Inhibition of Osteoclast-Like Cell Formation by Sodium Salicylate and Indomethacin in Mouse Bone Marrow Culture

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ABSTRACT—The present study examined the effect of sodium salicylate and indomethacin on the recruitment of osteoclast-like cells in vitro. When mouse bone marrow cells were cultured for 8 days with 1α,25-dihydroxyvitamin D₃ (1α,25-(OH)₂D₃, 10⁻⁸ M), prostaglandin E₂ (PGE₂, 10⁻⁶ M) and recombinant human interleukin-1α (rHIL-1α, 2 ng/ml), numerous tartrate-resistant acid phosphatase (TRAP-positive) multinucleated cells (MNCs) formed. Adding sodium salicylate or indomethacin inhibited the formation of TRAP-positive MNCs in a dose-dependent manner. This inhibitory effect was more pronounced when the drugs were given at a later stage in the culture period. Indomethacin appeared to be more potent than sodium salicylate. PGE₂ production was inhibited by sodium salicylate or indomethacin. Exogenous PGE₂ failed to overcome the inhibitory effect of both drugs. These results suggest that sodium salicylate and indomethacin have inhibitory effects on the recruitment of osteoclast-like MNCs, preferentially on the later stage, and that PGE₂ is not the only compound targeted by these drugs in reducing osteoclast-like cell formation in mouse bone marrow culture.

Keywords: Sodium salicylate, Indomethacin, PGE₂, Bone marrow culture (mouse), Multinucleated cell

The pharmacological action of non-steroidal anti-inflammatory drugs (NSAIDs) are commonly attributed to their inhibition of cyclooxygenase activity, the process that converts unsaturated fatty acids in the cell membrane to prostaglandins, which are unstable endoperoxide intermediates (1). It has been shown that prostaglandins are potent stimulators of bone resorption (2) and, in turn, enhance bone formation in vivo (3, 4). In 1988, a mouse bone marrow culture model was developed and was found to be suitable for the recruitment and differentiation process of osteoclast-like multinucleated cells (MNCs) from their progenitors (5).

Previous reports have shown that 1α,25-dihydroxyvitamin D₃ (1α,25-(OH)₂D₃) and recombinant human interleukin-1α (rHIL-1α) stimulate prostaglandin E₂ (PGE₂) production in mouse bone marrow cultures (6, 7). Other studies have shown that osteoclast-like cell formation is partially dependent upon PGE₂ synthetase and that indomethacin inhibits the formation of osteoclast-like cell formation (8, 9).

Research on the effects of sodium salicylate on bone in growing rats indicates that it inhibits the resorption process of bone (10–12). In other studies, flurbiprofen and ibuprofen (13–15) were shown to reduce bone resorption. Using a calvaria culture system, Ohya et al. (16) showed that sodium salicylate inhibits the bone resorption stimulated by both epidermal growth factor and parathyroid hormone. Sodium salicylates added continuously to the mouse bone marrow culture inhibited osteoclast-like cell formation stimulated by 1α,25-(OH)₂D₃ and decreased the number of pits and the bone-resorption area formed by osteoclast-like cells (17).

To clarify the mechanism of action of NSAIDs in osteoclast recruitment, the present study was undertaken to investigate the effect of sodium salicylate and indomethacin on osteoclast-like cell formation using a mouse bone marrow culture, and the potencies of these drugs were compared.

MATERIALS AND METHODS

Animals and drugs

Seven to 9-week-old male mice, ddY strain, were obtained from Nippon Bio Supply Center (Tokyo). 1α,25-(OH)₂D₃ was the generous gift of Teijin Pharmaceutical Co. (Tokyo). rHIL-1α was kindly supplied from Dainippon Pharmaceutical Co., Ltd. (Osaka). PGE₂ was purchased from Sigma Chemical Co. (St. Louis, MO, USA).
All other chemicals and reagents used in the present study were of analytical grade.

