Available online at www.sciencedirect.com
SCIENCE DIRECT®
Life Sciences 79 (2006) 898–904
Life Sciences
www.elsevier.com/locate/lifescie

Up-regulation of protease-activated receptor-2 by bFGF in cultured human synovial fibroblasts

Kazuki Abe [a], Akhmed Aslam [b], Andrew F. Walls [b], Toshitsugu Sato [a], Hideo Inoue [a,*]

[a] *Pharmacological Research Department, Minophagen Pharmaceutical Co.,2-2-3, Komatsubara, Zama-shi, Kanagawa-228-0002, Japan*
[b] *Immunopharmacology Group, University of Southampton, Southampton General Hospital, Southampton SO16 6YD, UK*

Received 15 September 2005; accepted 6 March 2006

Abstract

Protease-activated receptors (PARs) have been implicated in the development of acute and chronic inflammatory responses. We have examined the expression of mRNA for PARs and their regulation by growth factors and cytokines in synovial fibroblasts derived from patients with rheumatoid arthritis (RA). Messenger RNA for PAR-1, -2 and -3 was detected in these cells, but not that for PAR-4. Expression of mRNA for PAR-2 was up-regulated by bFGF in a concentration-dependent manner, whereas expression of mRNA for PAR-1 and PAR-3 was not affected. Levels of mRNA encoding PAR-1, PAR-2 and PAR-3 did not increase in response to IL-1β and TNF-α. Expression of mRNA for PAR-2 was maximal 12 h after addition of bFGF, and maximal levels of immunoreactive PAR-2 were reached after 24 h. Furthermore, PAR-2 agonist peptide ($SLIGKV\text{-}NH_2$), but not the inactive reverse peptide ($VKGILS\text{-}NH_2$), induced transitory cytosolic Ca^{2+} mobilization in cells, and its response was increased by pretreatment with bFGF. An important role could be played by bFGF in the regulation of functional PAR-2 expression in cultured RA synovial fibroblasts.

Keywords: Basic fibroblast growth factor; Interleukin-1β; Synovial fibroblasts; Protease-activated receptors; PAR-2 agonist peptide; Rheumatoid arthritis; Tumor necrosis factor-α

Introduction

Protease-activated receptors (PARs) belong to a novel family of seven-transmembrane G-protein-coupled receptors that are activated through the cleavage of their N-terminal domains by proteases (Macfarlane et al., 2001; Ossovskaya and Bunnett, 2004). The proteolytic cleavage of the N-terminal region of PARs unmasks a new N-terminal sequence that acts as a tethered ligand, binding and activating the receptor itself. Molecular cloning studies have led to the identification of four members of the family: PAR-1, PAR-2, PAR-3 and PAR-4. Thrombin activates PAR-1, PAR-3 and PAR-4. Trypsin activates both PAR-2 and PAR-4, and mast cell tryptase can induce PAR-2 activation. Cathepsin G released from neutrophils triggers PAR-4 activation. Activation of PARs requires the release or generation of an active protease in proximity to target cells expressing the requisite receptor. PARs are expressed on a variety of cells such as platelets, keratinocytes, endothelial cells and neutrophils, and can elicit various responses including platelet aggregation, chemotaxis, cell proliferation and rises in levels of intracellular calcium (Brass and Molino, 1997; Hou et al., 1998; Cocks et al., 2000). Activation of PARs by proteases has been suggested to be involved in pain, and in allergic and neurogenic inflammation.

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by synovial inflammation and hyperplasia leading to progressive cartilage and bone destruction (Harris, 1990). Many soluble mediators such as cytokines and growth factors are involved in the development of RA (Feldmann et al., 1996). In addition to inflammatory mediators, high levels of tryptase and thrombin have been detected in synovial fluid from patients with RA (Buckley et al., 1997; Nakano et al., 1999). Inhibitors of thrombin have been found to ameliorate the inflammatory reaction in models of experimental arthritis (Varisco et al., 2000). Also, there is evidence for a key role of PAR-2 in

* Corresponding author. Fax: +81 46 251 5871.
E-mail address: inoue@lab.minophagen.co.jp (H. Inoue).

0024-3205/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.lfs.2006.03.034

mediating chronic inflammation in arthritis induced by adjuvant in PAR-2 deficient mice (Ferrell et al., 2003). These findings highlight the potential for PARs and their activating proteases to participate in the pathogenesis of arthritis.

Synovial cells may be categorized into three main types; macrophages, fibroblasts and dendritic cells, each having a different morphology and expressing different surface antigens (Burmester et al., 1983). Synovial fibroblasts are a major component of RA pannus tissue and a significant contributor to the pool of chemical mediators such as IL-1β and TNF-α seen in inflammatory pathologies (Firestein, 1996). Basic fibroblast growth factor (bFGF) has also been implicated in joint destruction in RA (Manabe et al., 1999). Furthermore, bFGF induces the production of substance P (Inoue et al., 2001a), which causes neurogenic inflammation (Pernow, 1985; Holzer, 1988), and the expression of its receptor, neurokinin-1 receptors known as a seven-transmembrane G-protein-coupled receptor, in RA synovial fibroblasts (Akasaka et al., 2005). Previous studies indicate the possibility that bFGF may play a role of regulation in the expression of soluble factors and their receptors in human synovial fibroblasts.

