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The effects of the immunosuppressant rapamycin on the growth of rheumatoid arthritis (RA) synovial fibroblast

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SUMMARY

RA is a chronic inflammatory disease characterized by mononuclear cell infiltration and the overgrowth of synovial fibroblast. This invasive growth of synovial tissues corresponds with the progressive destruction of articular carilage and bone. Several immunosuppresive agents, such as cyclophosphamide, cyclosporin A and mizoribine, have been clinically used to control disease progresssion, though relatively little is known of their effects on rheumatoid synovium. Rapamycin exhibits a strong immunosuppressive activity by acting on T cell signalling pathways. In the present study we examined the effects of rapamycin on the growth of synovial fibroblast isolated from RA patients. Platelet-derived growth factor (PDGF) is a potent growth factor in synovial fibroblasts isolated from RA patients. PDGF and serum stimulation resulted in a rapid phosphorylation of tyrosine and activation of mitogen-activated protein kinase (MAP kinase), 70-kD-S6 kinase ($P70^{S6K}$) and 90-kD-S6 kinase ($P90^{rsk}$). Rapamycin, a macrolide immunosuppressant, inhibited completely growth factor-induced synovial fibroblast proliferation and $P70^{S6K}$ activation. In contrast, tyrosine phosphorylation and activation of MAP kinases and $P90^{rsk}$ were not influenced by rapamycin treatment. Our data demonstrate that growth factor-mediated $P70^{S6K}$ activation is closely related to the growth of synovial fibroblast, and suggest the efficacy of rapamycin for controlling synovial hyperplasia in RA.

Keywords rapamycin rheumatoid arthritis synovial fibroblast protein kinase

INTRODUCTION

The synovial tissue of patients with RA is characterized by mononuclear cell infiltration, neovascularization and proliferation of synovial fibroblasts [1–3]. The synovial hyperplasia is probably due to stimulation by various growth factors, such as IL-1, fibroblast growth factor (FGF) and platelet-derived growth factor (PDGF) [4–7]. PDGF-BB is locally produced in the RA synovium and may play an important role in the pathogenesis of RA [7,8]. In the present study we examined the PDGF-mediated intracellular signal transduction in synovial cell growth. PDGF arriving at the cell surface receptor is transmitted as a signal by activating protein kinases, and SRC-like protein tyrosine kinases (PTK), such as *src*, *fyn* and *yes*, have been shown to be associated with the PDGF receptor [9,10]. The activated receptor tyrosine kinases, in turn, trigger the activation of downstream serine/threonine kinases, including Raf, MAP, and S6 kinases, leading eventually to phosphorylation of nuclear tanscription factors [11,12].

Rapamycin, a macrolide antibiotic isolated from *Streptomyces hygroscopius*, exhibits a potent immunosuppressive activity by acting on the cell signalling pathway [13,14]. We demonstrate in the present study PDGF- and serum-stimulated tyrosine phosphorylation and activation of MAP kinase, p70 S6 kinase ($P70^{S6K}$) and 90-kD-S6 kinase ($p90^{rsk}$) in synovial fibroblasts isolated from RA synovium. We also demonstrate that rapamycin completely blocked $p70^{S6K}$ activation and growth factor-induced synovial cell proliferation, but not the activation of MAP kinases and $p90^{rsk}$. These findings suggest that $p70^{S6K}$ participates in the growth factor-induced signal transduction pathway in synovial cells and that rapamycin inhibits this critical element essential for synovial hyperplasia in RA.

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MATERIALS AND METHODS

Antibodies and reagents

Rabbit anti-MAP kinase antiserum was kindly provided by Dr J. Blenis (Harvard Medical School, Boston, MA). Anti-phosphotyrosine-specific MoAb (4G10), anti-$p70^{S6K}$ and anti-rskI S6 kinase ($p90^{rsk}$) were purchased from UBI (Lake Placid, NY). Rapamycin and recombinant human PDGF-BB were obtained from GIBCO, BRL (Gaithersburg, MD). Other reagents were purchased from Sigma Chemical Co. (St Louis, MO).