**Bone marrow culture**

The mice were killed by cervical dislocation, and the femurs and tibiae were aseptically removed and dissected free of adhering tissues. The bone ends were resected with scissors and the marrow cavity was flushed with 1 ml α-minimal essential medium (α-MEM; Gibco Lab., Grand Island, NY, USA), which was slowly injected into one end of the bone using a sterile 25-gauge needle. The marrow cells were collected into tubes, washed twice with α-MEM, and cultured for 8 days in 24-well plates (Corning Medical, Corning, NY, USA) containing 0.5 ml/well of α-MEM (1.5×10⁶ cells/ml) supplemented with 10% fetal calf serum (Filtron, Pty Ltd., Parkville, Australia). Cultures were fed every other day by replacing 0.4 ml of old media with fresh media.

The concentrations of sodium salicylate ranged from 10⁻⁶–10⁻⁴ M (first set of experiments), while that of indomethacin ranged from 10⁻⁸–10⁻⁶ M (second set of experiments). The following three different bone-resorbing stimulants were used in the two sets of experiments: 1α,25-(OH)₂D₃ (10⁻¹⁸ M), PGE₂ (10⁻⁶ M) and rHIL-1α (2 ng/ml). These compounds were added simultaneously to the media when it was refreshed and were replaced every other day. The drugs were added as follows: continuously in the first group (group A), during the last 4 days of culture in the second group (group B) and during the first 4 days only in the last group (group C). All cultures were maintained at 37°C in a humidified atmosphere of 5% CO₂ in air. After 8 days, cells adherent to the well surface were rinsed once with phosphate-buffered saline (PBS, pH 7.4) and then fixed with ethanol-acetone (50:50 vol./vol.) for 1 min. Culture plates were dried out at room temperature for 10 min, and the cells were then stained for tartrate-resistant acid phosphatase (TRAP), using the method described below.

**Histochemistry for tartrate-resistant acid phosphatase**

TRAP was used as a marker for osteoclasts (18–20). Staining for TRAP was performed according to the modified method of Burstone (21). In brief, the fixed cells were incubated for 20 min at room temperature in an acetate buffer (0.1 M sodium acetate, pH 5.0). The buffer contained naphthol AS-MX phosphate (Sigma Chemical Co.) as a substrate, 10 mM sodium tartrate and red violet LB salt (Sigma Chemical Co.) as a stain for the reaction product (22). TRAP-positive cells appeared as dark red cells under light microscopy. Cells containing three or more nuclei were counted as MNCs.

**Prostaglandin E₂ (PGE₂) assay**

Prostaglandin E₂ was assayed in the supernatants from some cultures using a commercially available ¹²⁵I radioimmunoassay (Du Pont Canada, Inc., NEN Research Products, Mississauga, Canada). Culture supernatant (100 ml) was taken for assay immediately before feeding or after terminating the cultures after 8 days and stored at −20°C until the assay.

**Measurement of bone resorption**

Slices of bovine cortical bone were used as a substrate for osteoclastic resorption. Bone slices (8×8×0.2 mm) were cut from cleaned, adult bovine femora using a water-cooled diamond saw (EXAKT-cutting grinding system, Norderstedt, Germany), cleaned by ultrasonication for 10 min and further sterilized by ultraviolet light radiation for 4 hr before use (23). The marrow cells were allowed to settle onto devitalized bovine bone slices and were then cultured for 8 days.

At the end of the incubations, the cells were fixed, reacted for TRAP, and the TRAP-positive MNCs were scored. The cells were then stripped by ultrasonication in 0.25 M NH₄OH. The bone slices were then stained with toluidine blue prior to processing for microscopy. The number of pits was counted, and the extent of bone resorption was measured by examining the whole surface of each bone slice. The area of resorption was quantified using a texture analyzing system on a Macintosh II microcomputer, running ULTIMAGE software (Graftek, Meudon-La-Forêt, France). The results were expressed as the percentage of surface area showing bone resorption to the whole surface of bone.

**Statistical analyses**

All values are expressed as the mean±S.D. Statistical significance was determined by Student’s t-test. A P value less than 0.05 was considered statistically significant.

