Induction of vascular endothelial growth factor expression in synovial fibroblasts by prostaglandin E and interleukin-1: a potential mechanism for inflammatory angiogenesis

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Abstract Inflammatory mediators such as prostaglandin E_2 (PGE_2) and interleukin-1 (IL-1) induce angiogenesis by yet undefined mechanisms. We demonstrate that PGE_2 and IL-1 induces the expression of vascular endothelial growth factor (VEGF), a selective angiogenic factor by rheumatoid synovial fibroblast cells. Transcripts for the EP_1 and EP_2 subtypes of PGE receptors are expressed in synovial fibroblasts. Activators of protein kinase A pathway stimulated the expression of VEGF whereas down-regulation of protein kinase C did not influence the PGE effect, suggesting that signalling from the EP_2 receptor via the protein kinase A pathway is important. The induction of VEGF expression by PGE_2 and interleukin-1α may be an important mechanism in inflammatory angiogenesis.

Key words: Angiogenesis; Interleukin-1α; Prostaglandin E_2; Rheumatoid arthritis; Vascular endothelial growth factor

1. Introduction

Angiogenesis is the process by which new blood vessels grow by sprouting from established ones. Blood vessel growth occurs during embryonic development but rarely in the adult. However, angiogenesis is important in the female reproductive system, in wound healing and in pathological processes such as solid tumor development and rheumatoid arthritis (RA) [1]. RA is characterized by pronounced tumor-like expansion of the synovium, primarily because of exuberant proliferation of synoviocytes and blood vessels [2]. RA synovial tissue, which is rich in blood vessels, invades the periarticular cartilage and bone and thus destroys the joint. Angiogenesis is an important component of chronic synovitis, and its regulation is thought to be driven by local production of angiogenic factors such as fibroblast growth factor and vascular endothelial growth factor (VEGF) [4]. In addition, inflammatory mediators such as interleukin-1 (IL-1), tumor necrosis factor-α, IL-8 and prostaglandins (PG) also are involved in the induction of the angiogenic response [3,4].

The synthesis of prostaglandins is initiated by the cyclooxygenase-catalyzed oxidation of arachidonic acid into prostaglandin H_2 [5]. The main products of the cyclooxygenase pathway, namely prostaglandin E_2, (PGE_2), prostaglandin $F_{2\alpha}$, thromboxane A_2 and prostacyclin are produced by rheumatoid synovial tissues [6]. PGE_2, may play a key role in the erosion of cartilage and juxtaarticular bone [7,8]. PGE_2 but not $PGF_{2\alpha}$ was shown to induce the angiogenic response [9], but the mechanisms by which prostaglandins induce angiogenesis are not understood.

IL-1 is a primary inflammatory cytokine produced by a variety of cells such as monocytes, fibroblasts and endothelial cells [10]. Systemic levels of IL-1 have been found to correlate with RA disease activity [11]. Injection of IL-1 into joints can induce inflammatory synovitis [12]. The majority of IL-1 is produced by macrophages present in inflamed rheumatoid synovia; however, synovial fibroblasts can also synthesize IL-1 [13]. In synovial fluid and peripheral blood of patients with RA, prostaglandins are formed as a result of the proinflammatory effects of IL-1 [14,15].

VEGF is a family of proteins which are specific endothelial mitogens in vitro [16]. Members of the VEGF family were discovered because they are potent inducers of microvascular permeability, a process important in inflammation [17]. Antigenic VEGF was significantly increased in RA and cells expressing VEGF were localized to the RA synovial lining layer and subsynovial macrophages [18]. This result suggests a role for VEGF in RA-associated angiogenesis. Recently, PGE_2 was shown to induce VEGF expression in osteoblast cells in culture [19]. Hyperplastic connective tissues are comprised primarily of fibroblasts-like cells and new blood vessels [20,21] and, vessels growth is directly proportional to total fibrovascular growth [22]. We investigated the possibility that PGE_2 may induce angiogenesis in RA synovium by inducing VEGF expression. Here we report that PGE_1, PGE_2 and IL-1 induce the expression of VEGF in RA synovial fibroblasts.