Preparation of synovial cells

The protocol was approved by the local ethics committees and a signed consent form was obtained. Synovial tissue was obtained from patients with RA who were operated for synovectomy. The synovial membranes were minced aseptically, then dissociated enzymatically with collagenase (4·0 mg/ml; Sigma) in RPMI 1640 for 4 h at 37°C. The obtained cells were plated in culture dishes and allowed to adhere. To eliminate non-adherent cells from the synovial cell preparation, the plated cells were cultured for 18 h with RPMI 1640, supplemented with 10% fetal calf serum (FCS) at 37°C in humidified 5% CO_2 in air. Cells were then washed extensively with PBS. Adhering synovial cells were removed by adding trypsine-EDTA and washed with PBS containing 2% FCS. The collected synovial cells were used at passages 3 or 4 for subsequent experiments. Synovial cell preparations were less than 1% reactive with MoAbs CD3, CD20 (Coulter Immunology, Hialeah, FL), Leu-M3 (Becton Dickinson, Mountain View, CA) and anti-human von Willebrand factor (Immunotech, Marseille, France), which respectively define an antigen on mature lymphocytes, monocytes/macrophage and vascular endothelial cells.

Synovial cell proliferation assay

Synovial cells (1×10^4/well) were seeded in 96-well flat-bottomed plates (Costar, Cambridge, MA) containing 200 μl RPMI 1640 medium, supplemented with 10% fetal bovine serum (FBS) and allowed to adhere for 24 h. The adhering cells were washed and incubated in 200 μl of a serum-free medium (RPMI 1640, containing 0·3% bovine serum albumin (BSA)) for 24 h. After this incubation, PDGF (20 ng/ml) was added to each well in the presence or absence of various concentrations of rapamycin for another 24 h. Eight hours before the termination of culture, 0·5 μCi of ^{3}H-thymidine (New England Nuclear, Boston, MA) was added to each culture. At the end of the incubation period, removal of unincorporated ^{3}H-thymidine was performed by washing dishes with PBS. Cells were later harvested on glass filters in a semi-automatic cell harvester (Lab Mash; Labo Science, Tokyo, Japan). Radioactivity was determined using a liquid scintillation β-counter.

Analysis of cell cycle

Synovial cells (5×10^5) were plated in culture dishes (Falcon 3003; Becton Dickinson) in RPMI 1640 supplemented with 10% FBS. For serum starvation, cells were washed with PBS and maintained in RPMI 1640 with 0·3% BSA for 24 h. After starvation, synovial cells were stimulated with PDGF (20 ng/ml) in the presence or absence of rapamycin (100 nM) for another 24 h. The synovial cells were fixed at −20°C with 70% ethanol, then washed and incubated at 37°C for 30 min with ribonuclease (100 μg/ml; Sigma). After centrifugation, cells were resuspended in 2·0 ml of propidium iodide (100 μg/ml; Sigma) in PBS for at least 1·0 h, then analysed by flow cytometry. An argon–ion laser flow cytometer (Profile model; Coulter) was used, with an excitation of 488 nm. Red fluorescence was collected with a photomultiplier masked with 610 nm long-band pass filter. The cells (2×10^4) were collected at a sample flow rate of 10 μl/min.

