Molecular Immunology 67 (2015) 532–539

Contents lists available at ScienceDirect

Molecular Immunology

journal homepage: www.elsevier.com/locate/molimm

Sprouty2 suppresses the inflammatory responses in rheumatoid arthritis fibroblast-like synoviocytes through regulating the Raf/ERK and PTEN/AKT signals

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ARTICLE INFO

Article history:
Received 19 May 2015
Received in revised form 23 July 2015
Accepted 26 July 2015
Available online 8 August 2015

Keywords:
Sprouty2
Rheumatoid arthritis
Fibroblast-like synoviocytes
Inflammatory responses
Signaling pathways

ABSTRACT

AKT and ERK pathways are known to be activated in human rheumatoid arthritis (RA) fibroblast-like synoviocytes (FLS), which play crucial roles in the pathogenesis and joint destruction of RA. Sprouty2 (SPRY2) has been known as a tumor suppressor by preventing both ERK and AKT signaling activations. Whether SPRY2 can function as a suppressor in tumor-like inflammatory FLS through negatively regulating AKT and ERK pathways, has not been reported. The purpose of this study was to determine whether SPRY2 might have antiinflammatory effects on RA FLS. The recombinant adenovirus containing SPRY2 complementary DNA (AdSPRY2) was used to deliver SPRY2 and express the protein in RA FLS. Adenoviral vector encoding green fluorescent protein (AdGFP) was used as the control. AdSPRY2 treatment suppressed the production of proinflammatory cytokines and matrix metalloproteinases (MMPs), and the cell proliferation, induced by TNFα in RA FLS. SPRY2 overexpression reduced AKT and ERK phosphorylation in TNFα-stimulated FLS, through mediating or interfering with the activity of PTEN or Raf respectively. These results suggest that using SPRY2 to block the AKT and ERK pathways suppresses the inflammatory responses of RA FLS, and the development of an immunoregulatory strategy based on SPRY2 may therefore have therapeutic potential in the treatment of RA.

1. Introduction

Rheumatoid arthritis (RA) is a chronic systemic autoimmune disease mainly affecting the joints, characterized by proliferative synovitis, hyperplasia of the synovial tissues, and destruction of cartilage and bone. Although the trigger is still unidentified, significant progress has been made in the understanding of the pathogenesis of RA. Among those inflammatory cell populations which may participate in the pathogenesis of RA, fibroblast-like synoviocytes (FLS) are considered crucial in the development of arthritis (Huber et al., 2006). The activated FLS are found predominantly in the synovial intimal lining, and their proliferation in RA joint is considered to be as fierce as that of tumor cells (Okamoto et al., 2007). Tumor-like proliferation of FLS and their secretions such as proinflammatory cytokines and matrix metalloproteinases (MMPs), play pivotal roles in the development of synovial hyperplasia, sustained inflammation and joint destruction in arthritic joints (Bartok and Firestein, 2010; Mor et al., 2005).

Increasing studies have implicated the activation of both PI3K/AKT and Ras/ERK pathways in the aggressive tumor-like FLS and RA. AKT activation is an important pathophysiologic change associated with the proliferating synovium in RA. FLS from patients with RA express higher levels of phosphorylated AKT than those from patients with osteoarthritis (Zhang et al., 2001). PI3K/AKT signal pathway in RA FLS can be activated by proinflammatory cytokines such as tumor necrosis factor α (TNFα), and AKT activation plays a crucial role in stimulating FLS proliferation and production of inflammatory cytokines (that perpetuate inflammation) and MMPs (that contribute to cartilage destruction) (Chen et al., 2006; Xu et al., 2007). Correspondingly, down-regulation of AKT activation results in the anti-proliferative and anti-inflammatory effects in RA FLS and rat arthritis (Wang et al., 2008). Other studies from experimental animal models of RA also demonstrate that antagonizing PI3K/AKT signaling cascades can suppress joint inflammation, suggesting that AKT pathway may be a potential therapeutic target for RA (Wang et al., 2008; Randis et al., 2008; Camps et al., 2005).