2. Materials and methods

2.1. Materials

Materials were obtained from Sigma Chemical Co. unless otherwise indicated.

2.2. RNA isolation and Northern blot analysis

Synovial fibroblasts from RA patients were isolated as previously described [23]. Growth medium is Dulbecco's modified Eagle's medium (Mediatech) supplemented with 10% fetal calf serum (HyClone), 10% human AB serum (Sigma) and antibiotics: penicillin (100 U/ml), streptomycin (100 U/ml) and fungizone (0.25 μg/ml) (JRH Biosciences).

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Abbreviations: IL-1α, interleukin-1; PGE_1, prostaglandin E_1; PGE_2, prostaglandin E_2; RA, rheumatoid arthritis; RT-PCR, reverse transcriptase-polymerase chain reaction; VEGF, vascular endothelial growth factor.

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Cells were plated in 75 cm^2 flask (Costar) and were allowed to reach 95% confluency (7 days), then harvested with trypsin (JRH Biosciences) and passaged 1:3. Cells were used between the third and the tenth passages. Before experiments, a 75 cm^2 flask (95% confluency) was split 1:5 and plated in 100 × 20 mm tissue culture dish (Falcon) with 10 ml growth medium. Medium was changed at day 3 and 5 after plating, experiments were conducted on the 8th day. Total RNA was isolated by the method of Chomczynsky and Sacchi [24], by using Trizol Reagent (Life Technologies). RNA (10 μg) was electrophoresed through 1% agarose gel containing 2.2 M formaldehyde and the integrity of the RNA analyzed by ethidium bromide staining. The RNA was then transferred onto zeta-probe membranes (Bio-Rad) and cross-linked by UV light (Stratalinker, Stratagene). The 0.6 kb cDNA of human VEGF consisting of the entire coding region for the precursor of the 165-residue form (a generous gift from Dr. J. Abraham, Scios Nova Inc.) was labeled to high specific activity using [α-^{32}P]dCTP and a random primer labelling system (Rediprime, Amersham). Filters were hybridized and washed according to Church and Gilbert's protocol [25]. The bands were visualized by autoradiography.

2.3. Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA (1 μg) was converted to cDNA by incubation at 37°C for 1 h in reverse transcriptase buffer (50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol), 400 units of Moloney-murine leukemia virus reverse transcriptase (Life Technologies Inc.), 40 units of RNAsin (Promega Corp.), 0.4 mM dNTPs, and 120 ng of random hexamers (Pharmacia LKB Biotechnology Inc.). The cDNA was diluted to 250 μl and RT-PCR was performed using 5 μl of diluted cDNA in 50 μl of 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 0.25 mM dNTPs, 1.5 mM $MgCl_2$, 0.5 μg of sense and antisense primers and 2.5 units of Taq polymerase (Boehringer-Mannheim). Samples were amplified for 40 cycles of PCR reaction in which denaturation was done for 1 min at 94°C and extensions was for 3 min at 72°C. Annealing time was 2 min however, the annealing temperature was 55°C for VEGF transcript, 59°C for EP_1, and 64°C for EP_2, EP_3, and EP_4. Amplified cDNAs were analyzed by 1.4% agarose gel electrophoresis with visualization by ethidium bromide staining. The primers to the human VEGF flanked the variable (carboxyl-terminal) portion of the coding regions [26]. The sense primer was GAGTGTGTGCCCACTGAGGAGTCCAAC, the antisense primer was CTCCTGCCCGGCTCACCGCCTCGGCTT. The primers to the human prostaglandin receptor EP_1 are: sense primer = TCTACCTCCCTGCAGCGGCCACTG (nucleotides 976–999), antisense primer = GAAGTGGCTGAGGCCGCTGTGCCGGGA (nucleotides 1179–1206) [27]. The primers to the human prostaglandin receptor EP_2 were chosen according to Regan et al. [28]: the sense primer was CTTACCTGCAGCTGTACG (nucleotides 740–757), and the antisense primer was GATGGCAAAGACCCAAGG (nucleotides 1090–1107). The primers to human EP_{31} receptor were chosen according to Adam et al. [29]: the sense primer was GAGCACTGCAAGACACACACGGAG (nucleotides 937–960), the antisense primer was GATCTCCATGGGTATTACTGACAA (nucleotides 1312–1335). The primers to human EP_4 were chosen according to Bastien et al. [30]: the sense primer was CTGGCGATCAACCATGCCTATTTC (nucleotides 742–765) and the antisense primer was TGAGCACCACCAGGGAGGTGGCAAAAT (nucleotides 1209–1236).

