Proprotein convertase subtilisin/kexin type 6 promotes in vitro proliferation, migration and inflammatory cytokine secretion of synovial fibroblast-like cells from rheumatoid arthritis via nuclear-κB, signal transducer and activator of transcription 3 and extracellular signal regulated 1/2 pathways

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Abstract. Our previous studies demonstrated that expression of proprotein convertase subtilisin/kexin type 6 (PCSK6) is greatly enhanced in rheumatoid arthritis fibroblast-like synoviocytes (RASFs), and that PCSK6 inhibition decreases cell proliferation, migration and invasion. The present study aimed to investigate the functional role of PCSK6 in the hyperplasia of RASFs. Cultured RASFs from RA patients were stimulated with recombinant human (rh)PCSK6. Subsequent changes in proliferation, invasion, migration and the secretion of inflammatory cytokines were measured in vitro using MTT, wound healing and Transwell assays, and ELISA. Cell cycle and apoptosis were analyzed by flow cytometry. Influence on downstream gene expression levels were analyzed using reverse transcription-quantitative polymerase chain reaction. Specific signaling pathways responsible for these effects were analyzed using western blotting and confirmed with pathway-specific inhibitors. It was demonstrated that rhPCSK6 significantly increased RASF cell invasion, migration and proliferation, which was influenced through both reduced cell cycle arrest and reduced apoptosis. Furthermore, rhPCSK6 stimulated RASFs to secrete the inflammatory cytokines interleukin (IL)-1α, IL-1β and IL-6, and exhibit altered expression of genes involved in angiogenesis, hypoxia, proliferation and inflammation. These cellular effects were mediated via the nuclear factor (NF)-κB, signal transducer and activator of transcription 3 (STAT3) and extracellular signal regulated (ERK)1/2 signaling pathways. The results demonstrated that signaling via NF-κB and STAT3 mediated cell cycle arrest, and signaling through NF-κB mediated apoptosis in RASF cells stimulated with PCSK6. PCSK6 can activate NF-κB, STAT3 and ERK1/2 signaling pathways in vitro to enhance cell proliferation, migration, invasion and inflammation in RASF cells. These findings suggest that PCSK6 may be an important therapeutic target in RA.

Introduction

Rheumatoid arthritis (RA) is a systemic autoimmune disease characterized by chronic inflammation of the synovium and hyperplasia of synovial fibroblasts, eroding adjacent cartilage and bone, thus causing subsequent joint destruction (1). The normal synovium forms a thin membrane of one to three cell layers at the edges of joints and provides lubrication and nutrients for cartilage. In RA, however, the synovial lining thickens and transforms into an inflammatory tissue known as the pannus (2). The major cells in the thickened lining and resultant pannus are activated RA fibroblast-like synoviocytes (RASFs). Notably, these cells exhibit tumor cell-like features, including increased proliferation, prolonged survival, resistance to apoptosis and invasiveness of adjacent tissues. Thus, RASFs are described as ‘tumor-like transformed’ cells (3,4).

Degradation of articular cartilage is one of the early features of RA and is mediated by increased activity of proteolytic systems (2). In particular, RASFs exhibit increased production of the matrix-degrading enzyme family of matrix metalloproteinases (MMPs) (3,5-7). MMPs can digest substrates, including fibrillar collagen I and II (8,9) and aggrecan, which predominantly exists in cartilage (10). MMPs are inactive precursors that must be processed by proprotein convertases, which cleave at single basic or paired basic residues of the proproteins to produce biologically active proteins (11). Proprotein convertase subtilisin/kexin type 6 (PCSK6) is a proprotein convertase that functions in proteolysis of various precursor proteins and regulation of protein maturation. Our previous study demonstrated that knockdown of PCSK6 can influence both expression and activity of MMP-2 and...
MMP-9 (12). These results revealed that PCSK6 contributes to hyperplasia of synovial lining cells, but the pathways involved remained unknown. Our previous study also revealed that the expression of PCSK6 is significantly higher in RASFs than in those of osteoarthritis, and silencing PCSK6 could inhibit the proliferation, migration and invasion of RASFs (12). Besides functioning in cells, PCSK6 can be secreted outside cells. However, the role and mechanism of secreted PCSK6 on RASFs have not been investigated. The aim of the present study was to investigate the involvement and functional role of PCSK6 in hyperplasia of RASFs, and the potential cell signaling pathways involved in this process.