In the present study, we have examined whether bFGF can regulate expression of mRNA for PARs in synovial human synovial fibroblasts. We report that cultured RA fibroblasts can express mRNA for PAR-1, PAR-2 and PAR-3 but not for PAR-4, and that bFGF can selectively up-regulate the expression of PAR-2.

Materials and methods

Materials and cell culture

bFGF, IL-1β and TNF-α were purchased from Becton Dickinson Labware (Bedford, MA, USA). PAR-2 agonist peptide (SLIGKV-NH_2) and the inactive reverse peptide (VKGILS-NH_2) were purchased from BACHEM (Bubendorf, Switzerland). Human RA synovial fibroblasts were obtained from Cell Applications (San Diego, CA, USA). Synovial fibroblasts used in this experiment were derived from two donors of RA patients. Fibroblasts were cultured in α-minimum essential medium (α-MEM) containing 10% heat-inactivated fetal calf serum (FCS) and 60 μg/ml kanamycin sulfate at 37 °C in 5% CO_2 humidified air. The cells at 6–12 population doubling levels (PDL) were used for subsequent experiments. Confluent cells were kept in α-MEM containing 0.5% FCS for 24 h and then exposed to bFGF, IL-1β or TNF-α for various times.

RT-PCR analysis

Total RNA was extracted from synovial fibroblasts (10^6 cells) by acid guanidine–phenol–chloroform extraction using ISOGEN (Nippon Gene, Toyama, Japan). About 1 μg of total RNA was subjected to reverse transcription using the RNA PCR Kit (AMV) Ver.2.1 (TaKaRa Biomedicals, Shiga, Japan) according to the manufacturer's protocol. The cDNA was amplified by PCR with the following primers: PAR-1, 5′-TGT GAA CTG ATC ATG TTT ATG-3′ (sense) and 5′-TTC GTA AGA TAA GAG ATA TGT-3′(antisense) (Lourbakos et al., 2001); PAR-2, 5-TCT TGG GAG ACA TGT TC-3 (sense) and 5-TTT ACA GTG CGG ACA CTT CG-3 (antisense); PAR-3, 5′-GAA AGC CCT CAT CTT TGC AG-3′(sense) and 5′-AGG TGA AAG GAT GGA CGA TG-3′ (antisense) (Hamilton et al., 2001); PAR-4, 5′-AAC CTC TAT GGT GCC TAC GTG C-3′ (sense) and 5′-CCA AGC CCA GCT AAT TTT TG-3′ (antisense) (Miike et al., 2001). The human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) 5′-ACC ACA GTC CAT GCC ATC AC (sense) and 5′-CCA CCA CCC TGT TGC TGT AG-3′ (antisense). PCR amplification was performed with an initial denaturation step at 95 °C for 9 min, followed by 30 cycles or for PAR-3, 25 cycles, and for GAPDH, 20 cycles; 94 °C for 30 s, 59 °C for 30 s, and 72 °C for 30 s and final elongation at 72 °C for 7 min. Each PCR product was separated by gel electrophoresis on 3% agarose gels and visualized with SYBR Gold (Molecular Probes, CA, USA). The identity of each amplified products was confirmed by sequence analysis. The oligonucleotides were synthesized by Hokkaido System Science (Hokkaido, Japan). No PCR product was amplified without a reverse transcription reaction. The fluorescence intensity of the band was measured with ATTO Lane and Spot analyzer ver.6 (ATTO, Tokyo, Japan).

Immunocytochemical analysis

Cells were grown to 70–80% confluence in four-well chamber slides (Nunc, NY, USA). Semiconfluent cells were kept in α-MEM containing 0.5% FCS for 24 h and then treated with bFGF for 24 h. After fixing in acetone for 15 min, endogenous peroxidase activity was blocked with 0.1% sodium azide and 0.3% hydrogen peroxide for 30 min, and non-specific protein binding sites were blocked with 10% FCS and 5% BSA in RPMI culture medium. Monoclonal antibody P2A which is specific for a peptide sequence of human PAR-2 (Aslam et al., 2002) was added to slides at a dilution of 1/400 (and incubated overnight at room temperature). As a control, the PAR-2 specific antibody was pre-adsorbed with the peptide immunogen (^{30}TNRSSKGR SLIGK^{42}VC). Biotinylated anti-mouse immunoglobulins (Sigma, Poole, UK) were applied as the secondary antibody for 2 h at room temperature, and after washing, streptavidin–biotin–peroxidase complexes (Biogenex, Berkshire, UK) were added for a further 2 h at room temperature. Immunostaining was developed with diaminobenzidine, and the slides were counterstained with Mayer's hematoxylin.

Measurement of calcium mobilization

To determine changes in intracellular calcium mobilization, Calcium kit-Fluo-3 (DOJINDO, Kumamoto, Japan) was used. Briefly, fibroblasts were plated on a 96-well plate at 1.5×10^3 cells/well and cultured for 48 h in the 10% FCS/α-MEM. Cells were changed to 0.5% FCS/α-MEM and incubated for 24 h, and then exposed to bFGF for 24 h. After bFGF treatment, the cells were loaded with fluorescent calcium indicator Fluo-3 acetoxy methylester (AM 5 μg/ml) in the loading buffer containing