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Analysis of Interleukin-8 Gene Promoter function in Human Osteoblast-like Cells: Regulation by Ca^{2+}-signaling and Cyclosporin A

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Abstract: We previously reported that an increase in intracellular calcium by calcium ionophore (A23187) and 4β-phorbol-12β-myristate-13α-acetate (PMA) induced the expression of IL-8 mRNA in human osteoblast-like HOS-TE85 cells and this induction was markedly suppressed by cyclosporin A (CsA). In this study we investigated whether the regulation by A23187, PMA and CsA was occured at a transcriptional level by reporter gene assays. A promoter region spanning from -1460 to +40 of IL-8 gene was PCR-amplified and inserted upstream of a luciferase reporter gene. Various deletion mutants were also constructed. Transfection of the plasmids into HOS-TE85 cells demonstrated that the nucleotides between -133 and -60 base pairs upstream of IL-8 gene are essential and sufficient for its induction by A23187/PMA, and suppression of this induction by CsA.

Key words: interleukin-8, cyclosporin A, human osteoblast

Interleukin (IL)-8 is a member of the CXC chemokine family and plays an important role as an activator and chemoattractant for neutrophil granulocytes and lymphocytes.[1)2)] It is a key factor in the pathogenesis of inflammatory joint diseases such as rheumatoid arthritis and osteoarthritis,[3)4)] as evidenced by its expression in subchondral osteoblasts isolated from patients with RA and OA.[5)] Analysis of the genomic structure of IL-8 revealed many potential targets for its regulation at both transcriptional and posttranscriptional levels.[6-9)] Within its 5′-flanking region, the IL-8 gene contains potential binding sites for transcription factors such as AP-1, AP-2, HNF-1, IRF-1, glucocorticoid receptor, NFκB, and NF-IL-6. In its 3′-flanking region, a repetitive ATTTA motif implicated in the destabilization of the transcript is present.[10,11)]

In a previous report, we demonstrated that the calcium dependent stimuli upregulate the IL-8 gene expression and CsA inhibit this induction in human osteobast-like HOE-TE85 cells.[12)] In the present study, we analyzed the promoter function of IL-8 gene by reporter gene assays.

Materials and Methods

1. Cell culture

HOS-TE85 cells [ATCC CRL-1543] established from human osteosarcoma[13)] were cultured in Dulbecco's modified Eagle medium (DMEM; Nissui, Tokyo) supplemented with 10% fatal bovine serum, penicillin (100 U/ml), and streptomycin (100 U/ml), and 292 μg/ml L-glutamine in a humidified incubator containing 5% CO_2 in air.

2. Plasmids

A fragment of the genomic IL-8 DNA spanning from -1460 to +40 bp (transcription start site was numbered as +1, GeneBank Accession number: M28130) was PCR-amplified and subcloned into pGEM-T Easy (Promega, Madison, WI, USA). The fragment was inserted into pGL3-Basic (Promega, Madison, WI, USA) which has multiple cloning sites just upstream of the firefly luciferase gene. A series of deletion fragments of the promoter (-651 to +40, -133 to +40, -60 to +40) were also generated and inserted into the pGL3-Basic.

3. Transfection and luciferase assay

HOS-TE85 cells were plated at a density of 2×10^5 cells on 35-mm tissue culture dish and cultured to 50% confluence. The plasmid DNAs were transfected into HOS-TE85 cells by the use of LIPOFECT AMINE (GIBCO BRL Life Technologies, Inc, Grand Island, NY, USA). In brief, each luciferase reporter construct (1.6 μg) and a plasmid expressing bacterial

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β galactocidase driven by cytomegalovirus promoter (pβgal-CMV; 0.1 μg) were placed in 100 μl of serum-free medium. LIPOFECT AMINE (5 μl) diluted in 100 μl serum-free medium was added and incubated at room temperature for 30 min, followed by addition of 0.8 ml of serum-free medium. After addition of the mixture, the cells were incubated at 37°C for 5 hours. Then DMEM containing 10% fetal bovine serum was added without removing the transfection mixture. The medium was replaced with fresh, complete medium at 24 h after the transfection. Twelve hour after the medium change, the cells were incubated with 2.5 μM calcium ionophore (A23187, Wako Pure Chemical Industries, Ltd., Osaka) and with 10 nM PMA (Sigma, St. Louis, USA). In experiments using CsA, the cells were pretreated with 5 μg/ml Cs A for 15 min and then exposed to calcium ionophore and PMA. After additional 24 h incubation with A23187 and PMA, the cells were harvested into 400 μl of extraction buffer (25 mM Gly-gly-KOH, 15 mM $MgSO_4$, 4 mM EGTA, 1 mM DTT, 0.2% Triton-X) and centrifuged (10,000 x g for 1 min). The resultant supernatant (50 μl) was used for the determination of luciferase activity with a Lumat model LB9507 luminometer (Berthold, Bad Wildbad, Germany). Levels of luciferase expression were normalized by β galactocidase activity. Each experiment was performed in triplicate and repeated three times.