Materials and methods

Synovial fibroblast isolation and treatment. Synovial tissues were collected during knee joint replacement surgeries from 7 patients with RA (5 females; 2 males; mean age=68.1 years). All patients met the American College of Rheumatology Classification Criteria (13). Participants provided written informed consent to participate in the study and to allow genetic analysis of biological samples. The Ethical Committee of Shandong Academy of Medicinal Sciences (Jinan, China) approved this study. The experiment was in compliance with the Helsinki Declaration.

RASFs were isolated from synovial tissues after collagenase digestion as previously described (12). The purity of the RASFs was determined by flow cytometry analysis using a FACSAria equipped with FACSDiva 2.0 (BD Biosciences, Franklin Lakes, NJ USA). Fourth generation RASFs were collected by centrifugation at 800 x g for 1 min at 4˚C and stained with anti-cluster of differentiation CD68 (fluorescein isothiocyanate (FITC)-conjugated; 1:20 dilution; 11-0689-41, eBioscience; Thermo Fisher Scientific, Inc., Waltham, MA, USA) primary antibodies on the bottom of the membrane were stained and analyzed by FACSAria equipped with FACSDiva 2.0 (BD Biosciences, Franklin Lakes, NJ USA) anti-human β-actin (1:1,000; cat. no. 4970s; Cell Signaling Technology, Inc., Danvers, MA, USA) anti-MMP9 (1:1,000; cat. no. ab137867; Abcam), anti-extracellular signal-regulated kinase (ERK)1/2 (1:2,000; cat. no. 8727s, Cell Signaling Technology, Inc.), anti-phosphorylated (p)-ERK1/2 (1:2,000; cat. no. 4370s, Cell Signaling Technology, Inc.), anti-phosphorylated-signal transducers and activators of transcription 3 (STAT3) (1:1,000; cat. no. 9131s; Cell Signaling Technology, Inc.); anti-STAT3 (1:1,000; cat. no. 4904p; Cell Signaling Technology, Inc.), anti-p-nuclear factor (NF)-κB p65 subunit (1:1,000; cat. no. 3033s; Cell Signaling Technology, Inc.) and anti-NF-κB p65 subunit (1:1,000; cat. no. 8242s; Cell Signaling Technology, Inc.). Antibodies were incubated with membranes at 4˚C overnight. After three washes, membranes were incubated with an anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:1,000; A0208; Beyotime Institute of Biotechnology, Beijing, China). Immunoreactivity was detected using an enhanced chemiluminescence detection system (GE Healthcare, Chicago, IL, USA). β-actin served as an internal loading control.

MTT assay. RASFs (2x10^4 cells/well) were seeded into 96-well culture plates and cultured at 37˚C to 80% confluence. Cultures were treated with 150 ng/ml rhPCSK6. After incubation for 24, 48 or 72 h, 20 μl 5 mg/ml MTT (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) in PBS were added to each well, and cultures were incubated for 4 h at 37˚C. MTT solution was removed and 150 μl dimethyl sulfoxide was added to extract MTT-formazan products at room temperature for 10 min. Absorbance was measured in triplicate at 490 nm using a spectrophotometer.

Cell cycle analysis. Cells were plated in 6-well culture plates at 1.0x10^5 cells/well and treated with rhPCSK6 as described above. After removal of culture medium, cells were harvested at 24 h by trypsinization, washed twice with ice-cold PBS and fixed overnight with cold 70% ethanol. Prior to analysis, fixed cells were rinsed, resuspended in PBS, and stained for 30 min at 37˚C with 1 ml 0.05 mg/ml propidium iodide solution (Beijing Dingguo Changsheng Biotechnology Co., Ltd., Beijing, China) containing 10 μg/ml RNase. Cells were detected with a Coulter Epics XL flow cytometer (Beckman Coulter, Inc., Brea, CA, USA) and DNA content was analyzed with Beckman Coulter EXPO32 software (Beckman Coulter, Inc.).

Apoptosis assay. For apoptosis assays, RASFs were serum-deprived in the presence or absence of rhPCSK6 (150 ng/ml) for 24 h and detected using an Annexin V-FITC apoptosis detection kit (BD Biosciences, San Jose, CA, USA) according to manufacturer’s protocol. Cells were detected with a Coulter Epics XL flow cytometer (Beckman Coulter, Inc.) and DNA content analyzed with Beckman Coulter EXPO32 software.

Invasion/migration assays. RASF invasion was measured using the Cyto Select 24-well cell invasion assay kit (basement membrane, colorimetric format; Cell Biolabs, Inc., San Diego, CA, USA) according to manufacturer's protocol. Invasive cells on the bottom of the membrane were stained and analyzed using light microscopy to count total cells in five random fields.
fields of each membrane at x20 magnification. The results are expressed as invasive cells per field.

