Phorbol 12-Myristate 13-Acetate (PMA)-Induced Oxyradical Production in Rheumatoid Synovial Cells

Takatoshi Tanabe1,2, Hitomi Otani1, Katsuyuki Mishima1, Ryokei Ogawa2 and Chiyoko Inagaki1,*

1 Department of Pharmacology, Kansai Medical University, Moriguchi, Osaka 570, Japan
2 Department of Orthopaedic Surgery, Kansai Medical University, Moriguchi, Osaka 570, Japan

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ABSTRACT—We successfully detected the oxyradical production in human synovial A (macrophage-like) and B (fibroblast-like) cells by phorbol 12-myristate 13-acetate (PMA) using the luminol-chemiluminescence method. The PMA (0.1 μg/ml)-induced photon generation was abolished by an O2− scavenger, superoxide dismutase, and an H2O2 scavenger, catalase, suggesting that the stimulus produced oxyradicals in synovial cells. Both of these responses were abolished by a protein kinase C (PKC) inhibitor, calphostine C, but unaffected by an intracellular Ca2+ chelator, BAPTA-AM, and Ca2+ removal from the extracellular medium. These findings suggest that synovial A and B cells produce oxyradicals through PKC-mediated and [Ca2+]-independent mechanisms, probably through the activation of NADPH oxidase.

Keywords: Synovial cell, Oxyradical production, Protein kinase C

Synovial cells have been suggested to play a major role in the destruction of joint cartilage and bone in the inflamed joints of patients with rheumatoid arthritis (1–3). Synovial cells consist of A (macrophage-like) and B (fibroblast-like) types. Both of the cells are known to produce a proteinase (transin/stomelysin) (4) or a growth factor (transforming growth factor-β) (5) that may contribute at least in part to joint pathology in chronic arthritis. Superoxide is a potent killing factor produced by phagocytic cells such as neutrophils, lymphocytes and fibroblasts in response to various inflammatory stimulators (6). However, the superoxide production in human synovial cells has not been detected. In this study, we attempted to measure oxyradical production in a single human A or B type synovial cell stimulated with phorbol 12-myristate 13-acetate (PMA).

Synovial tissues were isolated as described by Bathon et al. (7), from the knee joints of seven rheumatoid arthritis patients obtained during joint replacement surgery. The dissected tissue was minced into 2–3 mm3 pieces and then incubated in Hank’s balanced salt solution (HBSS: 136.8 mM NaCl, 5 mM KCl, 1.0 mM CaCl2, 0.8 mM MgSO4, 0.3 mM Na2HPO4, 0.4 mM KH2PO4, 20 mM HEPES, 4.1 mM NaHCO3, 5.5 mM glucose, pH 7.4) containing 0.2% collagenase (type II; Worthington Biochemical Co., Freehold, NJ, USA) for 60 min with occasional stirring. The cell suspension was centrifuged for 5 min at 600 × g, and the resulting pellets were washed and resuspended in Dulbecco’s modified Eagle’s medium (DMEM; Flow Laboratories, Irvine, UK) supplemented with 20% heat-inactivated fetal calf serum (FCS, Flow Laboratories), penicillin (100 units/ml), streptomycin (100 μg/ml) (ICN Biomedicals Inc., Costa Mesa, CA, USA) and L-glutamine (2 mM) (ICN Biomedicals Inc.). The cells were then plated on the 35-mm microwell dishes with a glass bottom (TK0179; MatTek Corp, Ashland, MA, USA) (1–2 × 104 cells/well), and cultured for 7–14 days at 37°C under 5% CO2: 95% air in a humidified chamber. The numbers of A and B cells after two weeks of culture were approximately 2.3–2.5 × 104 (12.5±5.7%, n=12) and 1.6–1.7 ×105 (87.5±9.3%, n=12) cells/well, respectively, as determined by differential counting for 20–30 cells in 10 different microscopic areas.

Luminol (1.0 mg; Sigma Chemical Co., St. Louis, MO, USA) was dissolved in a slightly alkaline solution that was then adjusted to pH 7.4 with HCl, and the volume was adjusted to 100 ml with distilled water and stored at −20°C. Calphostin C (Sigma) was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 1 mM and diluted with HBSS (pH 7.4). The final concentration of DMSO was less than 0.1%.

Luminol-dependent chemiluminescence from each synovial cell was measured by an ultrasensitive photon counting camera (C-2400-47; Hamamatsu Photonics,
Hamamatsu) equipped with a computer-assisted image processor. When an optical image was focused on the bialkali photocathode in the front camera head, it emitted photoelectrons in proportion to the input intensity from the microscope images (8). The spectral sensitivity of the photocathode is between 300 and 600 nm, and its maximum response is at 420 nm which nearly corresponds to the maximum photoemission band induced by luminol-dependent chemiluminescence (9). Ten minutes after the exposure to luminol (300 µM)-containing HBSS with or without test reagents, the cells were treated with PMA (0.1 µg/ml, Sigma). The dotted scintillating photonic images on the television monitor were digitally processed and were stored in a video frame memory via an image processor. To identify the photon-generating cells, the stored image of photon accumulation was superimposed upon the brightfield image. The intensity of scintillating photons on each synovial cell was analyzed. Experiments were performed using 8 to 12 each of A and B cells in 3 preparations derived from 3-4 each of different patients' synovial tissues. The Welch-test was used for the statistical analyses shown in Table 1. The differences between mean values with P values of less than 0.05 were considered to be significant.