Protein tyrosine phosphorylation and serine/threonine kinase activation

Synovial cells were grown to subconfluence on 10 cm culture dishes and starved by serum-free medium for 24 h. Various concentrations of rapamycin were added during the final 1 h before stimulation to inhibit the protein kinase. After starvation, synovial cells were stimulated with PDGF (20 ng/ml) for 5 min. Cells were washed with cold PBS and lysed by the addition of a lysis buffer (1% Nonidet P40/50 mM Tris, pH 7·5/100 mM NaCl/50 mM NaF/5 mM EDTA/20 mM β-glycerophosphate/1·0 mM sodium orthovanadate/10 μg/ml aprotinin/10 μg/ml leupeptin) for 20 min at 4°C . Insoluble material was removed by centrifugation at 15 000 ***g*** for 15 min at 4°C. The supernatant was saved and the protein concentration was determined using the BioRad protein assay kit. An identical amount of protein (50 μg) for each lysate was subjected to 10% SDS–PAGE. Proteins were transferred to a nitrocellulose membrane. The filters were blocked for 1·5 h using 5% non-fat dried milk in TBS (50 mM Tris, 0·15 M NaCl, pH 7·5) containing 0·1% Tween 20, washed in TBS and incubated at room temperature for 2 h in a 1:1000 dilution of mouse anti-phosphotyrosine MoAb (4G10), anti-p70^{S6K}, anti-p90^{rsk} or a 1:5000 dilution of rabbit anti-MAP kinase antiserum C2 [15]. Filters were later washed in TBS and incubated with 1:1000 dilution of donkey anti-mouse or anti-rabbit IgG antibodies, coupled with horseradish peroxidase. The enhanced chemiluminescence (ECL) system (Amersham; Aylesbury, UK) was used for detection. Filters were subsequently exposed to the film for 15 s and the latter was processed at a later stage. The change in serine/threonine kinase activity could be assayed in electrophoresis by the presence of a phosphorylated form of kinases with reduced mobility. Such a shifted form corresponds with an activated form resulting from phosphorylation [12,15–17].

In vitro *kinase assay for MAP kinase*

In vitro kinase assay was slightly modified from a previously described method [18]. After starvation, synovial cells were stimulated with PDGF (20 ng/ml) for 5 min. Incubation was terminated by lysis buffer (10 mM HEPES pH 7·8/15 mM KCl/1 mM EDTA/2 mM EGTA/10% glycerol/1 mM PMSF/1 mM DTT/10 μg/ml leupeptin/0.1 mM Na_3VO_4/20 mM β-glycerophosphate/0·2% NP-40). After centrifugation, supernatants were immunoprecipitated with anti-MAP kinase antiserum. The immune complexes were collected using protein A Sepharose beads, washed with lysis buffer and incubated with 0·4 μCi of γ-^{32}P-ATP (New England Nuclear, Boston, MA) in 40 μl of kinase buffer (30 mM Tris pH 8·0/20 mM $MgCl_2$/2 mM $MnCl_2$/10 μM cold ATP) using myelin basic protein (Sigma) as substrate for 15 min at 30°C. The mixtures were electrophoresed on a 10% Tricine–SDS polyacrylamide gel and autoradiographed.

RESULTS

Rapamycin inhibited PDGF-induced synovial cell proliferation and cell cycle progression

Previous studies have demonstrated the potency of PDGF as a growth factor for synovial cells [7]. To determine whether PDGF-induced mitogenic activity is dependent on the activation of serine/threonine kinases, we examined the effects of rapamycin on synovial cell growth. As shown in Fig. 1, rapamycin significantly blocked PDGF-induced synovial cell growth in a dose-dependent manner. We also analysed the effect of rapamycin on PDGF-induced synovial cell cycle progression. Synovial cells were synchronized in early G_1 phase by starvation for 24 h and then induced to enter the S-phase by 20 ng/ml of PDGF. The addition of 100 nM rapamycin to PDGF-stimulated synovial cells produced a

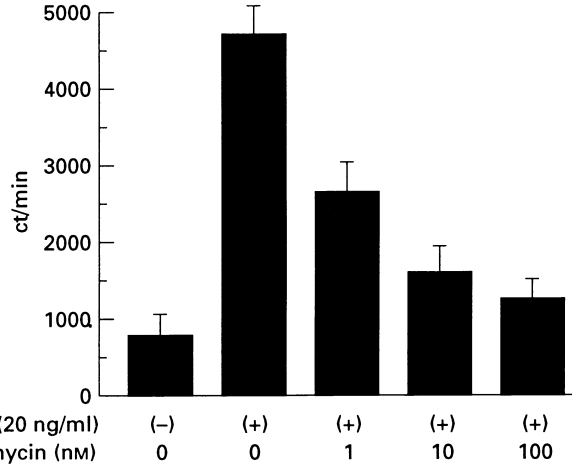

Fig. 1. Modulation of platelet-derived growth factor (PDGF)-induced synovial cell proliferation by rapamycin. Quiescent synovial cells were cultured with 20 ng/ml PDGF in the presence or absence of various concentrations of rapamycin for 24 h. Synovial cell proliferation was measured by ^{3}H-thymidine incorporation.

virtual inhibition of the S-phase entry and arrested the cell cycle (Fig. 2).