3. Results

3.1. IL-1α, PGE_2 and PGE_1 induce VEGF expression

Prostaglandins are important mediators of inflammation in RA [31,32]. IL-1 is a major proinflammatory cytokine present in synovial fluid and peripheral blood of RA patients. Because both agents have been implicated in inflammatory angiogenesis, we investigated the effects of PGE_1, PGE_2 and IL-1 on the induction of VEGF expression in RA synovial fibroblasts. The results obtained show that PGE_2, PGE_1 (1 μM) and IL-1α (10 ng/ml) induce a single VEGF transcript of approximately 3.8 kb (Fig. 1A). Induction of VEGF is specific to prostaglandin E_1 and E_2 since no induction was observed in prostaglandin $F_{2\alpha}$-treated cells. In addition, the VEGF transcript in human foreskin fibroblasts was not induced by PGE_2 whereas phorbol 12-myristate 13-acetate and $CoCl_2$ was capable of inducing it (data not shown). Four isoforms of VEGF coding regions have been described. A cDNA clone encoding a 189-amino-acid form of human VEGF ($VEGF_{189}$) has been isolated from U-937 cells [33]. cDNAs clones encoding $VEGF_{189}$ and 165-and 121-amino acid forms of human VEGF ($VEGF_{165}$ and $VEGF_{121}$, respectively) was isolated from phorbol ester-activated HL60 cells [34]. Another isoform of VEGF, 206 amino acids in length ($VEGF_{206}$) was found in fetal liver library [35]. To investigate the occurrence of the VEGF coding region heterogeneity in synovial fibroblasts, we used RT-PCR with primers flanking

