SC-19220, a prostaglandin E sub antagonist, inhibits osteoclast formation by 1,25-dihydroxyvitamin D in cell cultures

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Abstract

1,25 Dihydroxy vitamin D3 (1,25(OH)2D3), prostaglandin (PG) E2 and parathyroid hormone (PTH) induce osteoclast formation in cell cultures. Previously, we have shown that SC-19220, an antagonist of the EP1 subtype of PGE receptors, inhibited tartrate-resistant acid phosphatase (TRAP)-positive cell formation by PGE2 and PTH in adherent cell cultures taken from neonatal rats. Since 1,25(OH)2D3 has been shown to induce osteoclast formation through PGE2 synthesis, in this study we have examined the effect of SC-19220 on osteoclast formation induced by 1,25(OH)2D3 in cell cultures by measuring bone resorption as well as TRAP-positive cell formation. SC-19220 inhibited osteoclast formation by 1,25(OH)2D3 as well as by PGE2 in cell cultures. The addition of SC-19220 to the later half but not to the earlier half of the culture inhibited 1,25(OH)2D3-induced formation. In the culture in which hydroxyurea was added in the later half period, SC-19220 inhibited osteoclast formation by 1,25(OH)2D3. Under these conditions, 17-phenyl PGE1, an EP1 agonist, induced osteoclast formation. Thus, SC-19220 inhibits certain reactions in the later processes of osteoclast formation induced by 1,25(OH)2D3. In addition, SC-19220 also inhibited osteoclast formation induced by interleukin (IL)-11 and IL-6 as well as by PTH. It is suggested that the SC-19220 inhibiting reactions are shared by all the inducers including 1,25(OH)2D3 and are essential for osteoclast formation.

Introduction

1,25-Dihydroxyvitamin D3 (1,25(OH)2D3), prostaglandin (PG) E2 and parathyroid hormone (PTH) induce osteoclast formation in cell cultures (Takahashi et al. 1988, Lowik et al. 1989, Nijweide & Grooth 1992). In the case of 1,25(OH)2D3-induced osteoclast formation, PGE2 synthesis has been shown to be involved (Collins & Chambers 1992). In the case of PTH-induced osteoclast-like cell formation, we have previously suggested that PGE2 synthesis is also involved (Inoue et al. 1995). Thus, PGE2-related reactions have been shown or suggested to be involved in osteoclast formation by 1,25(OH)2D3 and PTH; however, the precise mechanism has not been clarified.

PGE receptors are classified into four subtypes: EP1, EP2, EP3, and EP4 (Coleman et al. 1994). In osteoclast formation, both hemopoietic osteoclast progenitors and osteoblastic stoma cells are involved. The EP1 PG receptor is found in osteoclasts and osteoblasts (Harada et al. 1994). SC-19220 is an antagonist of the EP1 receptor. We showed that SC-19220 inhibited osteoclast-like cell formation induced by both PTH and PGE2 (Inoue et al. 1995). This suggests that SC-19220 inhibiting reactions, probably EP1 related ones, are important for osteoclast formation induced by PTH, PGE2 and possibly by 1,25(OH)2D3.

The purpose of this study was to show the action of SC-19220 on the process of osteoclast formation by 1,25(OH)2D3 in cell cultures.

Materials and Methods

Cytokines and chemicals

1,25(OH)2D3 was purchased from Wako Pure Chemicals (Osaka, Japan). PGE2 and indomethacin were obtained from Sigma (St Louis, MO, USA). Parathyroid hormone, fragment 1–34 (Sigma) was used as PTH. SC-19220 was a gift from Searle & Co (Skokie, IL, USA) and was diluted to 15 mM in ethanol and stored at -20 °C. Murine recombinant interleukin (IL)-6 and human recombinant interleukin-11 were purchased from Boehringer (Mannheim, Germany). Murine recombinant granulocyte/macrophage-colony stimulating factor (GM-CSF) and anti
GM-CSF were obtained from Pepro Tech Inc. (Rocky Hill, NJ, USA).