**RESULTS**

Treatment of cultures with the bone-resorbing stimulants 1α,25-(OH)₂D₃ (10⁻¹⁸ M), PGE₂ (10⁻⁶ M) or rHIL-1α (2 ng/ml) alone for 8 days caused the appearance of TRAP-positive MNCs (Fig. 1). Adding sodium salicylate continuously for 8 days during the culture period (group A) or for the last 4 days of culture (group B) inhibited the number of TRAP-positive MNCs stimulated by 1α,25-(OH)₂D₃, PGE₂ or rHIL-1α in a dose-dependent manner (Fig. 1). In contrast, there was no inhibitory effect on the 1α,25-(OH)₂D₃-stimulated TRAP-positive MNCs by adding sodium salicylate at 10⁻⁴ M for the first 4 days of the culture period (group C). Increasing the concentration of sodium salicylate caused inhibition of...
the TRAP-positive MNCs, but the effect was weaker in group C compared with group A or B. Similar results were observed when the PGE$_2$ or rHIL-1$\alpha$ was used to stimulate the formation of TRAP-positive MNCs.

Adding indomethacin to the culture showed effects similar to those for sodium salicylate, and it inhibited the increase in the number of TRAP-positive MNCs stimulated by 1$\alpha$,25-(OH)$_2$D$_3$, PGE$_2$ or rHIL-1$\alpha$. Increasing the concentration of indomethacin inhibited the formation of TRAP-positive MNCs and, as was the case with sodium salicylate, the effect was less in group C than in group A or B (Fig. 2). Indomethacin had a stronger inhibitory effect on the formation of TRAP-positive MNCs than did sodium salicylate.

1$\alpha$,25-(OH)$_2$D$_3$ increased the production of PGE$_2$ released into the culture media (Fig. 3) during days 4–6, but the level of PGE$_2$ was found to be even higher at days 6–8. Sodium salicylate inhibited this increase, and this
effect was also more pronounced at days 6–8. rHIL-1α increased PGE2 production in the mouse bone marrow culture system, while sodium salicylate or indomethacin added during the last 4 days reduced it (Fig. 4). Indomethacin seemed to be more potent in reducing PGE2 production than sodium salicylate.

The effects of sodium salicylate on the process of pit formation on bone slices was examined to determine whether the drugs influence the bone-resorption activity of TRAP-positive MNCs. 1α,25-(OH)2D3 caused an increase in the number of TRAP-positive MNCs and pits, and in the

![Graph showing PGE2 production](image)

Fig. 3. Increase in PGE2 production in response to 1α,25-(OH)2D3 (10^-8 M). PGE2 assayed on days 4–6 (A) and days 6–8 (B) of the mouse bone marrow culture showed an increase in PGE2 production in response to 1α,25-(OH)2D3 (10^-8 M). PGE2 production was inhibited by adding 10^-5 - 10^-4 M of sodium salicylate continuously. Data are expressed as the means±S.D. of six cultures (*P<0.05, **P<0.01 versus 1α,25-(OH)2D3 treated alone).

![Graph showing PGE2 production](image)

Fig. 4. Increase in PGE2 production in response to rHIL-1α (2 ng/ml). PGE2 assayed at the end of the mouse bone marrow culture showed an increase in PGE2 production in response to rHIL-1α (2 ng/ml). PGE2 production was inhibited significantly by 10^-3 - 10^-4 M sodium salicylate ( ) or 10^-8 - 10^-6 M indomethacin ( ) on the last 4 days of culture. Data are expressed as the means±S.D. of six cultures (***P<0.001 versus rHIL-1α treated alone).

![Graph showing PGE2 production](image)

Fig. 5. Effects of sodium salicylate on the number of TRAP-positive MNCs and pits, and the bone-resorption area stimulated by 1α,25-(OH)2D3. Group A ( ) was administered sodium salicylate continuously, group B ( ) was administered sodium salicylate only on the last 4 days of culture, and group C ( ) was administered sodium salicylate only on the first 4 days of culture. The number of TRAP-positive MNCs and pits, and the bone-resorption area were inhibited by administering 10^-3 - 10^-4 M sodium salicylate. TRAP-positive MNCs, pits and the bone-resorption area were recorded after 8 days. Data are expressed as the means±S.D. of eight cultures (*P<0.05, **P<0.01, ***P<0.001 versus 1α,25-(OH)2D3 (10^-8 M) treated alone).
bone-resorption area. Increasing the concentration of the
drug appeared to cause less inhibition of TRAP-positive
MNCs, pits and bone-resorption area in group C than in
groups A or B.