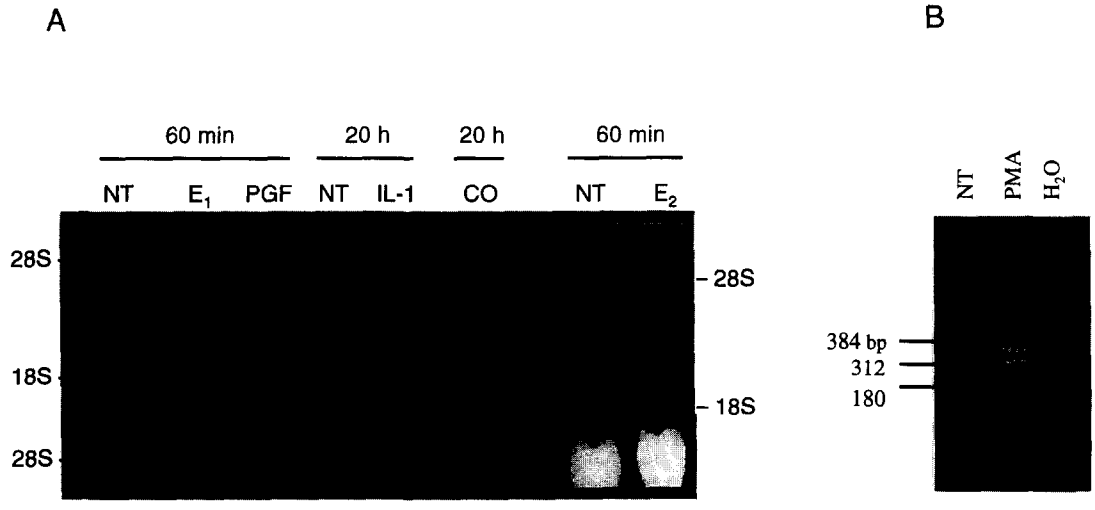

Fig. 1. Induction of VEGF expression in RA synovial fibroblasts. (A) Confluent monolayers of RA synovial fibroblasts were treated with 1 μM of PGE_1 (E_1), prostaglandin $F_{2\alpha}$ (PGF) and PGE_2 (E_2) for 1 h, or with 10 ng/ml IL-1 or 100 μM $CoCl_2$ for 20 h, or maintained under normal conditions (NT). Total RNA (10 μg) was analyzed by Northern blot utilizing a $VEGF_{165}$ radiolabeled probe. The locations of 28S and 18S rRNA are indicated. Autoradiographic were exposure is for 2 days. (B) Confluent monolayers of synovial fibroblasts were maintained under normal conditions (NT), or exposed for 6 h to 100 ng/ml phorbol 12 myristate 13-acetate (PMA). RNA (1 μg) was converted to cDNA and RT-PCR with the primers to the human VEGF was performed. PCR reaction in the absence of RT (H_2O) served as negative control.

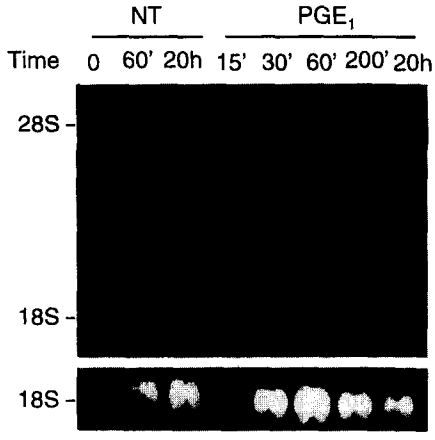

Fig. 2. Time-course of VEGF expression in response to PGE_1. Confluent monolayers of synovial fibroblasts were treated with PGE_1 (1 μM) for the indicated times or, maintained under normal conditions (NT). Total RNA (10 μg) was analyzed by Northern blot utilizing a $VEGF_{165}$ radiolabeled probe. The locations of 28S and 18S rRNA are indicated. Autoradiographic exposure is for 2 days.

the VEGF variable (carboxyl-terminal) portion [26]. RNA was extracted from synovial fibroblasts treated with phorbol 12-myristate 13-acetate (100 ng/ml, 6 h) and RT-PCR reaction was performed as described in section 2. The expected sizes of the PCR products are 435, 384, 312, and 180 for $VEGF_{206}$, $VEGF_{189}$, $VEGF_{165}$ and $VEGF_{121}$, respectively. In control untreated cells, the analysis revealed a minute presence of two bands of 312 and 384 bp corresponding to $VEGF_{165}$ and $VEGF_{189}$, respectively. In phorbol 12-myristate 13-acetate-treated cells however, there was a marked elevation in $VEGF_{165}$ and $VEGF_{189}$ as well as induction of a band of 180 bp, corresponding to $VEGF_{121}$ (Fig. 1B).

The kinetics of VEGF expression was determined at 15–20 h after treating the cells with PGE_1 and PGE_2 and, 5–48 h after treating the cells with IL-1. Treatment of synovial fibroblasts with 10^{-6} M PGE_1 resulted in transient increase in the level of VEGF mRNA which peaked at 60 min and declined within 3 h (Fig. 2). In contrast to the rapid response of the cells to prostaglandins, IL-1α induced an elevation in VEGF mRNA only after 8 h; this effect, however, was sustained up to 48 h (Fig. 3A). IL-1 is a potent and sustained inducer of PG synthesis by up-regulation of Cox-2 expression [41]. To determine if IL-1-induced PGE_2 is involved in the VEGF induction, RA synovial fibroblasts were treated with IL-1α in the presence of indomethacin, an inhibitor of the cyclooxygenase activity [6]. As shown in Fig. 3B, IL-1 induction of VEGF was not blocked by indomethacin, ruling out the involvement of autocrine PGE_2 action.

