Induction of apoptosis of monocyte-macrophage lineage cells by 5-S-GAD

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Abstract We found that 5-S-GAD, an insect-derived antibacterial peptide, inhibited murine osteoclast formation in vitro. We examined the specific time point of the inhibitory action of 5-S-GAD on osteoclast formation and found that it mainly suppressed differentiation of osteoclasts in the middle of the culture period. Using HL60 cells that are able to differentiate into multinucleated macrophage-like cells, we found that 5-S-GAD induced apoptosis of HL60 cells by producing H_2O_2. Thus, the inhibition of osteoclast formation by 5-S-GAD could be, in part, due to apoptosis of the cells of an osteoclast lineage.

Key words: Osteoclast; Hydrogen peroxide; Apoptosis; HL60 cell

1. Introduction

Insects are known to produce various defense molecules, such as antibacterial proteins, antifungal proteins and lectins, in response to body injury or microbial infection [1–4]. Previously we isolated a novel defense substance from immunized adult *Sarcophaga peregrina* (flesh fly), and identified it as *N*-β-alanyl-5-*S*-glutathionyl-3,4-dihydroxyphenylalanine (5-S-GAD) [5]. 5-S-GAD was originally found as an antibacterial substance. As its antibacterial activity was inhibited by catalase, which decomposes H_2O_2, the antibacterial activity of 5-S-GAD was suggested to be due to H_2O_2 derived from its catechol moiety.

On the other hand, we demonstrated that 5-S-GAD is a potent inhibitor of protein tyrosine kinases (PTKs) using a lysate of *v-src*-transformed NIH3T3 (mouse fibroblast) cells [6] and recombinant c-Src [7,8] in vitro. It is known that PTKs play crucial roles in the regulation of cell proliferation and differentiation, so 5-S-GAD was thought to be a useful tool for analyzing various biological events that involve protein tyrosine phosphorylation. Based on the fact that the targeted disruption of *c-src* resulted in osteopetrosis in mice [9], we examined the effect of 5-S-GAD on osteoclast formation in vitro using a co-culture system of osteoblasts and primary bone marrow cells. We found that 5-S-GAD markedly inhibited osteoclast formation in this system.

2. Materials and methods

2.1. Osteoclast formation in vitro

Osteoblast cultures were prepared from calvariae of neonatal ddy mice by sequential collagenase digestion as described by Takahashi et al. [10], and maintained in minimum essential medium, α-modification (α-MEM), containing 10% fetal calf serum (FCS, Gibco BRL). Primary bone marrow cells were obtained by flushing the medullary cavity of tibia with α-MEM, and then filtered through nylon cell strainers (100 μm pore, Falcon). The dispersed cells were overlaid at a density of 2.5×10^5 cells/cm^2 on 1×10^4 osteoblasts on 24-well plates. These cultures were maintained in α-MEM containing 10% FCS in the presence of 10^{-8} M 1α,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) for 8 days at 37°C, the medium being changed once or twice. Osteoclasts were identified by staining with tartrate-resistant acid phosphatase (TRAcP), and classified according to the number of nuclei [11]. When necessary, 350 μg/ml (610 μM) of 5-S-GAD was added.

2.2. Culture and morphological observation of HL60 cells

The human promyelocytic cell line, HL60, was obtained from the Human Science Research Resources Bank (JCRB0085). The cells (1×10^5 cells/ml) were cultured in RPMI 1640 medium (Gibco BRL) supplemented with 10% FCS at 37°C in 5% CO_2. The cells were treated for 6 h at 37°C in 5% CO_2 with 1 μg/ml of actinomycin D (Sigma), 50 μg/ml (1050 U/ml) of catalase (Sigma), and/or 57.3 μg/ml (100 μM) of 5-S-GAD, respectively, when necessary. Apoptotic cells were identified on the basis of the formation of apoptotic bodies.

2.3. DNA fragmentation assay

This was performed essentially as described by Hirt [12]. HL60 cells were washed and resuspended in lysis buffer (50 mM Tris-HCl, 10 mM EDTA and 0.5% sodium-*N*-lauroylsarcosinate) containing 50 μg/ml of RNase A and then incubated at 50°C for 30 min. Then 50 μg/ml of proteinase K was added to the cell lysate, followed by incubation at 50°C for 60 min. DNA was extracted from the cultured cells successively with phenol, phenol:chloroform:isoamyl alcohol (25:24:1), and chloroform:isoamyl alcohol (24:1), and then precipitated with 50% 2-propanol at room temperature for 15 min. After centrifugation, the precipitate was rinsed with 70% ethanol, dissolved in 15 μl of water, and then electrophoresed on a 2% agarose gel. DNA fragments were visualized by ethidium bromide staining.