Rapamycin did not inhibit PDGF-induced tyrosine phosphorylation and MAP kinase activation in synovial cells

To examine further the effect of rapamycin on the growth arrest of synovial cells, we examined the early biochemical events in PDGF-stimulated synovial cells. Stimulation of synovial cells with PDGF resulted in tyrosine phosphorylation of several proteins (Fig. 3). However, rapamycin did not influence such effect of PDGF. It has been demonstrated that PDGF stimulation activates several serine/threonine kinases, such as MAP kinases and S6 kinases [11,12]. Previous reports have indicated that serine/threonine kinases, including MAP kinases p70^{S6K} and p90^{rsk}, are phosphoproteins whose activities are increased by phosphorylation, and that these activated forms have reduced mobility on gel electrophoresis [12,15–17]. As shown in Fig. 3, MAP kinase activation could be analysed by a shift in ERK1 and ERK2. PDGF-induced activation of MAP kinases was not influenced by pretreatment with rapamycin. MAP kinase can be measured by immune complex kinase assay more precisely. In PDGF-stimulated synovial cells, an increase of MAP kinase activity was observed. Rapamycin pretreatment did not affect this PDGF-induced MAP kinase activation in synovial cells (Fig. 4).

Rapamycin inhibited P70 S6 kinase activity in synovial cells

In contrast to its effects on MAP kinase, 100 nM of rapamycin inhibited PDGF-induced activation of p70^{S6K} completely (Fig. 5). The status of p90^{rsk} after PDGF stimulation was examined in a similar manner (Fig. 5). PDGF stimulation induced a shift in p90^{rsk} due to phosphorylation, but rapamycin did not inhibit PDGF-induced p90^{rsk} activation.

To determine whether the inhibition of p70^{S6K} activity by rapamycin can be induced in other growth factor-stimulated synovial cells, we examined the effects of rapamycin using serum-stimulated synovial cells. As shown in Fig. 6, rapamycin selectively inhibited p70^{S6K} activation, but failed to modulate tyrosine phosphorylation, MAP kinase activation or p90^{rsk} activation.

DISCUSSION

Rapamycin, with a chemical structure similar to FK506 and cyclosporin A, is a potent inhibitor of T lymphocytes, although it