3.2. *Signal transduction mechanisms involved in PGE induction of VEGF transcript*

PGE_1 or PGE_2 mediates its action via interaction with four pharmacologically defined subtypes of prostaglandin receptors [27–30]. All these subtypes display high affinity for PGE_2 but show differences in their affinities for various agonists and antagonists and exert their actions through different signal transduction mechanisms. Thus activation of the EP_1 receptor has been associated with a rise in intracellular calcium [27], activation of the EP_2 and EP_4 receptors with increases in intracellular cAMP [28,30], respectively, and activation of the EP_3 receptor with a decrease in intracellular cAMP [36,37]. Identification of the coupling mechanisms between the prostaglandin receptors and the intracellular components requires the initial identification of the cellular receptor subtype. To this end, cDNA from synovial fibroblasts was amplified with four distinct receptor-specific oligonucleotide primers. Amplified products of human EP_1 and EP_2 were observed (Fig. 4) without detection of either EP_3 or EP_4. Adenylate cyclase and/or phospholipase C/protein kinase C pathways might therefore be involved in the induction of VEGF transcription by PGE_2.

To further investigate the signalling mechanisms, RA synovial fibroblasts were treated with PGE_2 in the presence or the absence of cholera and pertussis toxins, which modulate the activities of the G_s and the G_i proteins, respectively. Treatment with pertussis toxin did not influence the PGE effect, suggesting that the EP_3/G_i pathway is not involved. However, cholera toxin and forskolin, agents that activate the intracellular cAMP

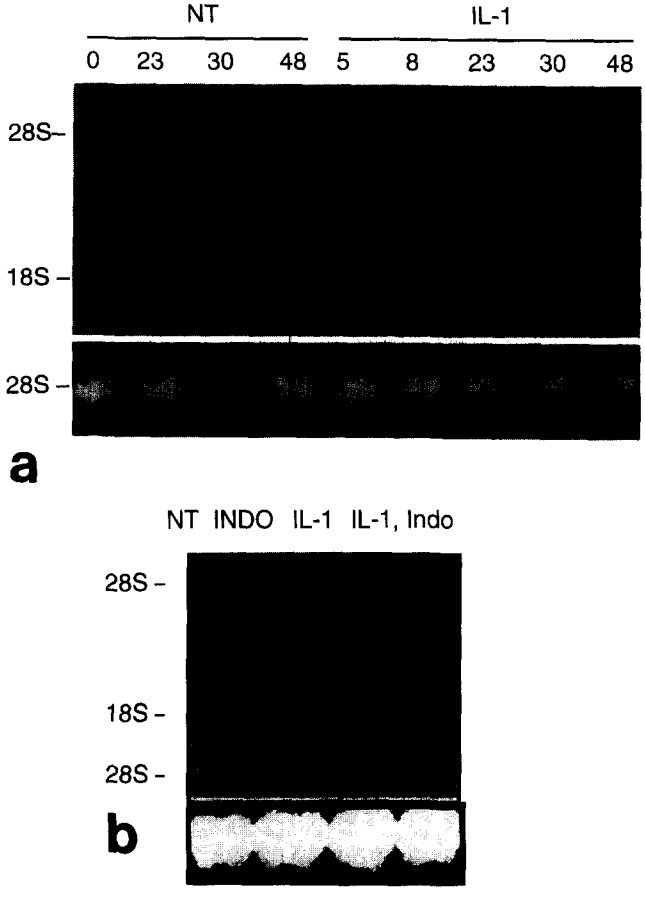

Fig. 3. Induction of VEGF expression by IL-1. (A) Confluent monolayers of synovial fibroblasts were treated with IL-1 (10 ng/ml) for the indicated times or maintained under normal conditions (NT). Total RNA (10 μg) was analyzed by Northern blot utilizing a $VEGF_{165}$ radiolabeled probe. The locations of 28S and 18S rRNA are indicated. Autoradiographic exposure is for 2 days. (B) Confluent monolayers of synovial fibroblasts were treated with IL-1 (10 ng/ml), indomethacin (2 μM) or the combination of IL-1 and indomethacin for 20 h or, maintained under normal conditions (NT). Total RNA (10 μg) was analyzed by Northern blot utilizing a $VEGF_{165}$ radiolabeled probe. Autoradiographic exposure is for 2 days.

