a decreased proportion of cells in the S phase, with a relative increase in the proportion of cells in the G0/G1 phase and/or the G2/M phase, was observed. This result was in agreement with observations in human umbilical vein endothelial cells,11 but in contrast with observations made in rat thoracic aorta vascular smooth muscle cells and human colon cancer cells, which indicated that aspirin arrests cells in G0/G1 phase of the cell cycle.27,48 This may indicate that the mechanisms of cell cycle control are complex and may differ for different cell types. At the same time, it also signifies that cell cycle regulation may not be a key mechanism of growth retardation in response to aspirin.

In conclusion, this is the first study to demonstrate an antiproliferative effect of aspirin on MSC, which may be regulated by the Wnt/β-catenin signal pathway. Further studies, such as examination of the effects of aspirin on MSC in an in vivo model, are required to fully demonstrate the effects of aspirin. The present findings highlight the use of aspirin and may facilitate the development of optimal doses of aspirin for use in the clinical setting of MSC transplantation therapy, as well as help advance our understanding of the molecular mechanism of action of aspirin.

ACKNOWLEDGEMENTS

This work was supported by grants from the National Natural Science Foundation of China (No. 30271290 and 30370524) and a grant from the National Science Fund for Distinguished Young Scholars (No. 30125039).

REFERENCES


