Activated Protein C Inhibits Proliferation and Tumor Necrosis Factor \( \alpha \)-Stimulated Activation of p38, c-Jun NH2-Terminal Kinase (JNK) and Akt in Rheumatoid Synovial Fibroblasts

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INTRODUCTION
Rheumatoid synovial fibroblasts (RSFs) are one of the major cell types in the synovial pannus and contribute actively to inflammation in rheumatoid arthritis (RA) (1–3). These cells respond to cytokines, such as interleukin (IL)-1\( \beta \) and tumor necrosis factor (TNF)-\( \alpha \) through the activation of multiple intracellular signaling pathways, including the mitogen-activated protein kinases (MAPKs) extracellular signal–regulated protein kinase (ERK), c-Jun NH2-terminal kinase (JNK) and p38, and the nuclear factor (NF)-\( \kappa B \) system (2,4). Activation of these pathways may result in exaggerated proliferation and expression of multiple cytokines including IL-1\( \beta \) (5–7), IL-6 (8) and TNF\( \alpha \) (6,7,9), as well as the secretion of matrix metalloproteinases (MMPs), which contribute to tissue destruction in RA (1–3).

Cell cycle control mechanisms include sequential activation and inactivation of the cyclin-dependent kinases. The Cip/Kip family contains proteins such as p21/WAF1 and p27/KIP that bind to cyclin/cyclin-dependent kinase complexes and prevent kinase activation. These cyclin-dependent kinase inhibitors play a key role in RA pathogenesis by affecting proliferation and inflammatory cytokine production in human RSFs and mouse synovial fibroblasts (11,12).

Recombinant human activated protein C (APC) is approved by the U.S. Food and Drug Administration as a treatment for patients with severe sepsis; however, it was recently withdrawn from the market after the PROWESS-SHOCK trial in patients with septic shock showed no clinical benefit in subjects who had received APC compared with placebo (13). Nonetheless, numerous studies have shown that APC exerts antiinflammatory and cyto-protective properties in various organs and that these effects are often...
mediated by endothelial protein C receptor (EPCR) and proteinase-activated receptors (PARs) (14–17).

In all cells tested to date, including keratinocytes, endothelial cells and tenocytes, APC promotes proliferation via regulating MAPK pathways (17–19). Considering that synovial fibroblast proliferation is a hallmark of the invasive pannus in the RA joint and APC is significantly elevated in human RA synovial fluid compared with normal or osteoarthritic (OA) synovial fluids (20,21), it is possible that APC may contribute to the damage in RA. No data are available on the effects of APC on RSF proliferation. In this study, we investigated the effects of APC on RSF proliferation and, surprisingly, our results show that APC inhibits proliferation of RSFs and that it acts via induction of p21/p27 and activation of ERK1/2.

MATERIALS AND METHODS

Isolation and Culture of Human RSFs

Human RA synovial tissues were obtained during joint replacement surgery from patients with RA according to the American College of Rheumatology criteria (22) from 11 patients comprising 7 female patients (mean ± standard deviation [SD] age 68.2 ± 5.1 years) and 4 male patients (age 70 ± 4 years). Ethical approval was granted by the Royal North Shore Animal Care and Ethics Committee and Northern Sydney Health Human Research Ethics Committee, and the written consent of every patient was obtained. We compared RSFs with low metabolic dermal fibroblasts, as previously described (23,24).

RSFs, human dermal fibroblasts (HDFs) and mouse dermal fibroblasts (MDFs) were obtained from RA synovium, dermis of neonatal foreskin and C57BL/6J mouse skin, respectively, by enzymatic digestions as described previously (25). Briefly, tissues were minced into small pieces and digested with 2 mg/mL collagenase (Sigma-Aldrich, St. Louis, MO, USA) in Dulbecco’s modified Eagle medium (DMEM) containing 100 U/mL penicillin, 100 μg/mL streptomycin, 10 mmol/L HEPES (all from Gibco BRL/Life Technologies, Carlsbad, CA, USA) and 3.7 g/L NaHCO3 at 37°C for 3 h, followed by digestion with 0.25% trypsin and 0.02% ethylenediaminetetraacetic acid (EDTA) at 37°C for 30 min. The cells were cultured in DMEM containing 10% fetal bovine serum (FBS) (ICN, Aurora, OH, USA) in 75-cm² flasks at 37°C in a humidified 5% CO2 atmosphere.

