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Interleukin-4 Inhibits the Gene Expression and Biosynthesis of Cytosolic Phospholipase A2 in Lipopolysaccharide Stimulated U937 Macrophage Cell Line and Freshly Prepared Adherent Rheumatoid Synovial Cells

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We recently reported that interleukin-4 (IL-4) inhibited prostanoid synthesis through inhibiting cyclooxygenase 2 biosynthesis. In the present study, we examined the effect of IL-4 on the expression of cytosolic phospholipase A_2 ($cPLA_2$). The amounts of protein and mRNA of $cPLA_2$ were determined by western blotting and reverse transcription polymerase chain reaction (RT-PCR), respectively. Although interleukin-1α (IL-1α) and tumor necrosis factor α (TNFα) had little effect on the biosynthesis of $cPLA_2$ in phorbol myristate acetate (PMA)-differentiated U937 cells, lipopolysaccharide (LPS) increased the protein level of $cPLA_2$ in a dose-dependent manner. IL-4 inhibited the increased synthesis of $cPLA_2$ at the mRNA level. In addition, IL-4 inhibited the biosynthesis of $cPLA_2$ in untreated or LPS treated freshly prepared rheumatoid synovial cells at the mRNA level. These findings suggest that IL-4 inhibits prostanoid synthesis through inhibiting the expression of both $cPLA_2$ and cyclooxygenase 2. © 1997 Academic Press

The initial step in arachidonic acid metabolism is the release of free arachidonic acid from cell membrane phospholipids by a family of phospholipase A_2 (PLA_2)(1). These enzymes can be categorized into two major groups, the secretory or low molecular weight forms, secretory PLA_2 ($sPLA_2$), and the cytosolic or high molecular weight forms, cytosolic PLA_2 ($cPLA_2$). The structural and mechanistic properties of the Ca^{++}-dependent 14 kD secretory forms of PLA_2 have been extensively studied. However, since these enzymes fail to selectively hydrolyze arachidonyl-containing phospholipids, their primary function is considered not to initiate the biosynthesis of arachidonic acid metabolites(2, 3). On the contrary, the recently cloned $cPLA_2$, that is active at micromolar calcium concentrations, selectively cleaves arachidonic acid at the *sn*-2 position of membrane phospholipids(4-6), and is coupled to hormonally regulated release of arachidonic acid(7). These findings suggest that $cPLA_2$ plays an important role in the production of prostaglandins and leukotrienes, the biologically active lipid mediators(5).

Interleukin-4 (IL-4), originally described as a B cell growth factor, is a 20 kDa product of activated T cells(8). Since IL-4 inhibits the ability of macrophage lineage cells to produce a group of inflammatory cytokines such as IL-1, tumor necrosis factorα (TNFα) and IL-6, it is considered as an anti-inflammatory cytokine(9-11). In addition, the inhibitory effect of IL-4 on PGE_2 production by monocytes(10, 12), macrophage(9), fibroblasts(13), and mesangial cell(14) has also been demonstrated. However, the mechanism of the inhibitory effect of IL-4 on PGE_2 production remains to be clarified. Recently, we have reported that IL-4 inhibited the expression of cyclooxygenase 2, that is the rate-limiting enzyme in prostanoid synthesis, in freshly prepared rheumatoid synovial cells(15). In the present study, we have examined the effect of IL-4 on the expression of $cPLA_2$ in U937 cells and freshly prepared rheumatoid synovial cells.

MATERIALS AND METHODS

Materials. Human recombinant IL-4 was kindly provided by Ono Pharmaceutical Co. (Osaka, Japan). Human recombinant IL-1α and TNFα were kindly provided by Dainippon Pharmaceutical Co. (Osaka, Japan). Lipopolysaccharide (LPS) was purchased from

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Sigma Chemical Co. (St Louis, MO). Dexamethasone (DEX) and indomethacin (IND) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

U937 cell line. U937 cells, derived from human histiocytic lymphoma(16), kindly provided by Japanese Cancer Research Resources Bank, were cultured in RPMI 1640 supplement with 10% heat-inactivated fetal calf serum (FCS) (ICN Biomedicals, Seven hills, Australia), penicillin 100 U/ml, N-2-hydroxyethylpiperazine-N-2-ethanesulphonic acid (HEPES) 12.5 mM, and L-glutamine 2 mM [RPMI/10%FCS]. U937 cells were differentiated into monocyte/macrophage cells by 48 hours of incubation with 100 nM of phorbol myristate acetate (PMA) (Sigma Chemical Co.). After differentiation, the cells became adherent to dishes, and expressed CD14 antigen on their surface (data not shown).

