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TNF-α modulates angiopoietin-1 expression in rheumatoid synovial fibroblasts via the NF-κB signalling pathway

B.B. Scott[a,*,1], P.F. Zaratin[a,2], A.G. Gilmartin[b,3], M.J. Hansbury[b,4], A. Colombo[a], C. Belpasso[a], J.D. Winkler[b,5], J.R. Jackson[b,3]

[a] *GlaxoSmithKline Pharmaceuticals, Neurobiology, Department of Neuroscience, Milan, Italy*
[b] *GlaxoSmithKline, Oncology Research, King of Prussia, PA, USA*

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Abstract

Angiopoietin-1 (Ang-1) is one of a family of ligands for the Tie-2 receptor which has been demonstrated to be involved in angiogenesis. Little is known about the regulation of Ang-1 gene expression. We have previously demonstrated that TNF-α is able to upregulate the expression of Ang-1 mRNA in synovial fibroblasts. This present study investigated the signal transduction pathways involved in the TNF-α induced expression of Ang-1. TNF-α signals primarily through the p38, JNK, MAP kinase, and IKK pathways resulting in the activation of the transcription factors AP-1 and NF-κB. Experiments with inhibitors and siRNA for these various signal transduction pathways revealed that TNF-α stimulation of Ang-1 expression occurs via the NF-κB signal transduction pathway.

Keywords: TNF-α; Angiopoietin-1; NF-κB; Tie-2 receptor; Synovial fibroblasts; Rheumatoid arthritis; siRNA

Angiogenesis has been shown to have an important role in tissue repair and in promoting the growth of new tissue in neoplasia and a number of inflammatory conditions such as rheumatoid arthritis (RA), psoriasis, and wound healing [1–4]. Chronic unregulated angiogenesis is a pathological feature of rheumatoid arthritis (RA) which is detectable in early disease [3]. Angiogenesis within the synovial tissue is required to support the inflammation and hyperplasia that occurs within the rheumatoid joint [4,5]. The synovial fibroblast is a key player in the RA disease process and has been demonstrated to constitutively express VEGF, which is up regulated by angiogenic stimuli within the RA synovial environment such as IL-1β and hypoxia [6]. Hence, the synovial fibroblast may be an important source of regulatory factors for the angiogenesis occurring within the RA joint.

The angiopoietin/Tie-2 receptor system is another important regulator of angiogenesis, and we and others have previously demonstrated that rheumatoid synovial fibroblasts constitutively express angiopoietins (Ang-1 and Ang-2), which are ligands for the Tie-2 receptor [7,8]. It is hypothesized that the role of Ang-1 is to differentiate and stabilize newly formed vessels, recruiting pericytes and smooth muscle cells to encapsulate and support the vessel, whereas Ang-2 functions to inhibit the effect of Ang-1 causing a decrease in vessel integrity,

* Corresponding author. Fax: +610 461 2006.
E-mail address: bscott@concurrentpharma.com (B.B. Scott).
[1] Present address: Concurrent Pharmaceuticals, Fort Washington, PA, USA.
[2] Present address: Serono SpA, Ivrea, Piemonte, Italy.
[3] Present address: GlaxoSmithKline, Department of Molecular Oncology, Collegeville, PA, USA.
[4] Present address: Incyte, Wilmington, DE, USA.
[5] Present address: Array Biopharma, Boulder, CO, USA.

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and allowing the angiogenic process to begin [9–11]. Although the biology of the angiopoietin-Tie-2 system has become clearer, still little is known about the regulation of expression of the components of this system.

In our previous work [7] we demonstrated that the expression of Ang-1 was modulated by TNF-α. In this study, we used this observation to attempt to dissect which signal transduction pathways may be involved in TNF-α induced up-regulation of Ang-1 expression in this inflammatory setting, to hopefully gain some insight into the regulation of Ang-1 expression, and to identify opportunities for pharmacological intervention. TNF-α and its receptors activate multiple signalling pathways involving NF-κB, protein kinases (MAPK/JNK/p38), and apoptosis through the engagement of various adaptor proteins [12–16]. Our findings suggest that TNF-α induces Ang-1 expression primarily through NF-κB activation in rheumatoid synovial fibroblasts.

Materials and methods

Cell culture

Rheumatoid arthritis synovial fibroblasts (RA-SF) were generated and cultured as described in detail previously [7]. Briefly, established synovial fibroblast cell lines were maintained in EMEM culture media (minimal essential media with Earle's salts and L-glutamine (Invitrogen Corp., Carlsbad, CA, USA)) with 10% FBS, penicillin, and streptomycin, and were used between passage 3 and 9 for studies.

Signal transduction pathway studies

Inhibitors. RA-SF were plated into 6-well tissue culture plates and grown to confluence in EMEM culture media. Cells were treated with individual compounds for 30 min followed by stimulation with TNF-α (2 ng/ml) for 24 h (R&D Systems, Minneapolis, MN, USA). Compounds used included SB203580, CEP-1347, Hymenialdesine, Sulfasalazine (Sigma, St. Louis, MO, USA), U0126 (Promega, Madison, WN, USA), and Dexamethasone (Sigma, St. Louis, MO, USA). Unless specified all compounds were generated in-house. Conditioned media from each time point were collected for experiments detailed below.

siRNA. Employing protocols for inhibition by siRNA in RA-SF, as described previously by Zhang et al. [17], RA-SF were cultured to subconfluence in 24-well plates. Cells were transfected with 25 nM siRNA for either NF-κB/p65, pool p38 or control siRNA (Cell Signaling Technology, MA, USA) in a final volume of 100 μl of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). The cells were cultured in a final volume of 500 μl for 48 h. The biologic effects were analyzed 24 h. after addition of TNFα. Western analyses were employed to measure protein expression and determine the effect of siRNA on target proteins using NF-κB and p38 antibodies (Cell Signaling Technology, MA, USA). Antibody to MAPKAP-2 was used as a control (Cell Signaling Technology, MA, USA) for specificity of siRNA effect.