We used RSFs at passages 1–4 (one passage per experiment) (26). Cultured RSFs abundantly expressed the fibroblast-specific marker cadherin-11 (27) and expressed barely detectable levels of the myeloid cell marker CD11b (Supplementary Figure S1) at passage 1 and passage 4. Cells were plated onto 24-well plates (Corning, Corning, NY, USA) in DMEM containing 10% FBS. To avoid serum effects (24), confluent cells were preincubated in DMEM without FBS (basal medium) for 24 h and then used for experiments with or without recombinant TNFα (Pepro Tech, London, UK), APC (Xigris; Eli Lilly, Indianapolis, IN, USA) or SB203580, PD98059 and U0126 (Calbiochem, San Diego, CA, USA) as indicated in the figure legends, by using fresh basal media.

Quantitative Reverse Transcriptase–Polymerase Chain Reaction (qRT-PCR)

Total RNA was isolated from RSFs of passage 1 and passage 4 by using RNAzol (Molecular Research Centre, Cincinnati, OH, USA) according to the manufacturer’s instructions (http://www.mrcgene.com/rnazol.htm). RNA concentration was determined by NanoDrop spectrophotometry (Thermo Scientific; Scoresby, Australia) and reverse-transcribed into complementary DNA (cDNA) using the cDNA synthesis kit (Bioline; Taunton, MA, USA). Subsequently, qRT-PCR was performed with the Rotor-Gene 6000 Real-Time PCR machine (Corbett Life Science, Mortlake, Australia) by using ImmoMix (Bioline; Taunton) and SYBR Green dye (Qiagen, Hilden, Germany). The reaction mixture consisted of 1 μg cDNA template, 0.75 μL each of forward and reverse primer, 12.5 μL ImmoMix and 2.5 μL SYBR Green. Cycling conditions comprised an initial activation step at 95°C for 10 min followed by 45 amplification cycles of 95°C for 15 s (denaturation), 58°C for 20 s (primer annealing) and 20°C for 45 s (extension). Specificity of the amplification reactions was verified by melting curve analysis.

Primers used in the assay were designed and checked for specificity by using the National Center for Biotechnology Information BLAST search tool (http://www.ncbi.nlm.nih.gov/tools/ primer-blast). The primer sequences for cadherin-11 (NM_001797; 163 bp) were 5′-ACCTCTACCATCAAGGTCTG-3′ (forward) and 5′-TCAGGTCACAAACATAGTCT-3′ (reverse) and CD11b (NM_001145808; 187 bp) were 5′-CAGACAGGAAGTAGCAGCT-3′ (forward) and 5′-CTTGTCATGTTGATAGGTTGCT-3′ (reverse). The amplification efficiency of each primer set was between 95% and 105%. Data were analyzed using the standard curve for absolute quantification method, and results were expressed as gene copies/μg cDNA, as described in a previous study (28). Fourfold serial dilution cDNA standard curves (1, 250, 62.5, 15.626 and 0 μg) were prepared by using preadipocyte (cadherin-11) and monocyte cDNA (CD11b) at the same time as experimental samples and assessed in the same real-time PCR run.

Cell Proliferation Assay

Cell proliferation was performed as described previously (17) with modification. Briefly, (1 × 10⁶ cells/well) confluent cells from 75-cm² flasks were seeded into a 96-well microplate (Corning) to a final volume of 200 μL and incubated for 4 h to allow cells to attach and then preincubated in basal media for a further 12 h. Cells were then treated with test agents in serum-free conditions by using fresh basal media. To avoid fibroblast contact inhibition (26), after incubation for 24 h (29,30), culture medium was removed and cells were stained with
1 μg/mL crystal violet (Sigma-Aldrich) dissolved in phosphate-buffered saline (PBS). The unbound dye was removed by washing with tap water, and cells were left to completely dry overnight. Bound crystal violet was solubilized with 0.1% sodium dodecyl sulfate in PBS. The optical density of each well was determined at a wavelength of 550 nm.