Abbreviations: FLS, fibroblast-like synoviocytes; MMP, matrix metalloproteinases; MOI, multiplicities of infection; PFU, plaque-forming unit; PTEN, phosphatase and tensin homolog; RA, rheumatoid arthritis; SPRY2, Sprouty2; TNFα, tumor necrosis factor α.

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http://dx.doi.org/10.1016/j.molimm.2015.07.033
0161-5890/

Ras/ERK signal pathway is also involved in the activation of RA FLS and the destruction of bone in arthritic joints (Yamamoto et al., 2003). ERK is constitutively expressed in RA synovium and rheumatoid FLS, and the expression of the activated phosphorylated forms is much higher in RA synovium than in that of OA (Bartok and Firestein, 2010). ERK signaling cascades are well known to play an important role in RA. Inhibition of Ras/ERK signal has been provided as a novel approach for RA treatment, by targeting FLS activation and bone destruction (Yamamoto et al., 2003). The Ras/ERK pathway inhibitor can block the production of inflammatory cytokines and MMPs by RA FLS, and the invasiveness of FLS, by antagonizing the activation of ERK (Ahn et al., 2012). Impaired ERK activation results in the reduced MMP-3 expression in FLS, and pannus formation and cartilage erosion in animal model (Peters et al., 2012). Additionally, ERK signal plays an important role in the regulation of IL-6 and MMP-1 production in RA FLS (Ahn et al., 2013). These studies demonstrate the important immunoregulatory role of Ras/ERK pathway in the pathogenesis of RA via inflammatory FLS. Overall, PI3K/AKT and Ras/ERK pathways are potential and attractive targets for the therapy of RA.

Sprouty proteins (SPRY1–4) are a family of negative regulators of receptor tyrosine kinase (RTK) characterized by a highly conserved cysteine-rich C-terminal sequence. Sprouty was originally identified in *Drosophila* as an antagonist of fibroblast growth factor (FGF) signaling (Hacohen et al., 1998). There are four SPRY genes in mammals. Mounting evidence reveals that SPRY2 is a tumor suppressor and its expression is universally repressed in a series of tumors including breast (Lo et al., 2004), hepatocellular (Fong et al., 2006), lung (Sutterlüty et al., 2007), colon (Feng et al., 2011), prostate (Gao et al., 2012; Patel et al., 2013) cancers, as well as melanoma (Tsavachidou et al., 2004). Correspondingly, overexpression of SPRY2 inhibits proliferation, migration, and invasion in cultured tumor cells (Lee et al., 2004). SPRY2 exerts its negative feedback role of RTK signaling mainly through the Ras/ERK pathway by inhibiting the activation of Raf (Yusoff et al., 2002). To date, SPRY2 is a well-established inhibitor of the Ras/ERK signaling cascade. In addition to the Ras/ERK pathway, SPRY2 can also inhibit PI3K/AKT signaling through phosphatase and tensin homolog (PTEN) by enhancing its activation and stability (Edwin et al., 2006), and reduction in SPRY2 expression results in hyperactivation of PI3K/AKT signaling to drive tumor cell proliferation and invasion (Gao et al., 2012). Hence, SPRY2 can act as an inhibitor of both Ras/ERK and PI3K/AKT signaling pathways, and as a matter of fact, SPRY2 deficiency sufficiently triggers the dual signaling activation of ERK and AKT pathways (Patel et al., 2013).

Considering SPRY2 is a dual suppressor by inhibiting both Ras/ERK and PI3K/AKT signaling cascades in tumor cells, and these pathways are potential targets in the treatment of RA, SPRY2 might serve as a potent inflammatory suppressor of tumor-like FLS, and thus RA. In this study, we explore the possible role and molecular mechanisms of SPRY2 overexpression in the inhibition of inflammatory responses of RA FLS.