Cell migration was detected using a scratch migration assay as previously described (12). To eliminate the effect of cell proliferation, RASFs were treated with 5 mg/ml mitomycin C (cat. no. M0503; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) for 4 h. Cell monolayers were scratched before stimulation and then incubated with 150 ng/ml rhPCSK6 for 24 h. Cells that migrated to wound areas were counted under a microscope at 20x magnification and expressed as a percentage of controls.

Measuring interleukin (IL)-1α, IL-1β, IL-6, IL-17 and tumor necrosis factor (TNF)-α levels with an enzyme-linked immunosorbent assay (ELISA). RASFs were treated with 150 ng/ml rhPCSK6 for 24 h, and the conditioned culture medium was collected and centrifuged at 1,200 g for 5 min at 4˚C. The supernatant (100 µl) was added to a 96-well microplate, and incubated overnight at 4˚C. Following gentle washing with PBS + 1% Tween-20, blocking was performed using 1% bovine serum albumin + 5% sucrose for 1 h at 37˚C. Following three PBS washes, antibodies diluted 1:1,000 against IL-1α (cat. no. ab92396), IL-1β (cat. no. ab2105), IL-17 (cat. no. ab79056), TNF-α (cat. no. ab667 or IL-6 (cat. no. ab6672) (all from Abcam) were applied to the plate for overnight incubation at 4˚C. The following day, the plate was washed and blocked again as aforementioned, and incubated with anti-rabbit IgG horseradish peroxidase antibody (1:2,000; cat. no. A0208, Beyotime Institute of Biotechnology) for 3 h at 37˚C. Staining was developed using a TMB kit (cat. no. PR1200, Beijing Solarbio Science & Technology Co., Ltd.). The absorbance at 450 nm was measured using a plate reader. Cell medium plus transfection reagent served as a control.

Pathway inhibitor analysis. To verify the induced pathways, specific inhibitors of the ERK1/2, STAT3 and NF-κB signaling pathways were used. Briefly, 1 µmol/l NF-κB activation inhibitor pyrroldine dithiocarbamate (PDTC; cat. no. ab141406; Abcam), the STAT inhibitor statividic (cat. no. ab210952 Abcam), or the ERK-1/2 inhibitor PD98059 (cat. no. ab120234, Abcam) was added to cells in the presence of 150 ng/ml rhPCSK6 for 2 h. RASF cells were then analyzed for changes in proliferation, migration, invasion, secretion of proinflammatory cytokines, cell cycle and apoptosis.

Statistical analysis. All results were confirmed in at least three independent experiments. One-way analysis of variance was used for multiple comparison between different groups, followed by a Bonferroni post hoc test. P<0.05 was considered to indicate a statistically significant difference. All statistical analyses were performed using SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA).

Results

rhPCSK6 stimulates RASF cell proliferation, migration and invasion in vitro. As PCSK6 has been reported to be significantly associated with RA, and as it is a secretory protein, the present study stimulated RASFs with rhPCSK6 to assess its effects on the biological activity of RASFs. MTT, wound healing and Transwell assays were performed. To determine the effective concentration, 1, 10, 50, 100 and 150 ng/ml rhPCSK6 was used. The MTT results demonstrated that 50, 100 and 150 ng/ml rhPCSK6 could induce the proliferation (only data of 150 ng/ml is presented, P<0.05 vs. control; Fig. 1A) of RASFs without cell death. As 150 ng/ml of rhPCSK6 has the most marked effect, it was used for subsequent experiments. In addition, the wound healing and Transwell results demonstrated that rhPCSK6 could promote the migration and invasion of RASFs (P<0.05; Fig. 1B and C, respectively).

To further analyze the effects of rhPCSK6 on the cell proliferation, flow cytometry was used and the results revealed that the ratio of G0/G1 phase cells was significantly lower in RASFs stimulated with rhPCSK6 after 24 h (Fig. 1D), indicating that PCSK6 could disturb the cell cycle in RASFs. However, proliferation changes could also result from reduced cell apoptosis; therefore, whether PCSK6 could protect from apoptosis was investigated by adding exogenous rhPCSK6 to the cultures of starved RASFs. Annexin V-FITC and propidium iodide staining revealed that exogenous rhPCSK6 significantly decreased the number of apoptotic cells induced by serum deprivation (Fig. 1E).

rhPCSK6 promotes the production of pro-inflammatory factors in RASFs. Pro-inflammatory cytokines serve prominent roles in RA. As reported, RASFs can secrete IL-1α, IL-1β, IL-6, IL-17 and TNF-α, and are closely associated with synovitis and joint destruction (15). Levels of these three pro-inflammatory cytokines were detected in the supernatants of RASFs following stimulation with 150 ng/ml rhPCSK6 or its vehicle controls by ELISA. Significantly, rhPCSK6 stimulation increased the secretion of IL-1α, IL-1β and IL-6 (P<0.05, Fig. 2A) from RASF cells, but not the secretion of IL-17 and TNF-α (data not shown).