Results were expressed as relative to control.

Cell Morphology and Cell Death

Passage 4 RSFs were seeded into a 24-well plate (Corning) at 1 x 10^5 cells/well to a final volume of 250 μL and incubated for 24 h to allow cells to attach and then preincubated in basal media for a further 12 h. At 90% confluency, cells were treated with or without 10 μg/mL APC in fresh basal media for 24 h. Micrographs were taken by an inverted phase contrast microscope (Nikon Eclipse TE2000-U) fitted with a DS-FI1 digital camera and DS-U2 camera control unit (Nikon, Tokyo, Japan). RSFs in culture with or without APC treatment appear elongated by phase light microscopy, sometimes oval or polygonal, with a few branched cytoplasmic processes (Supplementary Figure S2A). Cell death was measured by adding 10 μL 0.5% trypan blue (Sigma-Aldrich) in PBS to 100 μL cell supernatant, and cell viability was assessed by the trypan blue exclusion method on a hemocytometer and corrected for the total volume of media in the well.

Western Blotting

Western blotting was performed as described previously (17,31). The primary antibodies used were as follows: rabbit polyclonal antibodies directed against phosphorylated (P) forms of p38 and p38 (Santa Cruz Biotechnology, Santa Cruz, CA, USA); p21, p27, P-Akt (Ser473) and Akt; and P-p54, P-ERK1/2, P-ERK2 and ERK1/2 (Cell Signaling, Beverly, MA, USA). Immunoreactivity was detected by using the ECL detection system (Amer sham Biosciences, Buckinghamshire, UK). Anti-human β-actin (Sigma-Aldrich) antibody was included to normalize for unequal loading. Protein band intensity was evaluated by densitometry by using image analysis.

Statistical Analysis

The data are expressed as the mean ± SD. Statistical analyses were performed by using the Student t test or analysis of variance (ANOVA) followed by the Bonferroni post hoc test (where appropriate). Statistical significance was accepted at the p < 0.05 level.

All supplementary materials are available online at www.molmed.org.

RESULTS

APC Inhibits Cultured Human RSF Proliferation

To determine the relative effect of APC on human RSF proliferation, we used normal MDFs and normal HDFs as controls. Treatment with APC (0.1–10 μg/mL) for 24 h stimulated the proliferation of normal MDFs and normal HDFs by up to 60% (Figure 1A). However, when RSFs were treated with APC for 24 h, their proliferation was inhibited by up to 30% (Figure 1A). To determine the effect of APC on TNFα-stimulated RSFs, two different treatment regimens were used. APC (10 μg/mL) was delivered either 30 min before TNFα (Figure 1B, far right bar) or at the same time as TNFα. Both methods of delivery completely reversed TNFα-stimulated proliferation (Figure 1B). APC at a 10 μg/mL concentration has no effect on cell death (Supplementary Figure S2B).

APC Differentially Regulates p21/p27 in Cultured HDFs versus RSFs

In HDFs, APC (24-h treatment) had little effect on p21 protein expression, but downregulated p27 expression dose-dependently compared with control (Figure 2A). In contrast, in RSFs, APC robustly increased protein expression of p21 and p27 in a dose-dependent manner (Figure 2B). These data agree with the proliferation results in Figure 1 and suggest that inhibition of RSF proliferation by APC is linked to increased p21/p27 expression.

Increased Induction of p21/p27 and Inhibition of Proliferation in RSFs by APC are ERK Dependent

When RSFs were preincubated with an inhibitor to ERK (PD98059), the increase
in p21/p27 was reversed, whereas the p38 inhibitor SB203580 had a minimal effect (Figure 3A). Treatment of RSFs with APC alone (10 μg/mL) for 30 min strongly induced activation of ERK1/2 and ERK2 in a dose-dependent manner (Figure 3B and Supplementary Figure S3A, respectively), while it inhibited activation of p38 (Supplementary Figure S3A). APC also downregulated activation of p38 at 15 and 60 min (Supplementary Figure S3B). In addition, 1-h pretreatment with PD98059 and U0126 significantly blocked the inhibition of RSF proliferation by APC (Figure 3C and Supplementary Figure S4, respectively), whereas the p38 inhibitor SB203580 had no effect (Figure 3D). These results suggest that APC inhibits proliferation and induces cell cycle inhibitory proteins p21 and p27 via ERK in RSFs.