2.4. Measurement of intracellular H_2O_2 in HL60 cells by FACS analysis

HL60 cells were collected, washed and suspended in phosphate-

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Abbreviations: DCFH-DA, 2′,7′-dichlorofluorescein diacetate; EDTA, ethylene diamine tetraacetic acid; FACS, fluorescence-activated cell sorter; FCS, fetal calf serum; α-MEM, minimum essential medium, alpha modification; 1,25$(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3; PBS, phosphate-buffered saline; PMA, phorbol myristate acetate; 5-S-GAD, *N*-β-alanyl-5-*S*-glutathionyl-3,4-dihydroxyphenylalanine; TRAcP, tartrate-resistant acid phosphatase; TRAcP(+)MNC, TRAcP-positive multinucleated cells

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buffered saline (PBS) at a density of 1×10^6 cells/ml. The cells were treated with 5 μM 2′,7′-dichlorofluorescein diacetate (DCFH-DA; Eastman-Kodak Co., Rochester, NY, USA) at 37°C for 15 min. Then the cells were washed and resuspended in RPMI 1640 medium. To 100 μl of the cell suspension was added 100 μl of the sample solution, and then the incubation was continued for 10 min. The intercellular production of H_2O_2 was detected as the fluorescence emission at 530 nm using a FACScan flow cytometer, and the data were analyzed using Coulter ELITE software.

3. Results

3.1. Inhibition of osteoclast formation by 5-S-GAD in vitro

We investigated the effect of 5-S-GAD on osteoclast formation using a co-culture system of mouse osteoblasts and primary bone marrow cells derived from calvariae in the presence of 1,25-$(OH)_2D_3$. Under our culture conditions, the appearance of osteoclasts, assessed as the number of TRAcP-positive multinucleated cells [TRAcP(+)MNC], reached a plateau on day 8. The experimental design and results are summarized in Fig. 1. In each experiment, the medium was changed on the days indicated by the arrows. The solid arrow shows the time of addition of the medium with 5-S-GAD and the open arrow that without 5-S-GAD. We performed the same experiment in the absence of 5-S-GAD as a control and the percent inhibition of osteoclast formation was calculated relative to the control level. 5-S-GAD clearly inhibited osteoclast formation. However, there was a tendency that osteoclast formation was inhibited more when the cells were treated with 5-S-GAD for the last 4 or 5 days compared with those treated for the full 8 days (cf. lanes 1 and 3, 4 and 6, and 8 and 9), whereas the inhibitory effects of 5-S-GAD was much less when the cells were treated for the last 2 days (lane 10). The inhibitory effect of 5-S-GAD was also weaker when the cells were treated for the first 4–5 days (lanes 2 and 5). From these results, we concluded that the cells at days 3–6 are more susceptible to 5-S-GAD than those at other stages.

3.2. Induction of apoptosis by 5-S-GAD

As a primary culture of osteoclasts was not easy to handle, we further studied the effect of 5-S-GAD using HL60 cells, a human promyelocytic cell line, instead of osteoclasts, since osteoclast precursor cells are derived from similar progenitor cells to those that give rise to the cells of a monocyte-macrophage lineage. When HL60 cells were treated with phorbol myristate acetate (PMA) and 1,25-$(OH)_2D_3$, they differentiated into monocyte/macrophage-like cells. When 57.3 μg/ml of 5-S-GAD was present, however, the cells tended to die of apoptosis within 3–6 h irrespective of PMA and 1,25-$(OH)_2D_3$, since numerous apoptotic bodies were detected. As shown in Fig. 2A, apoptotic bodies were detected in nearly 60% of the 5-S-GAD-treated cells, and their formation was inhibited by the addition of 50 μg/ml of native catalase, but not the heat-inactivated form. When DNA derived from these cells was electrophoresed, DNA fragmentation was detected, coinciding with apoptotic body formation, as shown in Fig. 2B. Thus, the cell death evoked by 5-S-GAD was likely to be due to apoptosis caused by H_2O_2, and this is expected to happen during in vitro osteoclast formation. Therefore, it was difficult to conclude at this stage that inhibition of osteoclast formation in vitro is due to inhibition of c-Src by 5-S-GAD.

