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Acidic environments enhance the inhibitory effect of statins on proliferation of synovial cells

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ABSTRACT

Many previous studies in animal models and clinical investigations have suggested that statins are useful chemotherapeutics against rheumatoid arthritis, whereas in vitro experiments using synovial cell lines showed no significant effect of statins on cell proliferation until now. Since synovial fluid in rheumatoid joint knee was found to be acidic, we examined the effect of statins on human synovial sarcoma cell line SW982 cells in acidic medium. Statins suppressed the proliferation of SW982 cells at pH 6.7, while the suppression was very weak in pH 7.5 medium. It was shown that the suppression was caused by the decrease in geranylgeranyl diphosphate, suggesting that a geranylgeranylated protein(s) has an essential role in cell proliferation of SW982 cells under acidic conditions. Our present data clearly implied that statins had high efficacy against SW982 cells in acidic medium whose pH is close to that of rheumatoid arthritis loci in patients. These results lead us to anticipate that screening of chemicals having high therapeutic efficacy in acidic medium promotes the development of new microenvironment-dependent medicines for chemotherapies against rheumatoid arthritis.

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1. Introduction

Rheumatoid arthritis (RA) is a chronic, destructive, inflammatory disease of the synovial membrane. The thin synovial membrane is observed in healthy joints [1]. After onset of clinical RA, the synovial fibroblasts exhibit anchorage-independent growth and become hyperplastic. Ultimately this develops into an abnormal layer, which migrates onto the articular cartilage and promotes articular destruction [2]. A number of chronic inflammatory cells have been proposed to infiltrate into the hyperplastic synovial membrane and exasperate the pathology of RA. A high number of T cells was detected in the inflamed synovium, and the requirement for T cells was demonstrated in various animal models of arthritis [3]. Macrophages and monocytes are considered as important sources of synovial pro-inflammatory cytokines [4]. Several cytokines including TNF-α and IL-1 from these cells are implicated in the pathogenesis of RA [5].

In mammals, the pH values of blood and tissues are usually maintained in a narrow range around 7.4, mainly through regulation of respiration and renal acid extrusion [6,7]. Extracellular pH, however, drops to a value below 6 due to leaking of intracellular contents and the destruction of blood vessels, resulting in hypoxic metabolism and related lactic acid production during inflammation against the infection of pathogens [8]. Similar acidic environments were also associated with RA. The pH value of articular fluid in the rheumatoid human joint knee was around 6.6, compared to around 7.3 in normal knee-joints [9]. Other studies also showed the acidification of synovial fluid [10–12].

This inflammatory acidification may affect immune cell functions such as intracellular signaling. The induction of mature human dendritic cells and TNF secretion from macrophages were observed under acidic conditions [13–15]. Our group demonstrated that extracellular acidic environments enhanced T cell signalling induced by T cell receptor (TCR) stimulation, followed by the increase in phosphorylation of TCR signal proteins [16]. While data concerning the effect of acidosis on immune cell functions have accumulated, little is known about the proliferation of synovial fibroblasts under acidic conditions although the abnormal proliferation of synovial fibroblasts is one main pathogenesis.

In the present article, several molecular targeted inhibitors were tested in acidic conditions. We found that lovastatin, ionomycin, cantharidin suppressed the proliferation of synovial membrane cells preferentially at acidic pH. We further investigated the effect of statins and found that the attenuation of protein prenylation was the main reason for the inhibitory effect of statins on synovial cell proliferation. This pH-dependent efficacy of statins may lead us to expect that statins have therapeutic potential against RA with less effect on normal tissues. Furthermore, the present observations would promote the discovery of microenvironment-specific medicines for chemotherapies against RA.

2. Materials and methods

2.1. Cells and reagents

The human synovial sarcoma cell line SW982 was donated by T. Nakanishi (School of Pharmacy, Shujitsu University). SW982 cells were cultured at 37 °C under 5% CO_2 in DMEM containing 24 mM

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$NaHCO_3$, 10 μg/ml gentamicin, 5 μg/ml fungizone, and 10% fetal bovine serum (FBS).

SCADS inhibitor kits were kindly donated by the Screening Committee of Anticancer Drugs (SCADS), and this organization was supported by a Grant-in-Aid for Scientific Research on Priority Area "Cancer" from the Ministry of Education, Culture, Sports, Science, and Technology of Japan. [^{14}C] statins were kindly donated by Daiichi Sankyo Company, Ltd. Manumycin A (Wako) and YM-53601 (Sigma-Aldrich) were purchased. Cell Counting Kit 8 was purchased from Dojindo (Kumamoto, Japan). Statins were purchased from Wako (Osaka, Japan). In some experiments indicated, statins were converted to open ring form before use as described previously [17].

2.2. Media for cell proliferation under different pH conditions

Media at various pH values for the measurement of cell proliferation were prepared as follows. To minimize the pH change during the cell culture, 10 mM PIPES [piperazine-*N*,*N*′-bis (2-ethanesulfonic acid)] for acidic media or HEPES [*N*-(2-hydroxyethyl) piperazine-*N*'-2-ethanesulfonic acid] for alkaline media was added to DMEM instead of $NaHCO_3$. Medium containing FBS was often contaminated with germs when medium pH was adjusted, and it was hard to sterilize medium containing FBS. Therefore, medium pH was first adjusted by the addition of NaOH to medium without FBS. After sterilization of the medium by filtration, FBS was added. Since the medium pH was changed by the addition of FBS, the pH of medium without FBS was adjusted to a lower and higher value than the final pH in acidic and alkaline media, respectively. For example, when media of 6.7 and 7.5 containing 10% FBS were used, the pH values were adjusted to 6.4 and 7.6, respectively, before the addition of FBS. Media of other pH values were prepared in the same manner. An inhibitor was added after the addition of FBS when indicated.