Histological observations showed that most of the
TRAP-positive MNCs were typically located near the
resorption lacunae after being cultured for 8 days with
1α,25-(OH)2D3 (Fig. 6). After the cells were removed with
NH4OH and stripped by ultrasonication, pit formation
was less in the cultures that received sodium salicylate
(0.05 mM) continuously (Fig. 7B) than in those that
received only 1α,25-(OH)2D3 (Fig. 7A).

DISCUSSION

Three bone-resorbing stimulants, 1α,25-(OH)2D3, PGE2
and rHIL-1α were used in this present study to enhance
the recruitment of osteoclast-like cells. While all these
compounds are known to stimulate osteoclast-like cell
formation in a mouse bone marrow culture, their individual
mechanisms of action differ (5, 24). The addition of one
of these compounds to the present culture increased the
formation of TRAP-positive MNCs, pits and the bone-
resorption area. Adding sodium salicylate or indomethacin
inhibited the increased formation of TRAP-positive
MNCs stimulated by these three stimulants. Therefore,
the inhibitory effects on osteoclast-like cell formation
does not appear to be related to the specific stimulant of
osteoclast formation. Both sodium salicylate and indo-

methacin appear to act on a similar pathway that is im-
portant for osteoclast differentiation.

It is known that indomethacin and aspirin are potent
inhibitors of prostaglandins (PGs) biosynthesis and that
this effect is caused by a reduction in the PGs synthetase.
Indomethacin is reported to be more potent than aspirin
in reducing inflammatory responses and in inhibiting the
activity of cyclooxygenase (25). This is consistent with the
present findings that indomethacin is more potent than
sodium salicylate in its inhibitory effects on the formation
of TRAP-positive MNCs and in inhibiting the bone-
resorption activity of TRAP-positive MNCs.

The present finding that sodium salicylate and indo-
methacin strongly inhibited the formation of TRAP-posi-
tive MNCs more during the later stage (days 5–8) of the
culture period than during the earlier stage (days 1–4)
indicates that they have a stronger effect on the later stage
of osteoclast-like cell formation. Since the differentiation
of TRAP-positive precursor cells and TRAP-positive
preosteoclasts is reported to occur between days 4 and 6
of this culture system (5), sodium salicylate and indometh-
acin may either inhibit the differentiation of precursor
cells into TRAP-positive preosteoclasts or the differentia-
tion of TRAP-positive preosteoclasts into TRAP-positive
MNCs. A previous study (17) has shown that sodium
salicylate did not influence the proliferation of TRAP-
positive MNCs in the mouse bone marrow culture. There-
fore, both drugs appear to have a stronger inhibitory
effect on differentiation rather than proliferation of osteo-

Fig. 6. Micrographs of bone slices on which bone marrow cells were cultured. Mouse bone marrow mononuclear cells were
cultured with 1α,25-(OH)2D3 (10−8 M) on bovine bone slices for 8 days. Many dark red TRAP-positive MNCs (arrowheads) are
seen near the resorption lacunae (arrows).
Fig. 7. Micrographs of bone slices on which bone marrow cells were cultured. Mouse bone marrow mononuclear cells were cultured with \(1\alpha,25-(OH)_{2}D_{3}\) (10\(^{-8}\) M) (A and B) on bovine bone slices for 8 days. After the cells were removed by \(NH_{4}OH\), bone slices were stained with toluidine blue. A, numerous resorption lacunae (arrows) are seen after culture in \(1\alpha,25-(OH)_{2}D_{3}\) (10\(^{-8}\) M) alone. B, after simultaneous treatment with \(1\alpha,25-(OH)_{2}D_{3}\) (10\(^{-8}\) M) and sodium salicylate (0.05 mM), the resorption lacunae are shallower and are smaller in size and number compared with A.

Using the same culture system, Takahashi et al. (7) observed that calcitonin acts directly on the mononuclear precursors to inhibit their fusion, since there are more TRAP-positive mononuclear cells than TRAP-positive MNCs, under the influence of calcitonin. However, Soekanto et al. (17), found that the inhibitory action of sodium salicylate differs from that of calcitonin. Sodium salicylate may not directly inhibit the precursor fusion, since the TRAP-positive mononuclear cells and MNCs showed similar inhibition under the influence of this drug.