3.3. Measurement of intracellular H_2O_2 in HL60 cells treated with 5-S-GAD

To confirm the production of H_2O_2, we measured H_2O_2 in HL60 cells treated with 5-S-GAD. For this, HL60 cells were first treated with DCFH-DA and then with 5-S-GAD. The cells containing intracellular H_2O_2 were examined with a FACScan flow cytometer. As shown in Fig. 3, cells containing intracellular H_2O_2 increased with an increase in the concentration of externally added 5-S-GAD (A), but when catalase was present, H_2O_2-positive cells did not increase appreciably (B). Similarly, H_2O_2-positive cells increased when H_2O_2 was directly added to the medium (C). These results suggested

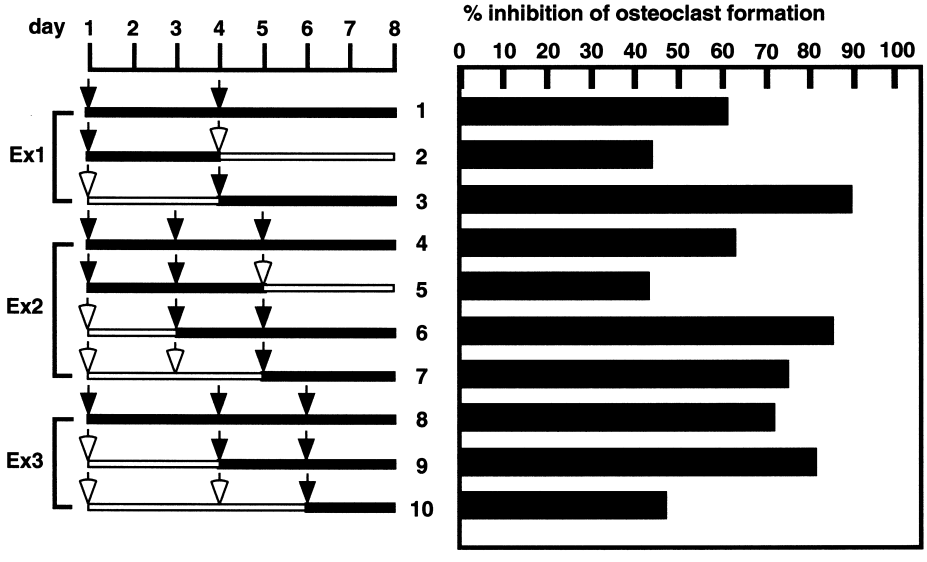

Fig. 1. The inhibition of osteoclast formation by 5-S-GAD. Three independent experiments were performed with different culture schedules. The cells were treated with 350 μg/ml (610 μM) of 5-S-GAD. The culture medium was changed on the days indicated by the arrows. Solid and open arrows show the medium with and without 5-S-GAD, respectively. The TRAcP(+)MNC were counted on day 8 and then the inhibition of osteoclast differentiation relative to the control (without 5-S-GAD) was calculated.

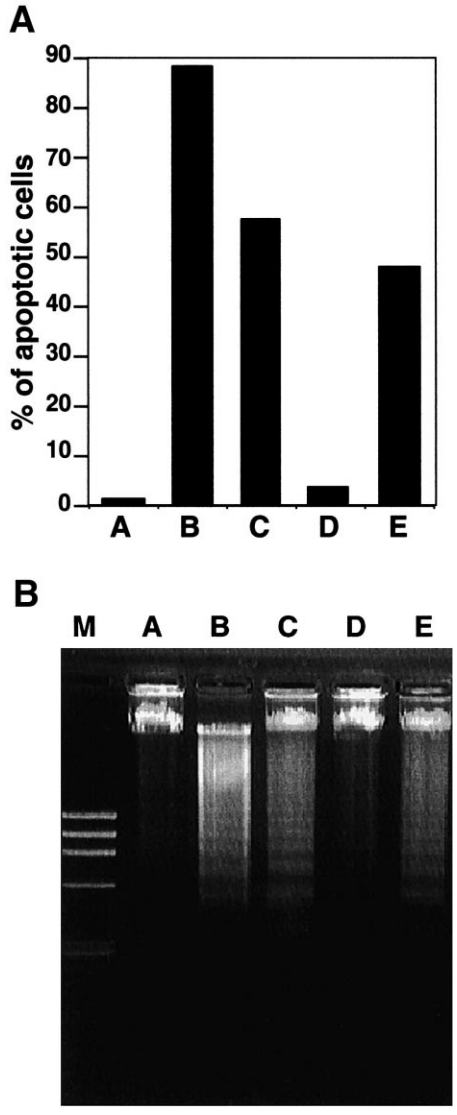

Fig. 2. Induction of apoptosis of HL60 cells by 5-S-GAD. A: HL60 cells were treated with 5-S-GAD under various conditions. The apoptotic bodies formed were counted and the number of apoptotic cells relative to normal cells in the control culture (without any addition) was calculated. B: DNA was extracted from the cultured cells and the electrophoretic profile was examined. Cells cultured: A, control (without any addition); B, with 1 μg/ml actinomycin D (positive control as to apoptosis); C, with 57.3 μg/ml (100 μM) of 5-G-GAD; D, with 5-S-GAD and 50 μg/ml of catalase; E, with 5-S-GAD and heat-inactivated catalase. M indicates molecular mass markers (φX174/*Hae*III).
←

that, when 5-S-GAD was added, H_2O_2 was produced extracellularly and penetrated into the cells, causing their apoptosis. It is noteworthy that the production of H_2O_2 was suppressed when the cells were treated with 5-S-GAD on ice or in the serum-free medium (D and E). Possibly, H_2O_2 is not formed or H_2O_2 does not penetrate into the cells under these conditions.