Adherent rheumatoid synovial cells. Rheumatoid synovial tissues were obtained from the knee joints of patients who were undergoing total joint replacement. Isolated rheumatoid synovia were minced, and enzymatically digested with 0.5-1 mg/ml of *clostridium* collagenase (Wako Pure Chemical Industries Ltd.) and 5-10 μg/ml of deoxyribonuclease 1 (Sigma Chemical Co.) for two to three hours. After digestion, the resultant single cells were washed, filtrated through nylon mesh, and resuspended in Dulbecco's modified Eagle's medium supplemented with 10 % FCS, penicillin, HEPES, and L-glutamine [DMEM/10%FCS]. The cells were cultured overnight to allow them to adhere to a plastic dish. The dish was washed to remove nonadherent cells, and the remaining adherent cells were used as freshly prepared adherent synovial cells in this study.

Western blot analysis. The cells prepared as described above, were lysed in a buffer (50 mM TRIS hydrochloric acid, pH 8.0, 1% Nonidet P-40, 0.5% deoxycholic acid, 150 mM sodium chloride, and 1 mM phenyl methylsulphonyl fluoride). Proteins were separated by electrophoresis on a 7.5% sodium dodecyl sulfate-polyacrylamide gel, and transferred onto an Immobilon-P membrane (Millipore, Bedford, MA). After blocking, the membrane was incubated with a mouse antihuman $cPLA_2$ monoclonal antibody (Santa Cruz Biotechnology Inc.,CA) then with a goat antimouse immunoglobulin antibody conjugated with horseradish peroxidase (Santa Cruz Biotechnology Inc.). Specific bands were visualized by an enhanced chemiluminescence detection system (ECL, Amersham Buckinhamshire, UK).

Extraction of total RNA, and reverse transcription polymerase chain reaction (RT-PCR). The total cellular RNAs were extracted by acid guanidine phenol/chloroform extraction(17) using Isogen (Wako Pure Chemical Industries, Ltd.). Two micrograms of the total RNAs was reverse transcribed to cDNA annealing with 25 pmol of random primer in the presence of 100 U of Moloney murine leukaemia virus (M-MLV) reverse transcriptase (Gibco BRL Life Technologies, Inc., Gaithersburg, MD), 10 U of ribonuclease inhibitor (RNasin, Promega Corp., Madison, WI), 1 mM dithiothreitol, and 10 pmol of each deoxynucleotide (Takara Shuzo Co. Kyoto, Japan) in a total volume of 10 μl for one hour at 37 °C. Two microlitres of the resultant cDNA preparation was used directly for each amplification reaction. Polymerase chain reaction (PCR) was performed in a 50 μl reaction mixture containing 20 pmol of each primer (see below), 20 pmol of each deoxynucleotide and 1.25 U Taq DNA polymerase (Takara Shuzo Co.). The primers used were: $cPLA_2$ sense primer, 5′-AAAGAACACATAAGGGAGAG-3′ (nucleotide 609′-628′); $cPLA_2$ antisense primer, 5′-AAAGAGGTAAAGGGCATTGT-3′ (nucleotide 1072′-1091′); glyceraldehyde-3-phosphate dehydrogenase (GAPDH) sense primer, 5′-CAAAAGGGTCATCATCTCTG-3′ (nucleotide 408′-427′); GAPDH antisense primer, 5′-CCTGCTTCACCACCTTCTTG-3′ (nucleotide 834′-853′). These primer sets yield PCR products of 502 bp and 446 bp for $cPLA_2$ and GAPDH, respectively. A reaction mixture was incubated in a Perkin-Elmer/Cetus DNA Thermal cycler for 25-30 cycles (denaturation, one minute, 94°C; annealing two minutes, 55°C; extension, one minute, 72°C). Aliquots of PCR products were run on a 1.2 % agalose gel in TAE buffer (40 mM TRIS acetate, 2 mM sodium EDTA), and PCR products were visualized by ethidium bromide staining. GAPDH was used as a house keeping gene(18).

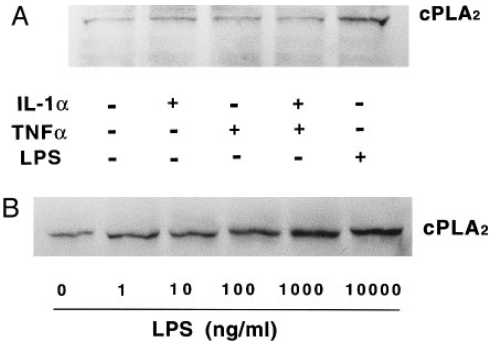

FIG. 1. Induction of cytosolic phospholipase A_2 ($cPLA_2$) in phorbol myristate acetate (PMA)-differentiated U937 cells by lipopolysaccharide (LPS). (A) Western blot analysis using a mouse anti-human $cPLA_2$ monoclonal antibody. PMA-differentiated U937 cells were cultured with interleukin-1α (IL-1α) 2 ng/ml, tumor necrosis factor α (TNFα) 20 ng/ml, or LPS (1 μg/ml) for 24 hr, and the cell lysates were used for western blotting as described in materials and methods. (B) Western blot. The PMA-differentiated U937 cells were cultured with LPS at the indicated concentrations, and the cell lysates were used for western blotting.