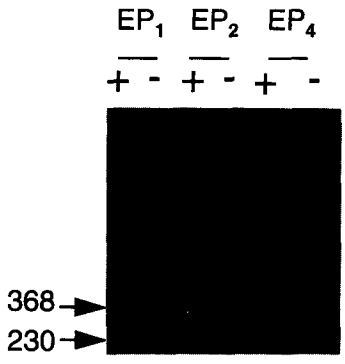

Fig. 4. Detection of the prostaglandin receptors in synovial fibroblasts. RNA (1 μg) from confluent monolayers of synovial fibroblasts was converted to cDNA. RT-PCR reaction with the primers to the human EP_1, EP_2 and EP_4 was performed. − = minus reverse transcriptase control.

levels potently activated the VEGF mRNA levels. (Fig. 5A). This suggests that EP_2/G_s/cAMP/protein kinase-A pathway is involved in the VEGF induction by PGE_2. To further investigate the possible influence of protein kinase-C, a major signalling pathway activated by EP_1 subtype of receptors, RA synovial fibroblasts were treated with phorbol myristic acetate (PMA) for 16 h to down-regulate the intracellular protein kinase C. Whereas PMA was able to upregulate VEGF expression in control cells, down-regulated cells did not respond to PMA treatment for 6 h. However, PGE_2 was able to activate VEGF expression in protein kinase-C down-regulated cells, suggesting that EP_1/G_q/phospholipase C/protein kinase C pathway is not involved in the PGE induction of VEGF expression (Fig. 5B).

4. Discussion

Spatially- and temporally-regulated formation of new blood vessels is required for the development and repair of all tissues. Angiogenesis involves the proliferation, migration and differentiation of endothelial cells and is regulated by polypeptide growth factors. VEGF is the only well-characterized secreted mitogenic factor which acts exclusively on endothelial cells [38]. In a mouse model of proliferative retinopathy, it was found that VEGF is expressed in the retina prior to the development of neovascularization, suggesting that it is a primary angiogenic factor [39].

While PGE_1 and PGE_2 are potent inducers of angiogenesis in vivo [9], their mechanism of action is unknown. PGE_1 and PGE_2 do not directly stimulate angiogenesis in vitro on endothelial cells (unpublished results). High levels of prostaglandins are produced by RA synovia and dysregulated angiogenesis is a major feature of the pathology of this chronic inflammatory process [8,9]. We therefore investigated whether PGE_1 and PGE_2 induced the VEGF expression by RA synovial fibroblasts. The results obtained demonstrate that PGE_1, PGE_2 and IL-1α induce the expression of VEGF in synovial fibroblasts, albeit with different kinetics. Interestingly, PGE_2 did not induce VEGF mRNA in human foreskin fibroblasts. Cell-type specific regulation of VEGF expression by PGE_2 could be related to the presence or absence of relevant PGE_2 receptor subtypes. Recently, it was shown that IL-1β induces VEGF in rat aortic smooth muscle cells [40]. IL-1 also induces the sustained synthesis of prostaglandins via induction the transcriptional induction and stabilization of the mRNA for the enzyme cyclooxygenase-2 [41]. Because indomethacin did not block the ability of IL-1 to induce VEGF expression, it is unlikely that autocrine stimulation by PGE_2 is involved. However, indomethacin does not