RNA isolation and cDNA synthesis

Cells were harvested by trypsinization and total RNA was isolated using RNeasy reagents (Qiagen, Chatsworth, CA, USA) according to the manufacturer's protocol and quantitated by spectrophotometry. cDNA was synthesized from 1 μg of RNA using the Superscript II (Invitrogen, Carlsbad, CA, USA) reverse transcriptase again following the manufacturer's protocols.

Quantitative PCR using Taqman system

The cellular levels of Ang-1 gene expression were quantified by real time quantitative Taqman PCR using an ABI PRISM 7700 (Perkin–Elmer, CA, USA). Specific primers and dual-labelled fluorescent probes were designed to detect Ang-1 mRNA using the Primer Express primer design program v1.01 (Perkin–Elmer, CA, USA). The constitutively expressed GAPDH was used as an internal control.

The primer and probe sequences were as follows: Ang-1 forward primer 5′-agctgtgatcttgtcttggcc-3′, Ang-1 reverse primer 5′-aaagtgt agctgcaggaccca-3′ and Ang-1 probe 5′-fluor label, 6-FAM-ctttcggaag agcatggacagcatagga-3′. GAPDH forward primer 5′-gaaggtgaaggtcg gagt-3′, GAPDH reverse primer 5′-gaagatggtgatgggatttc-3′, and GAPDH probe 5′-fluor label JOE-caagcttcccgttctca-3′.

PCR conditions were: *Taq* PCR buffer, $MgCl_2$ 5 mM, dNTPs (dATP, dCTP, and dGTP all 300 mM, dUTP 600 mM), 0.01 U/reaction UNG, and 1.25 U/reaction TaqMan gold. Primer concentrations were 300 nM and probe concentrations were 100 nM, except for GAPDH which were 100 nM for the primers and 50 nM for the probe. The thermal cycling was 50 °C for 2 min, 95 °C for 10 min followed by 45 cycles of 95 °C for 15 s and 60 °C for 90 s. All reagents for PCR including primer and probes were synthesized by Perkin–Elmer, CA, USA.

Data analysis

Data were analyzed using the cycle threshold (C_T) comparative method [18]. The PCR efficiency of the target (Ang-1 mRNA) and of the control (GAPDH) was equal to the cDNA concentrations used in these experiments. The amount of mRNA levels was given by $(1 + \text{E PCR efficiency})^{-\Delta\Delta C_T}$ where $\Delta\Delta C_T = [C_T \text{ Target} - C_T \text{ GAPDH}]_{RA} - [C_T \text{ Target} - C_T \text{ GAPDH}]_{control}$ and C_T = PCR cycle at which the amplification plot crosses the baseline threshold. The range given for the fold increase in target mRNA relative to control (level in the normal synovial fibroblast NSF-1) is determined by evaluating the expression: $2^{-\Delta\Delta C_T}$ with $\Delta\Delta C_{T+S}$ and $\Delta\Delta C_{T-S}$, where S = the standard deviation of the $\Delta\Delta C_T$ value.

Experiments where inhibitors were used were expressed as a percent of the TNF induced effect and plotted using XLfit 4.0 software (IDBS, Surrey, UK).

Autophosphorylation Western analysis

Serum-starved HUVECs (CSC System, Washington, USA) were treated with the conditioned media from the RA-SF or RA-SF treated for 24 h with TNF-α (2 ng/ml) with or without pre-treatment with inhibitors for 30 min. The conditioned media were diluted 50% into serum free HUVEC medium (CSC Systems) and applied to the HUVECs. The cells were incubated at 37 °C for 20 min and then placed on ice, and 500 μl of lysis buffer (RIPA lysis buffer: 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1.0% NP-40, 0.5% deoxycholate, 0.1% SDS + inhibitors: 1 mM sodium orthovanadate, 1 mM sodium fluoride, and 1 mM EDTA + Sigma mammalian cell protease inhibitor cocktail) was added to each well. Seven micrograms of anti-Tie-2 (R&D Systems, MN, USA, or Santa Cruz Biotech., CA, USA) was added to each lysate and incubated for 1 h at 4 °C. Twenty microliters of protein G–agarose slurry was then added and incubated for an additional hour at 4 °C. The agarose/antibody complexes were pelleted, washed with cold lysis buffer, and then resuspended in 40 μl of 1× SDS–PAGE reducing sample buffer. Thirty-five microliters of sample was run on a 4–12% Bis-Tris NuPAGE gels (Invitrogen, CA, USA).

The gel was then transferred to a nitrocellulose membrane (Invitrogen, CA, USA) for Western blotting. The blots were washed with PBS 0.05% Tween 20 and then blocked with 5% milk/PBS/0.005% Tween for 1 h at room temperature. The blots were then incubated with 1 μg/ml anti-phosphotyrosine antibody (Upstate Biotech., MA, USA) or anti-Tie-2 and HRP conjugated secondary antibodies