To gain further insight into the role of PCSK6 in RA pathology, expression of genes associated with cell proliferation, angiogenesis, hypoxia and invasion were assayed using qPCR. It was demonstrated the expression of various genes involved in angiogenesis (MMP-9 and nitric oxide synthase traffic inducer) hypoxia (hypoxia inducible factor-1A), proliferation (CHRNA4), and inflammation [C-X-C chemokine motif (CXCL9), CXCL10, NF-kB-α, IL-6, IL-10 and IL-26] were all upregulated after rhPCSK6 stimulation (Fig. 2B).

RASFs express MMP-2 and MMP-9. MMP-2 is constitutively expressed by most cell types, including synovial fibroblasts, while basal levels of MMP-9 are undetectable in most cell types except for malignant or inflammatory cells, such as RASFs (7). The present study only tested the effect of rhPCSK6 on MMP-9, because MMP-2 expression in RASFs is so high that no change can be detected after rhPCSK6 stimulation. Western blot analysis demonstrated that stimulation with rhPCSK6 increased the protein expression levels of MMP-9 (Fig. 2C).

rhPCSK6 induces activation of the ERK1/2, STAT3 and NF-κB signaling pathways. To investigate the pathway by which PCSK6 directly or indirectly regulates cell proliferation, migration, invasion, and secretion of proinflammatory cytokines, the present study examined ERK1/2, STAT3 and NF-κB activity in RASFs. Phosphorylation of ERK1/2
catalytically activates the protein, and ERK1/2 isoforms serve a major role in cell proliferation signaling (16). Western blot analysis demonstrated that rhPCSK6 increases levels of phosphorylated forms of ERK1/2 in RASFs (Fig. 2D).

In vivo and in vitro studies support the role of STAT3 in contributing to the chronicity of inflammatory arthritis (17). Therefore, the present study investigated STAT3 activation by PCSK6 and demonstrated that treatment of RASFs with rhPCSK6 increased levels of p-STAT3 (Fig. 2D). In addition, NF-κB is a critical activator of inflammatory processes (18). rhPCSK6 also induced the phosphorylated forms of NF-κB in RASFs (Fig. 2D).

To confirm the signaling pathways responsible for PCSK6 stimulation of RASFs, specific pathway inhibitors were used, including the NF-κB activation inhibitor PDTC, the ERK1/2 inhibitor PD98059 and the STAT3 inhibitor stattic. These chemical inhibitors exhibit no cytotoxicity at the concentrations used in these experiments (19). rhPCSK6-stimulated proliferation, migration, invasion and secretion of cytokines were markedly decreased in the presence of PDTC, PD98059 and stattic. Therefore, rhPCSK6 can stimulate RASF bioactivity via the NF-κB, ERK1/2 and STAT3 signaling pathways (Fig. 3). Furthermore, the effect of rhPCSK6 on cell cycle and apoptosis was assessed. The result revealed that PDTC and stattic can recover G0/G1 arrest of RASFs, respectively, indicating that PCSK6 can affect the cell cycle of RASFs via NF-κB and STAT3 activation. In RASF apoptosis assays, PDTC was demonstrated to recover reduced cell apoptosis induced by rhPCSK6, which highlights the role of NF-κB activation in PCSK6 stimulation on RASFs (Fig. 4).
Discussion

PCSK6 is a neuroendocrine-specific mammalian subtilisin-related endopeptidase (20) that exhibits a C-terminal cysteine-rich region (21) and functions in the secretory pathway. Our previous study demonstrated RASFs, but not osteoarthritis synovial fibroblasts, produce high levels of PCSK6 (12). As a secretory protein, the present study aimed to investigate the role of exogenous PCSK6 on RASF. rhPCSK6 was used in this study. The host is wheat germ, which eliminated the issue of lipopolysaccharide contamination. The present study demonstrated that PCSK6 may serve important roles in cell proliferation, migration, invasion and angiogenesis, because exogenous PCSK6 enhanced RASF survival, inflammation and invasion capacity (22). These findings suggested that PCSK6 may be an important therapeutic target in RA.