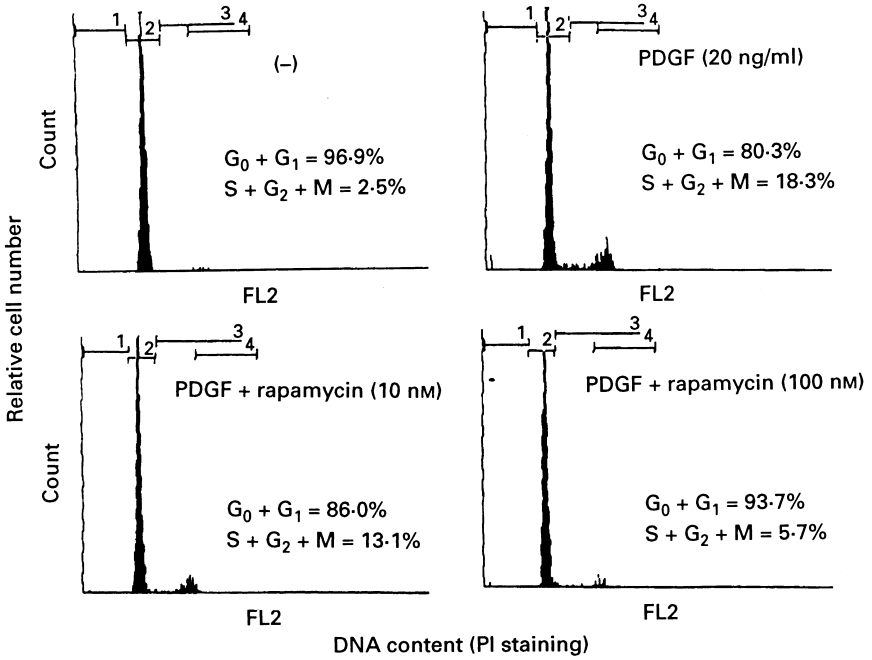

Fig. 2. Rapamycin arrests platelet-derived growth factor (PDGF)-stimulated synovial cell cycle progression in G_1phase. Quiescent synovial cells were unstimulated or stimulated with 20 ng/ml PDGF for 24 h. Ten or 100 nM of rapamycin were added to the PDGF-stimulated synovial cell culture. Cell cycle analysis was performed as described in Materials and Methods. The DNA histograms of synovial cells show the percentage of $G_0 + G_1$ and $S + G_2 + M$ phase.

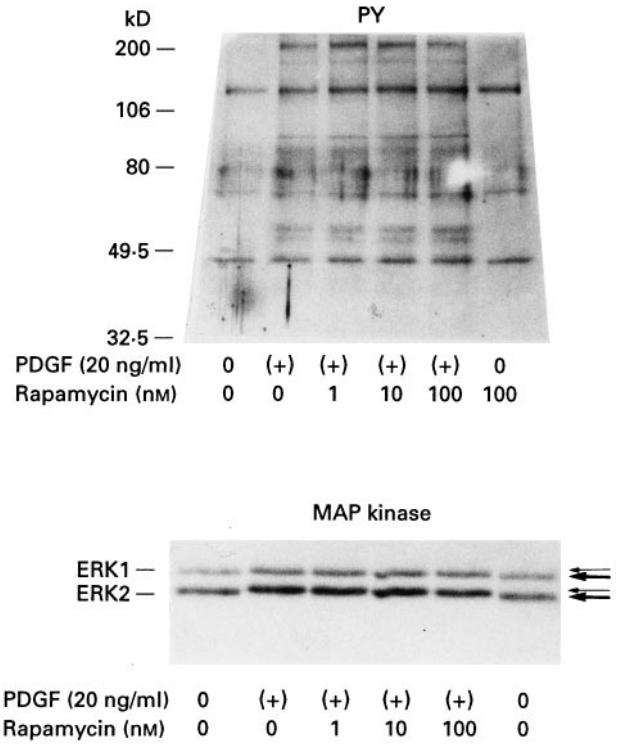

Fig. 3. Platelet-derived growth factor (PDGF) stimulation induces tyrosine phosphorylation and MAP kinase activation in synovial cells. Synovial cells were starved for 24 h with serum-free medium and pretreated with various concentrations of rapamycin for 1 h before stimulation. Quiescent synovial cells were stimulated with PDGF (20 ng/ml) for 5 min. Equal amounts of cell lysates were electrophoresed on 8% polyacrylamide gel and anlalysed by immunoblots using anti-phosphotyrosine antibody or anti-MAP kinase antiserum. The slower-migrating forms of ERK1 and ERK2 correspond to activated form. A representative example of four similar experiments.

