BASIC CALCIUM PHOSPHATE CRYSTAL INDUCTION OF COLLAGENASE 1 AND STROMELYSIN EXPRESSION IN HUMAN FIBROBLASTS OCCURS VIA A PROTEIN KINASE C DEPENDENT PATHWAY

Introduction
Synovial fluid basic calcium phosphate (BCP) crystals are common in osteoarthritic joints and associated with severe arthropathy. These crystals induce mitogenesis and the synthesis and secretion of matrix metalloproteinases (MMPs) by fibroblasts and chondrocytes. We previously reported that BCP induces the translocation of protein kinase C (PKC) activity from the cytoplasm to the membrane of these cells (1,2) and that BCP-induced mitogenesis and proto-oncogene expression are PKC-dependent events (1,2). However, the role of PKC in crystal-induced MMP synthesis was unknown. Here we report that PKC is also required for maximal BCP crystal induction of collagenase 1 (MMP-1) and stromelysin (MMP-3) mRNA and protein in human fibroblast (HF) cells.

Materials and Methods
Our studies employ the use of human foreskin fibroblasts as a model for the synovial fibroblasts that interact with crystals. These HF and synoviocytes exhibit similar biologic responses to crystals and growth factors in vitro (3). HF cultures were established from explants and transferred. They were grown and maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and containing 1% penicillin, streptomycin, and Fungizone.

Crystal preparation. BCP crystals were prepared by alkaline hydrolysis of brushite using a modification of the method of Bett et al. (4). These crystals have a calcium/phosphate ratio of 1.59 and contain partially carbonate-substituted hydroxyapatite mixed with octacalcium phosphate by FTIR spectroscopy. Crystals were crushed in an agate mortar and sieved to yield 10-20 µm aggregates which were sterilized and rendered pyrogen-free by heating at 200°C for at least 90 minutes.

Northern blot analysis. 100 mm tissue culture dishes of confluent cell monolayers were rendered quiescent by removing the medium, washing with phosphate buffered saline (PBS), and subsequently incubating in DMEM containing 0.5% heat-inactivated FBS for 24 hours. At the onset of the experiments, this medium was removed, the cells washed with PBS, and the medium replaced with serum-free DMEM. Cells treated with H7, staurosporine, or Bisindolylmaleimide I or V were pre-incubated for 30 minutes with the indicated concentrations. Cells were stimulated with BCP (50 µg/ml) for 24 hours. Media was then aspirated from the cell cultures and the RNA was harvested using the Trizol reagent following the manufacturer’s instructions. Total RNA (10 µg/lane) was electrophoresed through a 1% formaldehyde gel and transferred onto a nylon membrane using a downward capillary transfer system. Membranes were hybridized, stripped, and re-hybridized using the following probes: MMP-1, a 2.05 Kb HindIII/SmaI insert from the pTR1 clone, MMP-3, a 1.7 Kb EcoRI insert from the pTR1 plasmid, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) plasmid. The probes were randomly primed with α-32P dATP (50 µCi per labeling reaction, 6000 Ci/mmol). After washing, the hybridized membranes were exposed to x-ray film at -80°C with an intensifying screen.

Western blot analysis. Experimental conditions were the same as described for Northern blot analysis, except that the cells were stimulated with BCP (100 µg/ml) for 48 hours after a 30 minute pre-incubation with H7 or staurosporine. Samples of conditioned media were electrophoresed through a 10% SDS-polyacrylamide gel and then transferred onto a PVDF membrane. After transfer, the membrane was incubated for 3 hours at room temperature in TBST (20 mM Tris, 136 mM NaCl, 0.1% Tween-20) containing 5% non-fat dry milk, then washed several times with TBST. The membrane was next incubated overnight at 4°C with a polyclonal antibody against either MMP-1 or MMP-3 in TBST containing 5% bovine serum albumin.

The membrane was again washed several times with TBST and incubated with horseradish peroxidase-conjugated affinity purified goat anti-rabbit IgG in TBST/milk buffer for 1 hour at room temperature. After a final wash of the membrane several times with TBST, immunoreactive bands were visualized using a chemiluminescent substrate and x-ray film.

Results
BCP crystal-stimulated MMP-1 and MMP-3 mRNA expression is blocked by inhibitors of PKC. After 24 hours of treatment with BCP, Northern blotting experiments show that MMP-1 and MMP-3 mRNA expression is elevated significantly over basal levels detected in control cells. Concurrent treatment with the PKC inhibitors H7 (5 µM, 15 µM, 25 µM) and staurosporine (1 nM, 5 nM, 10 nM) results in a dose dependent reduction of BCP-induced mRNA for both of these MMPs. H7 and staurosporine appear to block crystal-stimulated MMP-1 mRNA expression to an equal extent, while staurosporine is more effective at blocking MMP-3 mRNA upregulation than H7. Another inhibitor of PKC, Bisindolylmaleimide I, but not its structural, non-PKC inhibitory analog Bisindolylmaleimide V (each at 10 nM, 100 nM, 1 µM, 10 µM), also blocks BCP induction of both MMP-1 and MMP-3 mRNA in a dose dependent manner. Treatment of BCP-stimulated cells with the highest dose of Bisindolylmaleimide I results in a nearly complete inhibition of crystal-induced mRNA for these MMPs.

PKC inhibitors block BCP-induced expression and secretion of MMP-1 and MMP-3 protein. When HF are stimulated with BCP for 48 hours, the levels of MMP-1 and MMP-3 protein expression present in cell conditioned media are considerably higher than observed in untreated cells, as determined by immunoblotting experiments with polyclonal antibodies specific for MMP-1 and MMP-3. Treatment with similar concentrations of H7 and staurosporine inhibits BCP crystal-induced MMP-1 and MMP-3 protein synthesis in a dose dependent manner.

Discussion
Here we show for the first time that BCP crystal stimulation of MMP-1 and MMP-3 mRNA and protein in HF is dependent upon a PKC signal transduction pathway in order to achieve maximal crystal-induced synthesis. We have also recently reported that BCP crystal stimulation activates nuclear AP-1 DNA binding activity in HF (1). The promoters of the MMP-1 and MMP-3 genes contain a TRE (TPA response element) DNA binding element for AP-1, a heterodimer typically comprised of Fos and Jun proteins, which mediates induction of transcription in response to TPA and other PKC-activating agents (5). We postulate that the BCP-induced PKC signal transduction pathway may play a role in the upregulation of AP-1 DNA binding activity, which would ultimately result in the increased expression of MMP-1 and MMP-3 mRNAs and protein. The PKC pathway may act independently of other BCP-induced signaling cascades that could also lead to induction of AP-1 DNA binding activity and upregulation of MMP-1 and MMP-3 (such as the p42/p44 MAP kinase pathway), or it may be integrated in some fashion with other signaling pathways.

REFERENCES

Session 21 - Matrix Metalloproteinases - VALENCIA A, Mon 11:30 AM - 1:00 PM

0121