Cultures for osteoclast formation

Two kinds of cell culture systems, bone marrow cell cultures and co-cultures, were used in the osteoclast formation. Bone marrow cells were prepared from ICR mice as described by Collins & Chambers (1992). In brief, the femora and tibiae were aseptically removed and dissected free of adherent tissue. The bone ends were cut, and the marrow cavity was flushed out with HEPES-buffered medium 199 by slow injection at one end of the bone using a sterile 21-gauge needle. The bone marrow cells were washed twice and resuspended (2 to 3 × 10^6 cells/ml) in Eagle’s minimum essential medium (MEM; Gibco BRL, Great Island, NY, USA) supplemented with 10% fetal calf serum (FCS), 1 µM hydrocortisone, and antibiotics. This suspension was placed in wells of 96-well plates, each of which contained a sterile bovine cortical bone slice. To the cultures was added 10 nM 1,25(OH)_2D_3 or 1 µM PGE_2 and the cells were incubated for 8 days. SC-19220 was added at the beginning of the culture. The cultures were fed every 3 to 4 days.

Co-cultures between osteoblastic cells and bone marrow cells were prepared as described by Girasole et al. (1994), with some modifications. Osteoblastic cells were prepared from 2- to 4-day-old mouse calvaria by collagenase digestion. The cells were cultured with α-modified MEM containing antibiotics and 10% FCS in 25-cm^2 flasks for 3–4 days in subconfluent conditions. Bone marrow cells were prepared as above. Osteoblastic cells (2.5 × 10^4/ml) and bone marrow cells (5 × 10^5/ml) were placed in 48-well culture plates and co-cultured in the presence of 0.1 µM dexamethasone, which induces IL-6 receptors on osteoblastic cells (Udagawa et al. 1995). To the medium was then added either 10 ng/ml IL-11 or 20 ng/ml IL-6. In the case of PTH, 0.25 nM hormone was added to the co-culture without dexamethasone. SC-19220 was added at the beginning of the culture. Dexamethasone did not seem to affect the inhibitory effect of SC-19220 on IL-11-induced osteoclast formation. All of the culture media were replaced every 2–4 days. In the study of post-mitotic reactions, the cells were co-cultured without any inducers. On the 4th day, 10 nM 1,25(OH)_2D_3 and 1 µM 17-phenyl-trinor PGE_2, with and without 75 µM SC-19220, were added to the cultures in the presence of 1 mM hydroxyurea, an inhibitor of mitotic reactions. In this co-culture system, mitotic processes occurred in the first 4 days of the co-culture, while post-mitotic processes occurred over the last 2 days (Tanaka et al. 1993).

Detection of osteoclast formation

Osteoclast formation in the cultures was judged from both tartrate-resistant acid phosphatase (TRAP)-positive cell formation and bone resorption. For TRAP staining, the adhered cells were washed twice with 10 mM phosphate-buffered saline (pH 7.2) at the end of the incubation. The cells were fixed with 3% formaldehyde, washed, incubated with ethanol, and then incubated with 20 nM sodium acetate buffer (pH 4.0) containing 20 nM sodium tartrate, naphthol AS MX phosphate, and fast red violet LB salt for 20 min at ambient temperature. TRAP-positive cells having multinuclear cells (over 3) in a well were scored. Cortical bone slices, 3-mm square and 0.1-mm thick, were prepared and used as the substrate for bone resorption, as previously described (Collins & Chambers 1992). At the end of the culture, the slices were incubated with sodium hypochlorite to remove the adherent cells, washed, dehydrated, and spatter-coated with gold. The surfaces of the slices were monitored with a scanning electron microscope at 100 × magnification. The percentage of resorbed area to the surface of the bone slice was calculated.