2.3. Measurement of viable cell numbers at different pH values

SW982 cells were suspended in pH 7.5 DMEM medium prepared as described above, and 50 μl of the cell suspensions were placed in 96 well plates at 7.5×10^2 cells/well. After incubation for 1 day, 100 μl of pH 6.1 DMEM medium containing the indicated inhibitors were added to the wells. The pH of the resulting mixture was 6.7. For incubation at pH 7.5, 100 μl of pH 7.5 DMEM medium containing the indicated inhibitors were added to the wells. Cells were incubated at 37 °C without a CO_2 supply but with an air supply to avoid hypoxia and constant humidity. Viable cell numbers were measured with a Cell Counting Kit 8 after incubation for 5 days. The Cell Counting Kit 8 contains WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt). Since WST-8 is converted into WST-8 formazan only by viable cells, the viable cell number can be estimated by measuring the amount of formazan produced by viable cells [18].

The proliferation of SW982 cells was also measured as follows. SW982 cells suspended in pH 7.5 DMEM medium prepared as described above were placed in 24 well plates (3×10^3 cells/well) and incubated for 1 day. The media were then exchanged with DMEM media of pH 6.7 or 7.5 containing inhibitors. After counting the number of cells, cells were incubated. Cell proliferation was determined by counting the number of viable cells per 1.44 mm^2 before and after incubation for 4 days, and the viable cell numbers were measured by trypan blue dye exclusion test. Cells were incubated at 37 °C without a CO_2 supply but with an air supply to avoid hypoxia and constant humidity.

2.4. Measurement of the amount of intracellular statin

SW982 cells spread in 6 well plates were incubated for 1 day, and the medium was changed into DMEM media of pH 6.7 or 7.5. After cells had been incubated for 1 day, [^{14}C] pravastatin (40 μM, 555 MBq/mmol) was added. Cells were incubated for 10 min, washed with PBS, and harvested with trypsine treatment. No significant increase in the accumulation was observed when cells were incubated for more than 10 min. The resulting cell suspension was mixed with Bray solution and radioactivity in the mixture was counted with a liquid scintillation counter. Cell numbers in the suspension before the addition of [^{14}C] pravastatin were measured. Cells were incubated at 37 °C without a CO_2 supply but with an air supply to avoid hypoxia and constant humidity.

2.5. Statistical analysis

The Student's *t*-test was utilized in this study.

3. Results

3.1. Inhibition of synovial cell proliferation at acidic pH

Synovial cells proliferate abnormally in acidic areas of RA [9–12]. SW982 synovial cells proliferated approximately 8-fold at pH 7.7 and 7.1 during 4 days' culture, and the medium pH decreased to 7.4 and 7.0, respectively (Fig. 1a). The cell number of SW982 increased 3-fold after 4 days' culture at pH 6.7 (Fig. 1a). The change in medium pH during 4 days' culture was less than 0.1 pH units in acidic media.

The effect of various molecular targeted inhibitors on synovial cell proliferation at pH 6.7, which was close to the pH value of RA loci, was examined using the SCADS inhibitor kits and compared with that at pH 7.5, which was close to the pH value of normal synovial environments. The SCADS inhibitor kits we used contained 273 inhibitors in total, and 70 inhibitors in the kits inhibited cell proliferation at the concentration of 1 μM. Lovastatin inhibited cell proliferation significantly at 1 μM in acidic conditions, while it did not reduce cell proliferation at pH 7.5 (Fig. 1b). Similar results were obtained with cantharidin (Fig. 1c) and ionomycin (Fig. 1d), but the other 67 inhibitors including Cdk1/2 inhibitor 3 decreased cell proliferation similarly at both pH values tested (Fig. 1e). The remaining 203 compounds in the SCADS inhibitor kits were not examined because the amounts of each inhibitor were insufficient for investigation of the effect on SW982 cell proliferation.

3.2. pH-dependent inhibition in statins

In addition to lovastatin, pravastatin and simvastatin suppressed cell proliferation preferentially at pH 6.7 (Fig. 2a–c). Pravastatin was shown to require a higher concentration as compared with lovastatin or simvastatin (Figs. 1 and 2). Kurakata et al. [19] showed that the half maximal inhibitory concentrations of simvastatin and pravastatin on sterol synthesis in lymphocytes were 0.013 μM and 5.6 μM, respectively. Around 100 mg/kg of pravastatin was shown to be effective for collagen-induced arthritis in mice [20], while simvastatin markedly inhibited collagen-induced arthritis in doses of 10 to 40 mg/kg in mice [21]. These data suggest that pravastatin requires a higher concentration for its inhibitory action as compared with simvastatin.

To examine whether or not the pH-dependent inhibition was due to the alteration of the statin accumulation under different pH conditions, the accumulation of [^{14}C] labeled pravastatin was determined at two pH values. The amount of pravastatin taken up by SW982 cells at pH 6.7 was lower than that at pH 7.5 (Fig. 2d), suggesting that the stronger inhibition at pH 6.7 is not due to the elevated accumulation.

Statins exist in two forms, lactone form and open ring hydroxy acid form [22]. It was reported that the lactone form is absorbed from the gastrointestinal tract and transformed into the active hydroxy acid form in vivo [23]. The preferential inhibition of statins at acidic pH appeared markedly in the open ring forms of lovastatin and simvastatin (Fig. 2e and f).

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