The finding that the amount of PGE\(_{2}\) stimulated by \(1\alpha,25-(OH)_{2}D_{3}\) production was higher on days 6–8 than on days 4–6 of the culture indicates that PGE\(_{2}\) production is higher when the precursor cells are committed to differentiation (24, 26). The mouse bone marrow culture consists of a mixed population of cells, including osteo-
Osteoclast precursors and other bone marrow cells. Among these cells, bone-derived stromal cells are known to have the capacity to support osteoclast differentiation. The addition of 1α,25-(OH)2D3 or rHIL-1α to a co-culture system with a bone marrow-derived-stromal cell line (ST2) and splenic cells (26, 27) may induce the development of osteoclasts by producing cytokines and other substrate(s) that regulate hematopoiesis (28, 29) and may increase PGE2 production (26, 30). The interaction with stromal cells and osteoclast progenitors is believed to be important in generating mature osteoclasts. PGE2 appears to be important in stimulating osteoclast formation through stromal cells. The finding that PGE2 production is stimulated by 1α,25-(OH)2D3 or rHIL-1α may explain, in part, the known bone-resorbing activity of these agents.

The present finding that PGE2 production on days 6–8 was reduced by sodium salicylate added continuously during the culture period and the observation that the production of PGE2 stimulated by rHIL-1α was inhibited by both drugs during the last 4 days of culture is consistent with the finding that the drugs had their strongest effect on the later stage of the culture period. Sodium salicylate or indomethacin may affect stromal cells by inhibiting the PGE2 induction stimulated by the bone-resorbing substances. These drugs may then inhibit the production of factors that induce the sequential differentiation process of osteoclast progenitors to mature osteoclast-like cells.

Although the present results show that PGE2 added exogenously fails to overcome the effect of sodium salicylate or indomethacin on the formation of osteoclast-like cells, Akatsu et al. (24) reported that indomethacin did not inhibit the formation of TRAP-positive MNCs induced by PGE2. However, since their results indicated a gradual decrease in the number of cells with increases in indomethacin, it is possible that this drug inhibits the effect of exogenous PGE2 on osteoclast-like cell formation. The present findings indicate that mechanisms other than PGE2 are involved in the inhibition of osteoclast-like cell formation caused by these drugs.

Several studies have shown that the salicylates and other NSAIDs also exert their effects through mechanisms not involving the inhibition of PGE2 synthetase (31–34). Salicylate has been shown to act directly as a scavenger of hydroxyl radicals (35), and bone resorption may be regulated by hydroxyl radicals by promoting the differentiation of osteoclast precursor cells to osteoclasts (36). In the in vitro culture system, indomethacin is a potent inhibitor of phosphodiesterase (37) and thus increases the intracellular concentration of cAMP. cAMP has been shown to suppress the formation of PG and to stabilize lysosomal membranes in polymorphonuclear leukocytes and macrophages (38). Since osteoclasts have a well-developed lyso-

somal system and secrete lysosomal enzymes to degrade the organic matrix of bone, any stabilizing action of drugs on the lysosomal system might reduce the bone-resorption activity of TRAP-positive MNCs generated in the mouse bone marrow culture.

Sodium salicylate is known to inhibit dentin formation in the rat incisor (39). This effect is thought to be related to its inhibition of the metabolic pathway of oxidation in odontoblast, since sodium salicylate and indomethacin are known to uncouple oxidative phosphorylation in mitochondria (24). Osteoclasts are known to be rich in mitochondria, and high energy production is required to dissolve the mineral and to degrade the organic matrix of bone during resorption activity. Therefore, the drugs might reduce oxidative phosphorylation and affect cell function or resorption activity. Moreover, electron-microscopy has revealed that the morphology of the osteoclasts changed markedly following exposure to sodium salicylate, indicating that their cellular function is somehow damaged by the drug (16). Thus, PGE2 is not the only factor targeted by these drugs.

The present studies showed an inhibitory effect of sodium salicylate and indomethacin on bone resorption and osteoclast-like cell differentiation in vitro. This inhibitory effect was more pronounced when the drugs were given during the later stage of the culture period (days 4–8). While these inhibitory effects are due, in part, to decreases in PGE2 synthesis, other mechanisms may contribute to the inhibition of bone resorption by NSAIDs.

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