Disaggregated osteoclasts

Disaggregated osteoclasts were prepared from six-day-old Wistar rats as previously described (McSheehy & Chambers 1986). The femora and tibiae were taken from the rats, cleaned of the surrounding soft tissue, and cut across the epiphysis. The shafts were split longitudinally with a razor blade and dipped in MEM, 250 µl per bone, in the absence of fetal bovine serum (FBS). The cells, disaggregated from the bone by scraping the endosteal side of the bone shafts, were suspended by pipetting in the medium. The disaggregated cells were placed on bone slices and incubated at 37 °C for 15 min under a 5% CO_2 atmosphere. The bone slices were washed briefly to remove less adherent cells. The cells on bone slices rich in osteoclasts were incubated in 100 µl 10% FBS in MEM with 50 U penicillin and 50 µg streptomycin for one day in both the presence and absence of 75 µM SC-19220.

Granulocyte and macrophage formation

The effect of SC-19220 on granulocyte/macrophage formation was examined by the monitoring of colony formation induced by GM-CSF. In colony assay, non-adherent bone marrow cells were prepared and suspended in MEM (1 × 10^7 cells/ml) containing 10% FCS and 2.4% methylcellulose, as previously described (Hattersley et al. 1991). The medium was supplemented with 1, 5 or 10 ng/ml murine recombinant GM-CSF, 1:200 anti GM-CSF, 30, 75, 150 µM SC-19220, and 0.1 µM dexamethasone, which blocks endogenous GM-CSF formation (Shuto et al. 1994). The cells were placed in 6-well culture plates and incubated for 7 days. Colonies were examined and phenotyped by colony morphology using an inverted microscope. Granulocyte/macrophage colonies were identified as a central granulocytic type of aggregate
with a peripheral rim of less cohesion and with larger cells. The number of granulocyte/macrophage typed colonies (>40 cells) and clusters (>10 cells) in each well were scored.

The effect on macrophage formation was confirmed by examination of non-specific esterase-positive cell formation by GM-CSF in the bone marrow cell cultures. For non-specific esterase staining, unfractionated bone marrow cells were cultured for 7 days in the medium as a colony assay without the addition of methylcellulose. After being cultured, the washed cells were fixed with ice-cold acetone containing formaldehyde, and incubated with 66 mM potassium phosphate buffer (pH 6.4) containing α-naphthyl butyrate and fast garnet GBC sulfate salt (Sigma) for 30 min at 37 °C. Cells showing positive for non-specific esterase were stained dark brown.

Statistics

All results were obtained from two to four separate experiments, each of which used three to four cultures. The data were examined using analysis of variance followed by Fisher’s protected least significant difference test. *P*<0.05 was considered significant.

Results

SC-19220 inhibited osteoclast formation induced by 1,25(OH)$_2$D$_3$ or PGE$_2$ in bone marrow cell cultures, judging from both the decrease in TRAP-positive cell formation and bone resorption. In the range of 3–150 µM SC-19220, the reagent inhibited the formation by 1,25(OH)$_2$D$_3$ or PGE$_2$ in a dose-dependent manner (Fig. 1). Approximately 75 µM SC-19220 was enough for complete inhibition of formation in both cases. We confirmed that 75 µM SC-19220 did not affect the number of pre-existing disaggregated osteoclasts from neonatal rats; however, the reagent inhibited bone resorption by the cells (Table 1).

The reactions inhibited by SC-19220 were studied for the osteoclast formation induced by 1,25(OH)$_2$D$_3$. To examine the period in which SC-19220 inhibited 1,25(OH)$_2$D$_3$-induced osteoclast formation, SC-19220 was added to bone marrow cultures for the first or second half periods. The addition of SC-19220 for the second half but not for the first half inhibited 1,25(OH)$_2$D$_3$-induced osteoclast formation (Fig. 2). Thus, the reactions inhibited by SC-19220 are involved in the second half period of formation. Since 1,25(OH)$_2$D$_3$ and PGE$_2$ induce osteoclast formation by acting on the post-mitotic processes and are not necessary during the mitotic processes (Takahashi et al. 1994), the effects of SC-19220 on osteoclast formation in the presence of hydroxyurea, which suppresses the mitotic processes in the co-culture system (Tanaka et al. 1993), were examined. Figure 3 shows that SC-19220 inhibited the osteoclast formation induced by 1,25(OH)$_2$D$_3$ in co-culture, while 17-phenyltrinor PGE$_2$, an agonist of EP1 (Coleman et al. 1994),
induced osteoclast formation in the presence of hydroxyurea and absence of 1,25(OH)\textsubscript{2}D\textsubscript{3}.