2. Material and methods

2.1. RA FLS

Human rheumatoid FLS isolated from inflamed synovial tissue from patients with RA were obtained from Cell Applications. FLS were cultured in synoviocyte growth medium, prepared by adding synoviocyte growth supplement (Cell Applications, San Diego, CA, USA) in synoviocyte basal medium (Cell Applications) at 37 °C in 5% CO_2, and subcultured with 0.25% trypsin/EDTA solution (Sigma–Aldrich, St. Louis, Mo, USA). Disposable 25 or 75 cm^2 sterile cell culture flasks (Corning, Tewksbury, MA, USA) were used for FLS culture. All cell culture plates were obtained from Costar. FLS were used from passages 3 to 5 for all experiments.

2.2. Expression of SPRY2 messenger RNA (mRNA) in RA FLS

Total RNA from RA FLS was extracted using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the instructions. Complementary DNA was synthesized using a reverse transcription system (Takara, Dalian, China), and the SPRY2 transcript was detected by PCR amplification using SPRY2-specific primers (upstream: 5′-GAGGCCAGAGCTCAGAGTGGCAACGGGTCG-3′; downstream: 5′-TGTTGGTTTTTCAAAGTTCCTAGGGGGGAC-3′). Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) was used as an internal reference gene control using specific primers (upstream: 5′-TCCACCACCCTGTTGCTGTA-3′; downstream: 5′-ACCACAGTCCATGCCATCAC-3′). Real-time quantitative PCR was performed using 1 μl of complementary DNA per well, TaqMan Master Mix (Applied Biosystems, Foster City, CA, USA), and 0.25 μM each of upstream and downstream primers (SPRY2: upstream, 5′-CCTCTGTCCAGATCCATAAG-3′, downstream, 5′-GCAGCAGCAGGCCCGTGGGA-3′; G3PDH: upstream, 5′-GGGAAGCTTGTCATCAATGG-3′, downstream, 5′-TGGACTCCACGACGTACTCA-3′). Results were evaluated using the ΔDCT method and the calculated number of copies was normalized to that of G3PDH copies in the same sample.

2.3. Recombinant adenovirus

The complementary DNA (cDNA) clone of human SPRY2 (Genebank accession No. NM_005842) was obtained from Origene technologies. The PCR product of SPRY2 gene containing a 6 × His tag sequence at 5′ end was subcloned into a shuttle vector pShuttle-CMV (Stratagene, Santa Clara, CA, USA). The resultant plasmid was linearized with PmeI, and transformed into *Escherichia coli* strain BJ5183 together with an adenoviral backbone vector pAdEasy-1 (Stratagene) by electroporation with a Bio-Rad Gene Pulser. The recombinant plasmid was obtained and validated by PCR and PacI digestion. The adenovirus plasmid was linearized with PacI and transfected into human embryonic kidney 293 (HEK293) cells with lipofectamine 2000 (Invitrogen Life Technologies). 293 cells were cultured in Dulbecco's modified Eagle's medium (Hyclone, Logan, Utah, USA) supplemented with 10% fetal bovine serum (PAA, Shanghai, China). The recombinant virus AdSPRY2 was propagated in 293 cells and purified by cesium chloride density gradient ultracentrifugation. Control virus AdGFP was generated using the same procedure. Virus titers (plaque-forming unit, PFU) were determined by plaque assay.

2.4. Cell lysates

The collected cells were suspended in Laemmli sample buffer (Bio-Rad, Hercules, CA, USA) containing 5% β-ME, followed by boiling for 5 min. The supernatants were collected as cell lysates, and the protein concentration was measured using BCA protein assay kit (Pierce, Appleton, WI, USA).

2.5. Expression of AdSPRY2

293 cells (4×10^5 cells/well) or FLS (1×10^5 cells/well) were cultured in 6-well plates and infected with different multiplicities of infection (MOI) of recombinant AdSPRY2. Following incubation for 48 h, cells were collected and cell lysates were prepared. AdSPRY2 expression in cell lysates was examined through Western blot analysis by probing for His tag, and equal loading was examined by β-actin.