4. Discussion

We demonstrated that 5-S-GAD inhibited murine osteoclast formation in vitro. We found that 5-S-GAD induced the apoptosis of HL60 cells. It is known that H_2O_2 induces the apoptosis of HL60 cells [13]. Moreover, H_2O_2 derived from dopamine-related compounds is also known to induce the apoptosis of catecholaminergic cells [14]. Thus, our present results obtained using 5-S-GAD coincided with these results. It is clear that the inhibition of PTKs by 5-S-GAD, if any, does not directly participate in the apoptosis of HL60 cells induced by 5-S-GAD, because catalase almost completely prevented the inhibitory effect of 5-S-GAD. HL60 cells can differentiate into multinucleated macrophage-like cells. Thus, in a sense, HL60 cells are similar to osteoclast precursor cells. However, whether or not 5-S-GAD induces apoptosis of murine osteoclast precursor cells by the same mechanism as that of HL60 cells remains to be elucidated. It is also possible that

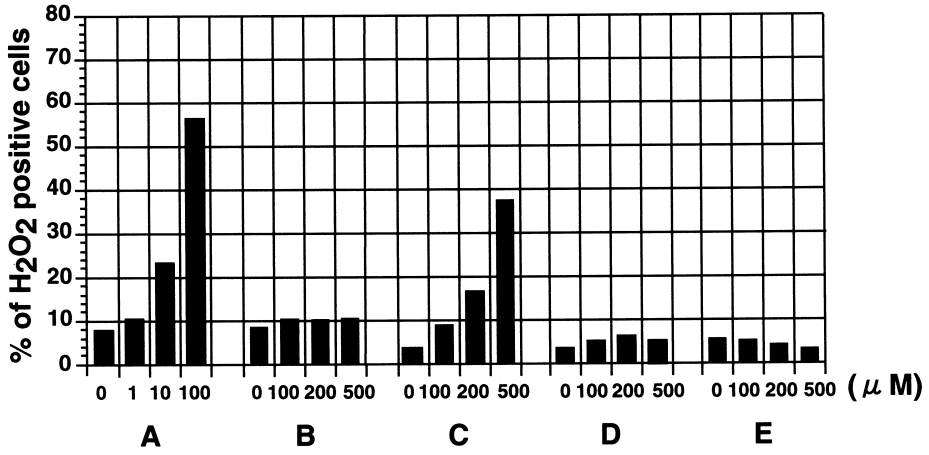

Fig. 3. Detection of intracellular H_2O_2 in HL60 cells treated with 5-S-GAD. HL60 cells were treated with increasing amounts of 5-S-GAD for 10 min and then the proportion of intracellular H_2O_2-positive cells was determined by FACS analysis. The cells were pre-treated with DCFH-DA prior to the treatment with 5-S-GAD to make intracellular H_2O_2 detectable. As a positive control, cells were treated with H_2O_2. The additions and culture conditions were: A, 5-S-GAD; B, 5-S-GAD with catalase; C, H_2O_2; D, 5-S-GAD, the cells were kept on ice; E, 5-S-GAD, FCS was omitted from the medium.

5-S-GAD inhibits a *src* family PTK that is related to survival signaling, resulting in the apoptosis of osteoclast precursor cells.

A few points may need discussion. The inhibitory effect of 5-S-GAD on osteoclast formation was more obvious when it was added in the middle of a culture rather than when it was present throughout the culture period. There seemed to be a specific stage at which osteoclast precursor cells become more sensitive to 5-S-GAD than at other stages, and this specific stage is days 3–6 under our culture conditions. The progression of osteoclast formation in a murine marrow cell culture with 1,25-$(OH)_2D_3$ is provisionally divided into three stages: proliferation stage of precursor cells (days 1–4), differentiation stage of TRAcP-positive cells (days 4–6), and fusion stage to form TRAcP(+)MNC (days 6–8) [15]. Possibly, premature TRAcP-positive cells are very sensitive to 5-S-GAD.

In our culture system, H_2O_2 was produced on treatment with 5-S-GAD only in the presence of FCS. It is likely that the hydrogen released from 5-S-GAD is converted to H_2O_2 on the surface of cells, and FCS seems to contain a certain substance that catalyzes the production of hydrogen from 5-S-GAD.

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