Furthermore, RA is characterized as a chronic inflammatory disorder, in which pro-inflammatory cytokines including IL-1α, IL-1β and IL-6 contribute to synovitis and joint destruction. Our previous results demonstrated that silencing PCSK6 in RASFs could regulate the production of these parameters (12); the present study also revealed that rhPCSK6 treatment exerts the same effects on RASFs, suggesting that PCSK6 exacerbates inflammation by regulating expression or secretion of cytokines.

RASFs are a dominant cell type in RA synovium and mediate persistent inflammation as well as cartilage and bone destruction (23). In the present study, MMP-9 expression in RASFs increased after stimulation with rhPCSK6,
Figure 3. Effect of pathway inhibitors on rhPCSK6-stimulated proliferation, migration and invasion of RASFs. RASF cells were treated with specific pathway inhibitors (1 μmol/l) and the resulting effects of rhPCSK6 stimulation were measured with (A) MTT, (B) secretion of cytokines, (C) migration and (D) invasion assays. Inhibitors used were the nuclear factor-κB activation inhibitor PDTC, the extracellular signal-regulated kinase1/2 inhibitor PD98059 and the signal transducer and activator of transcription 3 inhibitor stattic. Data are expressed as the mean ± standard error of three independent experiments conducted in triplicate. *P<0.05 vs. 150 ng/ml rhPCSK6. rhPCSK6, recombinant human proprotein convertase subtilisin/kexin type 6; RASFs, rheumatoid arthritis fibroblast-like synoviocytes; OD, optical density; PDTC, pyrrolidine dithiocarbamate; IL, interleukin.

Figure 4. Effect of pathway inhibitors on rhPCSK6-stimulated cytokine secretion, cell cycle and apoptosis of RASFs. RASFs were treated with specific pathway inhibitors (1 μmol/l) and the resulting effects of rhPCSK6 stimulation were analyzed through (A) cell cycle and (B) apoptosis assays. Inhibitors presented are the nuclear factor-κB activation inhibitor PDTC, the extracellular signal-regulated kinases/2 inhibitor PD98059 and the signal transducer and activator of transcription 3 inhibitor stattic. Data are expressed as the mean ± standard error of three independent experiments conducted in triplicate. *P<0.05 vs. 150 ng/ml rhPCSK6. rhPCSK6, recombinant human proprotein convertase subtilisin/kexin type 6; RASFs, rheumatoid arthritis fibroblast-like synoviocytes; Q1, non-viable non-apoptotic cell; Q2, late apoptotic cell; Q3, viable cell; Q4, early apoptotic cell.
STAT3 and ERK1/2 signaling pathways may mediate the RA through its effects on RASFs. Furthermore, the NF-κB of PCSK6 on RASF cells.

In the present study, rhPCSK6-stimulated proliferation, invasion, migration were significantly prevented by influencing both cell cycle arrest and protect RASFs from apoptosis, demonstrating that rhPCSK6 promotes RASF cell proliferation. In our previous study (12), increased expression of the cyclin-dependent kinase inhibitor p27KIP1 was observed in PCSK6-silenced RASFs, consistent with a previous finding in prostate cancer (27). A lack of mitogenic activity is associated with higher cell quiescence states characterized by the increased expression of p27KIP1 (28). In addition, previous studies have demonstrated that mitogen-activated protein kinase (MAPK) p42 and p44 (ERK1/2) are required for fibroblast proliferation to pass the G1 restriction point and enter S-phase (29). Overall, the results of the present study indicated that PCSK6 promotes the proliferation of RASFs by disturbing cell cycle arrest via targeting p27KIP1 or the activity of the MAPK signaling pathway. This study also demonstrated that rhPCSK6-mediated activation of RASFs is likely via the NF-xB, STAT3 and ERK1/2 signaling pathways, as rhPCSK6 treatment induced the phosphorylation levels of these pathways. Furthermore, specific inhibitors of these pathways significantly prevented RASF proliferation by influencing both cell cycle arrest and apoptosis. Consistently, previous data (12) has demonstrated that the ERK1/2 isoforms serve a major role in cell proliferation signaling, while STAT3 contributes to the chronicity of inflammatory arthritis and NF-xB is a critical activator of inflammatory processes. In the present study, rhPCSK6-stimulated proliferation, invasion, migration and secretion of proinflammatory cytokines were inhibited in the presence of NF-xB, STAT3 and ERK1/2 inhibitors, confirming a role of these pathways in mediating the effects of PCSK6 on RASF cells.

In conclusion, the present study revealed that after secretion into the joint, PCSK6 could exacerbate the progression of RA through its effects on RASFs. Furthermore, the NF-xB, STAT3 and ERK1/2 signaling pathways may mediate the pro-inflammatory role of PCSK6 in RA. Therefore, PCSK6 may be a potential therapeutic target for RA.

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