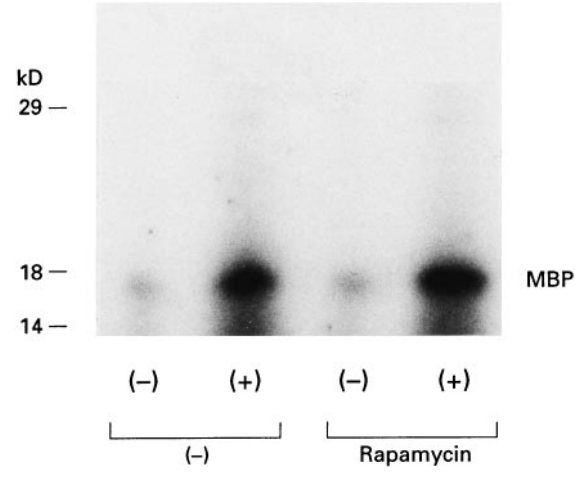

Fig. 4. Rapamycin treatment did not affect platelet-derived growth factor (PDGF)-induced MAP kinase activation of synovial cells. Control and rapamycin-treated (100 nM, for 1 h) synovial cells were stimulated with PDGF (20 ng/ml) for 5 min. MAP kinase was immunoprecipitated from each lysate and kinase assay with myelin basic protein (MPB) as a substrate was set up on the immunoprecipitates, and MPB phosphorylation was visualized by autoradiography on 10% Tricine–SDS polyacrylamide gel. A representative example of three similar experiments.

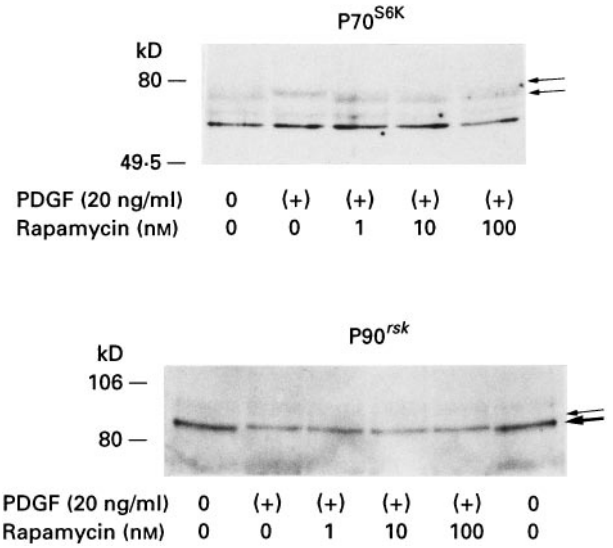

Fig. 5. Platelet-derived growth factor (PGDF) stimulation induces phosphorylation of $p70^{S6K}$ and $p90^{rsk}$ in synovial cells. Synovial cells were starved for 24 h by serum-free medium and pretreated with various concentrations of rapamycin for 1 h before stimulation. Quiescent synovial cells were stimulated with PDGF (20 ng/ml) for 5 min. The phosphorylation of $p70^{S6K}$ and $p90^{rsk}$ was examined by immunoblot analysis. The slower migrating species on SDS–polyacrylamide gel correspond to phosphorylated and activated forms. A representative example of four similar experiments.

acts by a mechanism different from both FK506 and cyclosporin A [19]. Recently, it was shown that rapamycin exhibits the capacity to inhibit not only the growth of T cells, but also the proliferation of cells with different cell lineage stimulated by 'non-immune' growth factors [20].

In the present study, the effects of rapamycin were examined on human synovial fibroblast growth stimulated by PDGF and serum. The major finding of our study was that stimulation of human synovial fibroblasts with PDGF and serum resulted in tyrosine phosphorylation and activation of MAP kinase, p70 S6 kinase and $p90^{rsk}$. Furthermore, we also demonstrated that rapamycin blocked PDGF-induced synovial cell growth and activation of p70 S6 kinase, but not that of MAP kinase and $p90^{rsk}$.

It has been previously established that stimulation by PDGF activates receptor tyrosine kinase and that this signal is ultimately translated into phosphorylation of several serine/threonine kinases, such as MAP kinase and S6 kinase [9–12]. Two distinct families of growth factor-related S6 kinases, p70 S6 kinase and $p90^{rsk}$, have been identified [21,22]. Activation of these kinases by phosphorylation on serine and threonine residues resulted in 40S ribosomal protein S6 phosphorylation. This process is important for cell cycle progression in a variety of cells [23,24].