Macrophages are the closest cells to osteoclasts in cell lineage. The effects on macrophage formation were examined in two types of experiments: colony formation and nonspecific esterase staining. SC-19220 did not inhibit the colony formation induced by GM-CSF, but slightly increased it (Fig. 4). This reagent appeared to enhance the formation of nonspecific esterase-positive cells induced by GM-CSF in bone marrow cultures (data not shown).

IL-11 (Girasole et al. 1994) and IL-6 (Ishimi et al. 1990, Tamura et al. 1993) induced osteoclast formation in co-cultures of osteoblastic cells and bone marrow cells. SC-19220 (75 µM) also inhibited osteoclast formation induced by IL-11, IL-6 and PTH (Fig. 5).

**Discussion**

In this study, we have shown that SC-19220 inhibited osteoclast formation by 1,25(OH)\textsubscript{2}D\textsubscript{3} in cell culture. SC-19220 added in the second half period of the culture inhibited the osteoclast formation induced by 1,25(OH)\textsubscript{2}D\textsubscript{3}, even in the presence of hydroxyurea. Therefore, the step inhibited by SC-19220 appeared to be one of the post-mitotic steps in osteoclast formation. The possibility that SC-19220 enhanced the degradation of osteoclasts can be excluded, because SC-19220 did not decrease the number of disaggregated osteoclasts (Inoue et al. 1995) and we confirmed this in the present study. SC-19220 inhibited the bone resorption by disaggregated osteoclasts as shown in Table 1. Therefore, the inhibitory effect of SC-19220 on bone resorption in cultures for osteoclast formation accounted for both the direct inhibition and the inhibition through reduced osteoclast formation.

**Table 1**

<table>
<thead>
<tr>
<th>Addition</th>
<th>Mean osteoclast number/bone slice</th>
<th>Mean resorbed area/bone slice (µm\textsuperscript{2}×10\textsuperscript{3})</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>19.9 ± 1.7</td>
<td>13.4 ± 2.9</td>
</tr>
<tr>
<td>SC-19220</td>
<td>21.8 ± 2.6</td>
<td>8.2 ± 2.3*</td>
</tr>
</tbody>
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*P<0.005 (vs None).

**Figure 2**

Effects of SC-19220 on the initial and second half processes of osteoclast formation induced by 1,25(OH)\textsubscript{2}D\textsubscript{3} in mouse bone marrow cell cultures. Bone marrow cells were cultured with 1,25(OH)\textsubscript{2}D\textsubscript{3} in the presence (D3+SC) or absence (D3) of 75 µM SC-19220. On the 4th day, the wells containing the cultured cells were washed 3 times by replacing all of the medium and cultured with 1,25(OH)\textsubscript{2}D\textsubscript{3} in the presence (D3+SC) or absence (D3) of SC-19220. 1,25(OH)\textsubscript{2}D\textsubscript{3} and SC-19220 were not added to the control (cont.). After incubation, the number of TRAP-positive cells (solid bars) and the extent of bone resorption (%) (open bars) were measured. Results were obtained from twelve wells or bone slices in three separate experiments. Bars show s.e.m. *P<0.001 vs D3 and D3 (n=12).