APC Downregulates TNFα-Stimulated P-p38, P-JNK and P-Akt in Human RSFs

To determine the effects of APC on TNFα-stimulated MAPKs and Akt, two different treatment regimens were used: APC was either added 30 min before or at the same time as TNFα (data not shown). Using RSFs, TNFα significantly stimulated P-p38 (Figure 4A) and P-JNK (Figure 4B) but had no significant stimulatory effect on P-ERK1/2 (Figure 4D), compared with control. Pretreatment with APC significantly inhibited TNFα-stimulated P-p38 (Figure 4A) and P-JNK (Figure 4B) and conversely activated P-ERK1/2 (Figure 4D). In addition to the MAPKs, TNFα-induced Akt activation, and this was completely reversed by APC (Figure 4C).

DISCUSSION

This is the first report to show that APC can inhibit cell proliferation. This effect directly opposes the stimulatory effect of APC on other cells including endothelial cells, keratinocytes (14,17,18), normal MDFs and HDFs. The reason why APC selectively inhibits RSF prolif-
Inhibition of RSF proliferation by activated protein C

Proliferation is not entirely clear, but may relate to the native rate of proliferation in each cell type. RSFs play an active role in joint destruction in RA by contributing to synovial inflammation (1,2) and pannus invasion (1,3). A critical feature of these cells is excessive proliferation, which expands the synovial pannus to cause progressive cartilage destruction (3,32). In addition to increasing differentiation and migration of mesenchymal stem cells, imbalance between proliferation and apoptosis of RSFs largely contributes to this cellular excess (3). Although studies have failed to find evidence of markedly increased proliferation of RSFs in the RA synovium (33), distinct clonal expansion of cultured RSF has been reported (34). More interestingly, RSFs retain their resistance to apoptosis in vitro (35) and are able to invade and “metastasize” in vivo (32,36). In contrast, normal MDFs and HDFs (24,37) are relatively slow-growing cells unless activated in situations such as wound healing. Our results show that APC appears to selectively control the cell proliferation regulators p21 and p27 by replenishing their very low levels in RSFs. Cyclin-dependent kinase inhibitors play a vital role in controlling excessive proliferation and inflammation in RA. Gene transfer of p21 ameliorates arthritis by exerting antiproliferative effects and anti-inflammatory effects in human RSFs (11,38). Loss of p21 expression in RSFs and prevention of p27 upregulation in hypoxic RSFs contribute to excessive invasion and subsequent joint destruction (12,39). In addition, p21 knockout (KO) mice exhibit increased and prolonged inflammatory arthritis and elevated cytokine levels, and p21 mediates a reduction in arthritis severity and suppression of cytokines production via inhibition of p38-MAPK in murine RA model (40).