Result

As shown in Fig. 1, promoter regions responsible for IL-8 gene activation by A23187/PMA in HOS-TE85 cells were analyzed by the determination of luciferase activity. Transfection of a reporter gene construct driven by a sequence from -1460 to +40 resulted in approximately 6 fold increase in luciferase activity above that by promoter less pGL3-Basic.

Addition of A23187/PMA markedly increased the luciferase activity in cells transfected with -1460, -651, and -133 Luciferase constructs. The increase with all the constructs attained by A23187/PMA was inhibited by the addition of CsA.

Note that deletion of a sequence from -1460 to -60 completely abolished the response to A23187/PMA, suggesting the presence of cis-acting element(s) responsive to Ca^{2+}-signaling within the region from -133 bp to -60 bp of IL-8 gene.

Discussion

The present study demonstrated that cis-acting element(s) responsive to Ca^{2+}- signaling is present within the region from

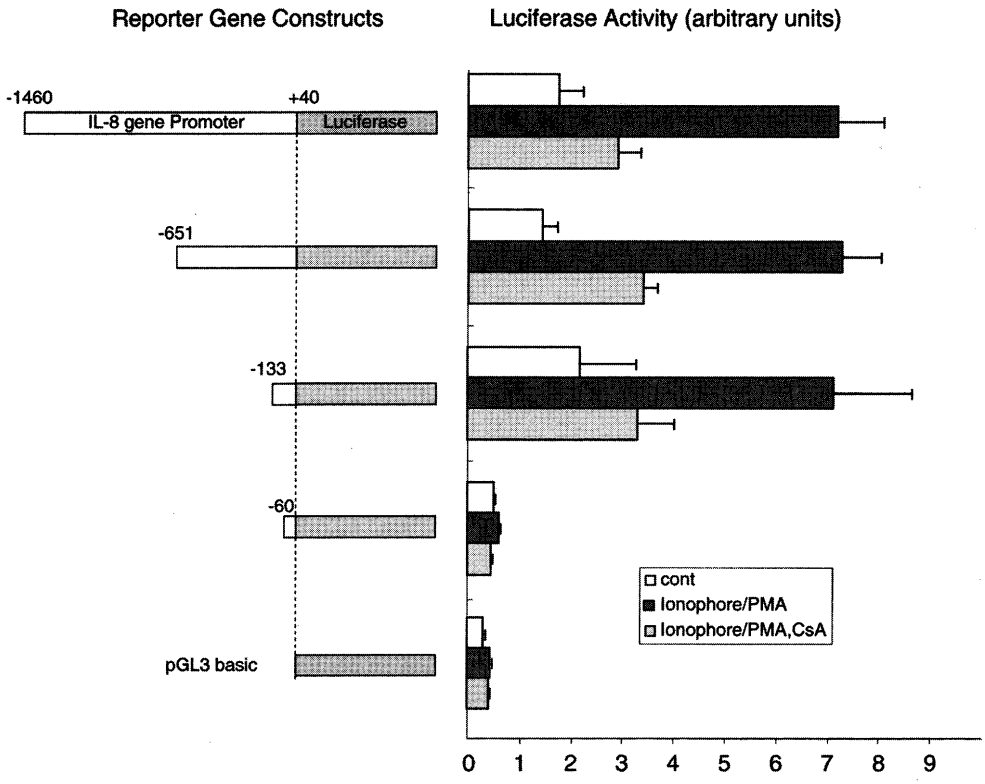

Fig. 1 Effect of calcium ionophore/PMA and cyclosporin A on IL-8 gene expression in osteoblast-like HOS-TE85 cells.
HOS-TE85 cells were transfected with pGL3-Basic plasmid and plasmids containing serial deletions in the 5′-flanking promoter region of the IL-8 gene. After 36 h of transfection, cells were incubated for 24 h with either medium alone (control) or A23187 (2.5 μM)/PMA (10 nM) in the absence or presence of CsA (5 μg/ml). Levels of luciferase expression were normalized by β galactocidase activity. The data were expressed as mean±SD.

-133 bp to -60 bp of IL-8 gene. In this region, there are three possible cis-acting elements, AP-1 (-126 bp to -120 bp), NF-IL-6-like (-94 bp to -81 bp) and NFκB-like (-80 bp to -71 bp) sites. Mukaida et al. reported that the transcription of IL-8 gene requires the activation of either NFκB or AP-1, or that of both NFκB and NF-IL-6, depending on the cell types. Although activation of NFκB is shown to upreglate IL-8 gene transcription in any type of cells examined,[7,14-19] Okamoto et al. reported that NFκB-like site was not always bound by NFκB.[20] It is thus difficult to assign a transcription factor(s) for the Ca^{2+}-signaling in osteoblasts. However, involvement of NFAT in the IL-8 gene regulation is suggested since CsA inhibits activation of the transcription factor by calcineurin.[21] Further experiments such as site-directed mutagenesis of each element and electrophoretic mobility shift assay are required to define the transcriptional regulation by Ca^{2+}-signaling in osteoblasts.

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