Luminol (300 µM) was used as a chemiluminescence probe to visualize the release of oxyradicals, i.e., superoxide anions (O$_2^-$) and/or hypochlorous anions (OCl$^-$) (10, 11). Panels a and c in Fig. 1 show the optical phase contrast microscopic cellular images of synovial A and B cells, respectively, on the 7th culture day. Panels b and d in Fig. 1 show the superimposed images (microscopic cellular image plus scintillating photonic images of luminol-dependent chemiluminescence) of synovial A and B cells, respectively, obtained 3 min after the exposure to the protein kinase C (PKC) activator PMA (0.1 µg/ml). Most photon particles overlapped with the distribution of A and B cells, indicating that synovial cells are the major source of PMA-triggered chemiluminescence activities. More than 95% of both synovial A and B cells showed such chemiluminescence activities.

Figure 2 shows the time course of PMA (0.1 µg/ml)-induced oxyradical production in a single A- or B-type synovial cell. Both types of PMA-treated synovial cells time-dependently produced photon accumulation. In each synovial cell, the amounts of photon increased rapidly during the first 2 min after the stimulation and then slowly reached a plateau level within 3 min. In the following experiments, therefore, the amounts of photon accumulated during the first 3 min were determined. The maximum photon intensity in A cells was approximately 2 times higher than that observed in B cells. Such photon generation was abolished in the presence of an O$_2^-$ scavenger, superoxide dismutase (400 units/ml), plus an H$_2$O$_2$ scavenger, catalase (150 units/ml), but not by a xanthine-xanthine oxidase inhibitor, allopurinol (0.2 µM, Sigma), showing that the photons generated reflect the production of oxyradicals such as O$_2^-$ and/or OCl$^-$ probably via activation of an oxyradical production system such as NADPH oxidase (9).

Several stimulants such as PMA, N-formyl-Met-Leu-Phe (fMLP) and substance P reportedly induce oxyradical production in neutrophils via PKC activation and/or [Ca$^{2+}$], elevation (12, 13). Therefore, we examined the involvement of PKC and [Ca$^{2+}$] in the PMA-induced oxyradical production by using the PKC inhibitor calphostin C (14), the intracellular Ca$^{2+}$ chelator BAPTA-AM, and Ca$^{2+}$-free medium containing EGTA.

As shown in Table 1, pretreatment with calphostin C (1 µM) abolished PMA-induced oxyradical production in both types of synovial cells, while BAPTA-AM and Ca$^{2+}$-free medium did not cause significant changes in the response to PMA. Although PMA increased [Ca$^{2+}$], with a lag of 3-4 min in both types of synovial cells (d[Ca$^{2+}$]/dt: approximately 31.3±3.2 and 29.6±5.2 nM/min for A and B cells, respectively, n = 6), the latter treatments markedly inhibited the increases in d[Ca$^{2+}$]/dt (2.1±0.4 and 1.9±0.8 nM/min after BAPTA-AM treatment, 1.1±0.3 and 1.0±0.2 in Ca$^{2+}$-free with EGTA medium, for A and B cells, respectively, n = 6), indicating that BAPTA-AM and Ca$^{2+}$-free medium effectively inhibited [Ca$^{2+}$], elevation in synovial cells. These findings suggest that PMA induces oxyradical production through Ca$^{2+}$-independent and PKC-activated mechanisms in rheumatoid arthritis synovial cells. Since NADPH oxidase has been reported to be phosphorylated by PKC (13), stimulation of PKC by PMA may directly activate this enzyme.

In the present study, the stimulus-induced oxyradical

| Table 1. Effects of calphostin C, BAPTA-AM and Ca$^{2+}$ removal from extracellular medium on PMA-induced oxyradical production in synovial A and B cells |
|-------------|---------|
| Vehicle     | 0.8±0.2 |
| PMA (0.1 µg/ml, control) | 20.9±1.1 |
| Calphostine C (1 µM) + PMA | 2.4±0.8* |
| BAPTA-AM (50 µM) + PMA | 18.5±0.5 |
| Ca$^{2+}$ free + EGTA (10 mM) + PMA | 17.3±1.0 |

Each value represents a mean±S.E.M. (n = 6-12). *P<0.01, compared with the corresponding control (PMA alone) by Welch's test. The production of oxyradicals was estimated from the amount of photons produced during the first 3 min after the addition of PMA.
Fig. 1. Visualized oxyradical production in human synovial A and B cells. Panels a and c are the brightfield images of synovial A (macrophage-like) and B (fibroblast-like) cells, respectively. Panels b and d are photon accumulation images superimposed upon the cellular images a and c, respectively. The photon accumulation images of luminal-dependent chemiluminescence from a synovial A (b) and B cell (d) were obtained under the stimulation with 0.1 μg/ml PMA for 10 min in the presence of 300 μM luminol.
production in human synovial cells was first demonstrated using the luminol chemiluminescence method that enabled the detection of oxyradical production 10–20 times lower than that in neutrophils (191±12.8 photon counts/3 min/cells as measured by the same method). The present study also showed that synovial A cells had higher oxyradical production than B cells. Such PMA-induced oxyradical production was also observed in the synovial cells from non-rheumatoid joints (trauma of joint), at a 15–20% lower level than those in rheumatoid arthritis. Possible increases in the population of such A cells and/or endogenous stimulants in the inflammatory joints probably accelerate the oxyradical production near the cartilage and bone.

Although endogenous inducers of oxyradical production in synovial cells are currently unclear, interleukin (IL)-1, IL-6 and substance P may be candidates for such inducers because of their abundance in the inflamed synovial tissues (15, 16) and abilities to produce oxyradicals in the immune-related cells such as neutrophils (12, 17). The pathophysiological importance of synovial oxyradical production should be further analyzed, especially in relation to their roles in the destruction of joint cartilage and bone.

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