RESULTS

The effect of IL-1α, TNFα, or LPS on the biosynthesis of $cPLA_2$ in PMA-differentiated U937 cells. As the previous studies reported that $cPLA_2$ expression was induced by IL-1, TNFα and LPS in a variety of cells(19-25), the effect of these stimuli on the biosynthesis of $cPLA_2$ in U937 cells was examined. PMA-differentiated U937 cells were treated with IL-1α, TNFα or LPS for 24 hours. As shown in Fig. 1, LPS increased the $cPLA_2$ protein level in the U937 cells in a dose-dependent manner. On the contrary, IL-1α, TNFα and both have little effect on the expression of $cPLA_2$.

The suppressive effect of IL-4 on the biosynthesis and gene expression of $cPLA_2$ in the U937 macrophage cell line. To determine the effect of IL-4 on the expression of $cPLA_2$ in PMA-differentiated U937 cells, the cells were treated with LPS in the presence of various concentrations of IL-4 for 24 hours. IL-4 inhibited the biosynthesis of $cPLA_2$ in a dose-dependent manner (Fig 2-A). Next, to clarify further whether IL-4 might inhibit the synthesis of $cPLA_2$ at the mRNA level, we examined the effect of IL-4 on the gene expression of $cPLA_2$ in U937 cells. The cells were treated with LPS in the presence of various concentrations of IL-4 for 8 hours. As shown in Fig 2-B, LPS increased the level of $cPLA_2$ mRNA. IL-4 also inhibited the increased expression of $cPLA_2$ mRNA.

The suppressive effect of IL-4 on the biosynthesis and gene expression of $cPLA_2$ in freshly prepared adherent rheumatoid synovial cells. It is interesting to clarify

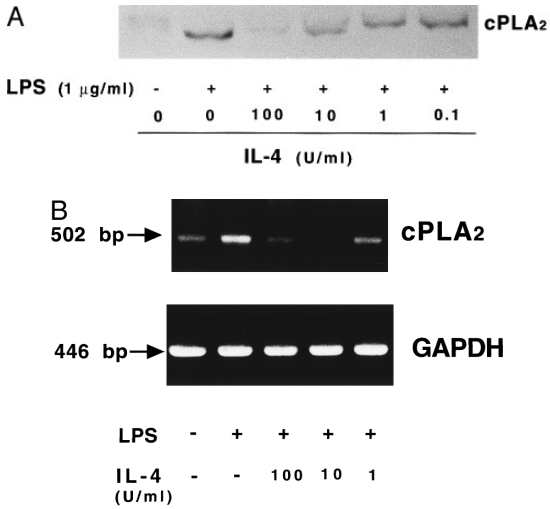

FIG. 2. Suppressive effect of interleukin-4 (IL-4) on the biosynthesis and gene expression of cytosolic phospholipase A_2 ($cPLA_2$) in lipopolysaccharide (LPS) stimulated U937 cells. (A) Western blot analysis using a mouse anti-human $cPLA_2$. Phorbol myristate acetate (PMA)-differentiated U937 cells were cultured with LPS 1 μg/ml in the presence of IL-4 at the indicated concentrations for 24 hr, and the cell lysates were used for western blotting. (B) Reverse transcription and polymerase chain reaction (RT-PCR) using primer sets specific for $cPLA_2$ and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The PMA-differentiated U937 cells were cultured with LPS 1 μg/ml in the presence of IL-4 at the indicated concentrations for 8 hr. The total cellular RNAs were extracted from these cells, and used for RT-PCR. GAPDH was used as a housekeeping gene.

whether IL-4 also might inhibits the expression of $cPLA_2$ in inflammatory cells obtained from disease sites. As freshly prepared rheumatoid synovial cells are known to produce large amounts of PGE_2(26), we examined the effect of IL-4 on the expression of $cPLA_2$ in these cells. Since both DEX and IND are known to inhibit the production of PGE_2(27, 28), the effects of these agents were also examined. Freshly prepared synovial cells obtained from rheumatoid synovia were treated with or without LPS in the presence of IL-4, DEX, or IND for 24 hours. As shown in Fig 3-A,B, the expression of $cPLA_2$ protein in freshly prepared rheumatoid cells was detected at the mRNA and protein levels without stimuli. IL-4 and DEX inhibited the spontaneous expression of $cPLA_2$ mRNA. On the contrary, IND did not modify the expression (Fig 3-B). In addition, both IL-4 and DEX inhibited the LPS stimulated biosynthesis and gene expression of $cPLA_2$ in freshly prepared synovial cells (Fig 3-A,C).