ERK activation is primarily triggered by certain receptors to cytokines and growth factors, whereas lipopolysaccharide, proinflammatory cytokines and osmotic shock activate p38 and ultraviolet light, protein synthesis inhibitors and cytokines such as TNFα stimulate the JNK pathway (2). Interestingly, our results show that the increased protein levels of p21 and p27 triggered by APC were abrogated by inhibition of ERK but not by p38 inhibition, suggesting that ERK is required for induction of p21 and p27 by APC in nonstimulated RSFs. Although ERK and MEK1/2 inhibitors have been shown to be beneficial in collagen-induced arthritis (41,42), a recent study showed that P-ERK was not increased in collagen-induced arthritis (43). The capacity of pharmacological inhibitors to MEK1/MEK2 in cell-based experiments is discordant (44,45). In addition to MEK1/2, both U0126 and PD98059 have been shown to inhibit MKK5 in vitro and in vivo, and U0126 has been stated to inhibit as many as 25 kinases (44,45). To minimize the risk of nonspecific effects in this study, we used both PD98059 and U0126. ERK signaling is usually associated with stimulation of cell proliferation; however, in our study, the ERK inhibitors PD98059 and U0126 block the antiproliferative effect of APC. Similar results have been described in cancer research. The active-site inhibitor mTOR (KU63794) causes a marked increase in ERK activation yet inhibits cancer cell proliferation more effectively than rapamycin, which does not affect ERK activation (46). Further, mTORC1 inhibition can activate...
ERK in vitro, in mouse models and in cancer patients, yet it downregulates cell proliferation, cancer growth and Ki 67 expression in vivo (47). The amplitude of the p38 signal plays a key role in regulating the cell cycle (48). Moderate ERK1/2 activation is required for the expression of cyclin D1 and cell cycle reentry, whereas high-intensity ERK1/2 activation can induce cell cycle arrest or senescence as a result of the high expression of p21CIP1 (49). Consistent with our results, TNF-dependent activation markers relevant to RA are relatively insensitive to ERK inhibition in human RSFs (50). In agreement, IL-1β, but not TNFα, significantly stimulates ERK activation in human RSFs and rabbit synovial fibroblasts (51,52). Nonetheless, the effect of TNFα on RA inflammation appears to be dose-dependent (53), and when used in vitro at considerably higher doses (15–50 ng/mL) than the current study, TNFα can stimulate ERK activation (54,55). Anti-TNFα treatment can prevent RA joint destruction without affecting the clinical signs and symptoms of inflammation (56–58). In our previous studies, the ERK inhibitor PD98059 did not suppress IL-1β–stimulated MMP-13 or MMP-1 and -3 production in human RA chondrocytes (59) or human RSFs (25), respectively, whereas it significantly suppressed MMP-13 in OA chondrocytes (59), suggesting that ERK may contribute more to OA than RA pathogenesis.

TNFα stimulates proliferation of synovial fibroblasts and activates signaling molecules such as Akt and MAPKs (2,10), which leads to increased production of inflammatory cytokines that contribute to the pathogenesis of RA (2,4,60). Consistent with previous work (51,52,61), we found that TNFα significantly stimulated the phosphorylation of p38 and JNK in human RSFs. APC reversed the effect of TNFα on p38, JNK, and Akt (Figures 4A–C) in human RSFs. In agreement, our previous studies showed that APC completely suppresses lipopolysaccharide-stimulated NF-κB activation, a pivotal regulator of inflammation, hyperplasia and tissue destruction in RA (62), and attenuates TNFα protein by >40% in RA monocytes (63).

MAPK specific inhibitor studies in vitro, in vivo and in clinical trials suggest that the most relevant MAPKs in RA pathogenesis appear to be p38 and JNK (4,64,65). Recent studies show that the p38 network predicts the response of anti-TNFα therapy (66) and JNK activation predicts the development of erosive arthritis (67). In addition, Akt is critical for survival of RSFs (10), by regulating cellular activation, chemotaxis and inflammatory responses (68).

Mechanism(s) of action of APC on ERK induction and downregulation of TNFα-stimulated p38, JNK and Akt in RSFs remain to be elucidated. In our previous studies, we reported that APC receptors EPCR, PAR-1 and PAR-2 are expressed by RA synovium (63,69) and APC inhibits the migration and activation of RA monocytes via EPCR (63). It is possible that ERK induction and downregulation of TNFα-stimulated p38, JNK and Akt in RSFs by APC will also be mediated by EPCR and PARs.

However, further studies are required using synovial fibroblasts from normal and OA patients as well as RA synovium from different joints to confirm the selective effects of APC. This study provides the first evidence that APC may inhibit inflammation in the rheumatoid joint by acting on the synovial fibroblasts. As summarized in Figure 5, APC acts on RSFs to inhibit proliferation via p21, p27 and ERK and reduce TNFα-induced inflammatory signals of JNK, Akt and p38. This evidence, together with our previous report (63), suggest that, through its cytoprotective actions on signaling molecules, APC prevents proliferation and invasion in RA and impedes the inflammatory cascade to limit arthritic disease (14).

CONCLUSION

A key finding of this study is that APC can inhibit proliferation of RA synovial fibroblasts from patients in vitro, and this effect is mediated by an ERK-dependent increase of the cell cycle regulators p21 and p27. Furthermore, APC downregu-