**Figure 3**

Effects of SC-19220 on the post-mitotic processes of osteoclast formation induced by 1,25(OH)\textsubscript{2}D\textsubscript{3} in co-culture of osteoblastic cells and bone marrow cells. On the 4th day, 1,25(OH)\textsubscript{2}D\textsubscript{3} (D3) with or without 75 µM SC-19220 (SC), or with 1 µM 17-phenyl-trinor PGE\textsubscript{2} (17P) alone were added to co-cultures in the presence of 1 mM hydroxyurea and cultured for a further 4 days. 1,25(OH)\textsubscript{2}D\textsubscript{3} and 17-phenyl-trinor PGE\textsubscript{2} were not added to the control cultures (cont.). The number of TRAP-positive cells (solid bars) and the extent of resorbed area (%) (open bars) were measured after the 4-day culture period. Results were obtained from eight wells or bone slices in two separate experiments. Bars show s.e.m. *P<0.005, **P<0.0005 vs D3 (n=8), ***P<0.05, ****P<0.005 vs cont. (n=8).
SC-19220 has been shown to inhibit PTH- or PGE$_2$-induced osteoclast-like cell formation as described previously (Inoue et al. 1995). In this study, we confirmed this previous observation by means of conventional culture systems. We also showed that IL-11-induced osteoclast formation was inhibited by SC-19220. These results are in accordance with the report that indomethacin inhibits osteoclast formation by IL-11 (Girasole et al. 1994). The present results in which SC-19220 inhibited IL-6-induced as well as IL-11-induced osteoclast formation were made possible because of the fact that the receptors for both IL-6 and IL-11 share the same membranous protein, gp130, which is thought to be involved in osteoclast formation (Tamura et al. 1993). Since SC-19220 inhibited osteoclast formation by IL-6 and IL-11 as well as by PTH, some of the PGE$_2$-related reactions, sequential reactions evoked by PGE$_2$ binding to the receptors, are most likely involved in the mechanism of osteoclast formation by these inducers.

The inhibitory effect of SC-19220 seems to be selective for osteoclasts in myeloid cell formation. It has been reported that the progenitors of osteoclasts are multipotential cells. SC-19220 did not inhibit colony formation induced by GM-CSF (Fig. 5), nor the formation of nonspecific esterase-positive cells, which are macrophages in mice (Takahashi et al. 1994) induced by GM-CSF in bone marrow cultures. Osteoclasts and macrophages share progenitors in the early stages of their formation pathway. The observation that SC-19220 did not inhibit macrophage formation is in accordance with its lack of inhibitory effects in the early stages of osteoclast formation, as shown in Fig. 2. We also confirmed that SC-19220 did not inhibit the osteoclast differentiation of calvarial cells (T Tsujisawa, H Inoue, T Fukuzumi & C Uchiyama, unpublished observations).

Since SC-19220 is classified as an antagonist for EP1, the inhibition of 1,25(OH)$_2$D$_3$-induced formation by SC-19220 could be caused by the blockade of some of the EP1-mediating reactions in the later processes of osteoclast formation. The involvement of EP1-mediating reactions in the later processes was further supported by the observation that 17-phenyl-trinor PGE$_2$, an agonist of EP1 (Coleman et al. 1994), induced the later processes (Fig. 3). Interestingly, when 17-phenyl-trinor PGE$_2$ was added from the beginning of the culture period, no osteoclasts formed (data not shown). Since 17-phenyl-trinor PGE$_2$ added in the second half period of the culture induced osteoclast formation, this agent may inhibit the earlier processes. As SC-19220 inhibited osteoclast formation...
induced by all the inducers examined, it is suggested that some of the EP1-mediated reactions, which are blocked by SC-19220, are critical in osteoclast formation in the later processes.

An increase in intracellular calcium ion levels is shown to be involved in the cascade of EP1 stimuli in the rabbit cortical collecting duct (Hebert et al. 1991), and in UMR–106–01 osteoblastic cloned cells (Muallem et al. 1989). SC-19220 antagonized calcium-induced contractions of the potassium depolarized aorta in rabbits and rats (H Inoue, unpublished observation). The reaction(s) inhibited by SC-19220 may cause an increase in intracellular calcium ion levels. Since an extracellular calcium ion is shown to induce osteoclast formation (Kaji et al. 1996, Takami et al. 1997), the involvement of calcium related reactions in the SC-19220 inhibiting process should prove an interesting problem for future study.

References


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