DISCUSSION

PGE_2 is a potent mediator of the pain and edema associated with rheumatoid synovitis, and involved in bone resorption(26). IL-4 is known to inhibit the overproduction of PGE_2 in rheumatoid synovial cells(15, 29). However, the mechanism(s) of the inhibitory action of IL-4 on PGE_2 production remains to be clarified.

In this study we demonstrated that LPS stimulated the biosynthesis of $cPLA_2$ in PMA-differentiated U937 cells. Although IL-1α and TNFα are reported to stimulate the expression of $cPLA_2$ in a variety of cells(19-23, 25), these cytokines did not increase the protein of $cPLA_2$ level in our study. It is possible that PMA treatment may modulate the response of U937 cells to IL-1α and TNFα, or that the stimulatory effect of these two cytokines may be too small to detect by western blotting. IL-4 inhibited the LPS-stimulated biosynthesis of $cPLA_2$ in U937 cells at the mRNA levels. The inhibitory effect of IL-4 on the expression of $cPLA_2$ has been controversial. IL-4 is reported to inhibit $cPLA_2$ expression in *c-kit* ligand-stimulated immature mouse

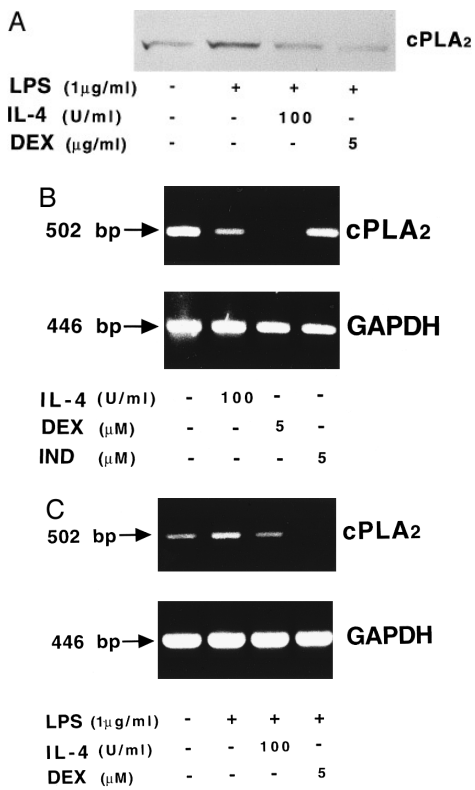

FIG. 3. Suppressive effect of interleukin-4 (IL-4) on the biosynthesis and gene expression of cytosolic phospholipase A_2 ($cPLA_2$) in freshly prepared rheumatoid synovial cells. (A) Western blot analysis using anti-human $cPLA_2$ antibody. The freshly prepared synovial cells obtained from rheumatoid synovia were cultured with or without lipopolysaccharide (LPS) 1 μg/ml in the presence of IL-4 100 U/ml or dexamethasone (DEX) 5 μM for 24 hr, and the cell lysates were used for western blotting. (B) Reverse transcription and polymerase chain reaction (RT-PCR) analysis. The freshly prepared rheumatoid synovial cells were cultured with IL-1α 100 U/ml, DEX 5 μM, and indomethacin (IND) 5 μM for 24 hr. The total cellular RNAs were extracted, and used for RT-PCR. (C) RT-PCR analysis. The freshly prepared rheumatoid synovial cells were cultured with LPS 1 μg/ml in the presence of IL-4 100 U/ml or DEX 5 μM for 8 hr. Their total cellular RNAs were used for RT-PCR analysis.

mast cells(30) and in neonatal mouse parietal bone culture(31). On the contrary, Onoe et al(32). have reported that IL-4 did not inhibit the expression of $cPLA_2$ mRNA in IL-1α stimulated mouse osteoblasts. The discrepancy among these results might be explained by the differences in the cell types or stimuli.

The effect of IL-4 on $cPLA_2$ expression has never been examined in human. We demonstrated that the inhibitory effect of IL-4 on $cPLA_2$ expression was also observed in freshly prepared rheumatoid synovial cells from the patients. In addition, we confirmed the previous reports showing the inhibition of induction of $cPLA_2$ by dexamethasone (19, 22). It has been reported that cyclooxygenase 2 and $cPLA_2$ play critical roles in overproduction of PGE_2 by human synovial cells(19) or rheumatoid synovial cells(21). We have found that IL-4 is capable of inhibiting cyclooxygenase 2 in rheumatoid synovial cells(15). These findings suggest that IL-4 inhibits PGE_2 production through inhibiting both expressions cyclooxygenase 2 and $cPLA_2$ in rheumatoid synovia.

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