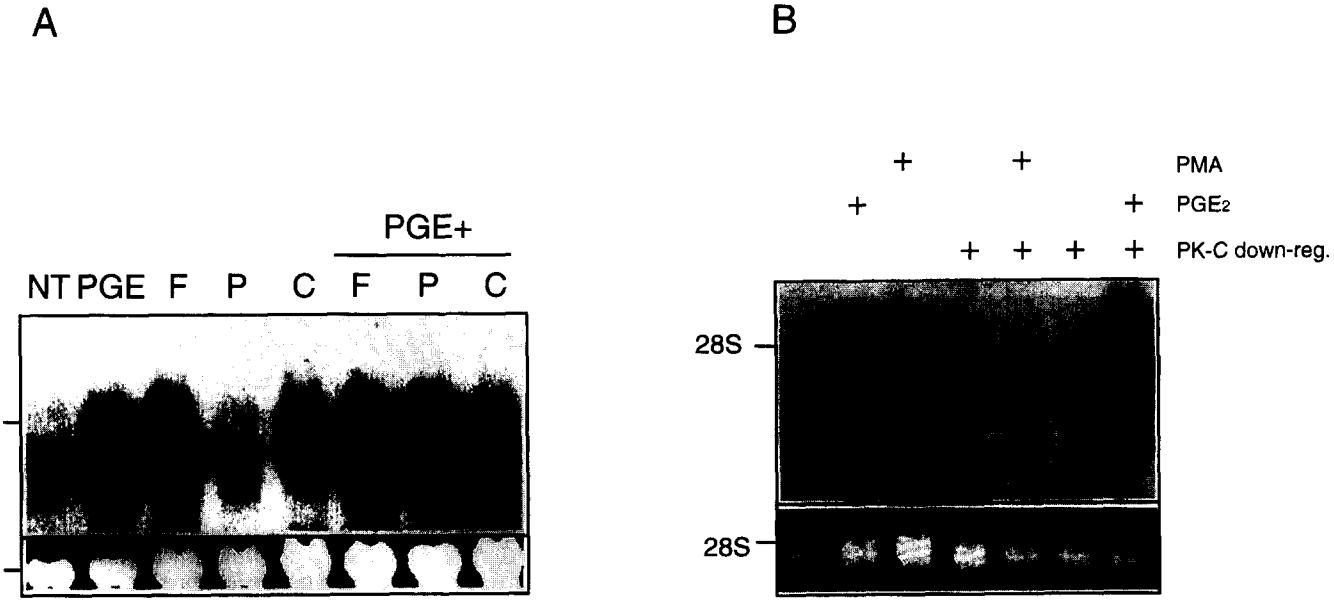

Fig. 5. Signalling mechanisms in PGE induction of VEGF mRNA. (A) Confluent monolayers of synovial fibroblasts were treated with PGE_2 (1 μM), forskolin (10 μM), pertussis toxin (pertussis) (20 ng/ml), cholera toxin (cholera) (50 ng/ml), or the combination of PGE_2 with forskolin, pertussis toxin and cholera toxin for 1 h, or, maintained under normal conditions (NT). Total RNA (10 μg) was analyzed by Northern blot utilizing a $VEGF_{165}$ radiolabeled probe. Autoradiographic exposure is for 2 days. (B) Confluent monolayers of synovial fibroblasts were treated with PGE_2 (1 μM) for 1 h, PMA (100 ng/ml) for 6 h or, maintained under normal conditions (NT). Pretreatment of the cells with 100 ng/ml PMA for 16 h was used as a procedure to down-regulate PKC. Cells were then treated with PGE_2 for 1 h or with PMA for 6 h or, without any further addition. Total RNA (10 μg) was analyzed by Northern blot utilizing a $VEGF_{165}$ radiolabeled probe. Autoradiographic exposure is for 2 days.

block the peroxidase activity of Cox-2 and other IL-1-inducible genes which may be important.

PGE_2, which is a potent stimulator of angiogenesis [9], induces a rapid induction of VEGF mRNA in synovial fibroblasts. We also found that RA synovial fibroblasts express the prostaglandin receptors, EP_1 and EP_2. Various prostaglandin receptors were found in human erythroleukemia cell line [42]. EP_1 elevates intracellular Ca^{2+} levels in injected *Xenopus* oocytes [27] probably due to coupling phospholipase C. EP_2 stimulates the formation of cAMP in EP_2-transfected Cos-7 cells [28]. Because forskolin and cholera toxin also stimulated VEGF expression and because down-regulation of protein kinase C is not important for the PGE induction of VEGF mRNA, EP_2/G_s/adenylate cyclase/protein kinase-A may be an important signalling pathway in RA synovial fibroblasts. Induction of VEGF by protein kinase A and protein kinase C pathways was also observed in osteoblasts [19] and preadipocytes [43].

IL-1, PGE_2 and PGE_1 but not $PGF_{2\alpha}$ strongly stimulate the expression of VEGF in RA synovial fibroblasts. Since these agonists are capable of inducing angiogenesis [9,44,45], the induction of VEGF may be an important mechanism in inflammatory angiogenesis. Pharmacologic modulation of this pathway may be potentially useful in controlling RA.

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