Recent studies have demonstrated a selective blockage of p70 S6 kinase activation cascade by rapamycin [14,17,25]. Our data confirmed the selective blocking of PDGF-induced p70 S6 kinase activation by rapamycin in RA synovial fibroblast. Our data also demonstrated that inhibition of rapamycin-mediated growth and cell cycle arrest correlated with the kinase activity of p70 S6 kinase, but not with that of MAP kinases and $p90^{rsk}$. Based on these results, we suggest that activation of p70 S6 kinase is a critical event for growth factor-induced human synovial cell growth. Resent work has shown that $p90^{rsk}$ is a substrate of MAP kinase

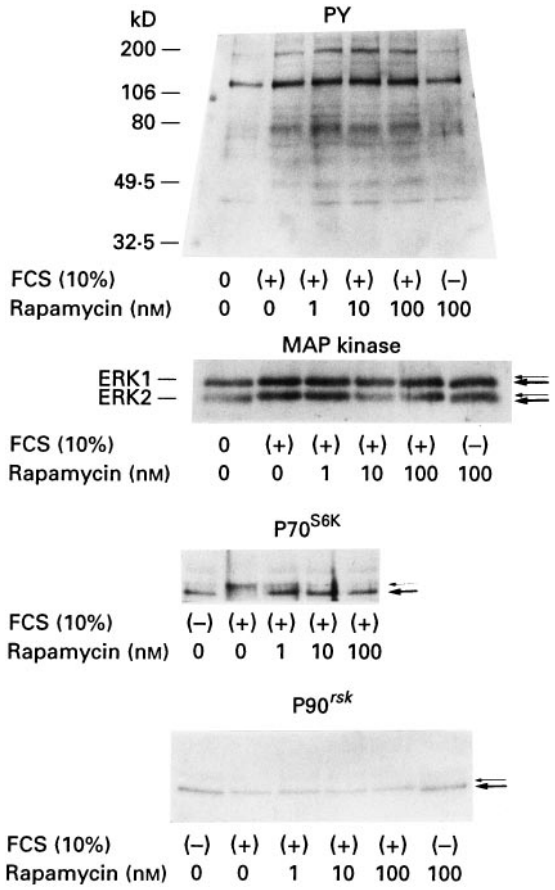

Fig. 6. Immunoblot analysis of tyrosine phosphorylation and MAP kinase, $p70^{S6K}$, and $p90^{rsk}$ kinases in serum-stimulated synovial cells. Synovial cells were starved for 24 h with serum-free medium and pretreated with various concentrations of rapamycin for 1 h before stimulation. Quiescent synovial cells were stimulated with fetal calf serum (FCS) (10%) for 5 min. Equal amounts of cell lysates were examined by immunoblots using anti-phosphotyrosine antibody anti-MAP kinase, anti-$p70^{S6K}$, and anti-$p90^{rsk}$ antiserum. A representative example of three similar experiments.

and its kinase activity is regulated by MAP kinase [15]. So, our results suggest that p70 S6 kinase and MAP kinase/$p90^{rsk}$ are a distinct signalling cascade in PDGF-stimulated synovial fibroblast. In human synovial fibroblasts, rapamycin may affect its target, p70 S6 kinase or upstream kinase, and block growth factor-mediated signals.

The synovium of RA is characterized by tumour-like proliferation and neovascularization induced by growth factors generated during the inflammatory process [1–7]. The underlying mechanisms of growth factor-mediated signal transduction are, therefore, relevant to the pathogenesis of RA. Furthermore, synovial fibroblasts of RA overexpress certain oncogene products, such as c-myc and c-fos [26], that are known to augment cell growth and facilitate joint destruction. It has also been shown that stimulation of PDGF induces c-fos and c-myc mRNA [27] and that a signalling pathway via S6 kinase contributes towards the activation of c-fos transcription [28]. Our results identified a rapamycin-sensitive signalling pathway via $p70^{S6K}$ that may regulate the transcription factor for the overgrowth of human synovial fibroblasts. In this regard, rapamycin also blocks T cell signalling pathway as a strong immunosuppressant [29]. Thus, these findings suggest that the pharmacological inhibition of T cell activation and synovial cell growth by rapamycin may help in identifying a new and potent therapy for RA.

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