Increased phosphorylation of ezrin/radixin/moesin proteins contributes to proliferation of rheumatoid fibroblast-like synoviocytes

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Abstract

Objectives. Increasing evidence indicates that ezrin/radixin/moesin (ERM) proteins may play a critical role in cell proliferation. This study examined the role of ERM proteins in proliferation of fibroblast-like synoviocytes (FLS) from patients with RA.

Methods. Synovial tissues (STs) were obtained from 18 RA and 6 OA patients. The expression of ERM and its phosphorylated proteins in cultured FLS and ST was assessed by western blots or IF staining. Small interference RNA (siRNA)-mediated ERM knockdown was used to inhibit phosphorylation of ERM. Proliferation of FLS was measured by bromodeoxyuridine (BrdU) incorporation into cell DNA and by PCNA immunoblotting.

Results. Our study showed that increased phosphorylation of ERM proteins was found in ST and FLS from patients with RA as compared with OA patients and non-arthritis controls. Treatment with TNF-α, IL-1β or PDGF-induced phosphorylation of ERM proteins in dose- and time-dependent manner by RA FLS, but did not affect the expression of total ERM protein. Rho kinase and p38MAPK signal pathways were involved in TNF-α-induced ERM phosphorylation. We further showed that inhibition of ERM phosphorylation by siRNA-mediated ERM knockdown suppressed TNF-α- or IL-1β-induced BrdU incorporation and PCNA expression in RA FLS.

Conclusions. This study provides the novel evidence that increased phosphorylation of ERM proteins may contribute to proliferation of RA FLS, suggesting that specific inhibition of ERM phosphorylation may be a new therapeutic approach for RA.

Key words: Rheumatoid arthritis, Fibroblast-like synoviocyte, Signal transduction, Ezrin/radixin/moesin, Pathogenesis, Cytokine, Synovium, TNF-α, Cytoskeletal protein.

Introduction

RA is an autoimmune disease of joints characterized by chronic inflammation, synovial hyperplasia and erosion of bone and cartilage. A body of evidence suggests that fibroblast-like synoviocytes (FLS) are key players in the pathophysiological process of RA [1]. Aberrant proliferation of resident FLS is considered as a critical contributor to rheumatoid synovial hyperplasia, and eventually results in the destructive phase of disease, causing damage to cartilage and bone. Targeting the proliferative fibroblast could facilitate regeneration of synovial joints [2].

Ezrin/radixin/moesin (ERM) are a family of proteins that act as cross-linkers between the cytoskeleton and plasma membrane by binding to the membrane proteins at their NH2-terminal (N) domains and to filamentous actin (F-actin) at its COOH-terminal (C) domain [3, 4]. ERM proteins are critical modulators in formation of microvilli, cell adhesion, cell motility, maintenance of cell shape and...
membrane trafficking [5, 6]. Recently, increasing evidence has shown that ERM proteins are not only involved in alteration of cellular architecture but also in a number of cell signalling pathways [7]. These findings brought about the identification of other biological functions of ERM, such as cell proliferation and apoptosis and their regulation by various stimuli in a cell-type-specific manner [7–10]. However, the role of ERM in the pathogenesis of RA remains undetermined.

One critical mechanism for modulating ERM protein functions is through phosphorylation on a conserved threonine residue in C terminus. The linker between membrane and cytoskeleton is regulated by the phosphorylation of each ERM protein. Recent evidence indicates that several kinase pathways, such as p38MAPK and Rho kinase (ROK), may be involved in phosphorylation of ERM on the conserved threonine residue in some cell lines [4, 5, 8, 11, 12]. In the present study, we explored the possible role of phosphorylation of ERM proteins in proliferation of RA FLS and its signal mechanisms.

**Materials and methods**

**Reagents and antibodies**

Recombinant human TNF-α, IL-1β and PDGF were obtained from R&D Systems (Minneapolis, MN, USA). DMEM/F12, FBS, antibiotics, Trypsin-EDTA, PBS and other products for cell culture were purchased from Invitrogen (Carlsbad, CA, USA). Anti-β-actin antibody was obtained from Sigma Chemicals (St Louis, MO, USA). SB203580 and Y27632 were purchased from Calbiochem (San Diego, CA, USA). Antibodies for phospho-ERM, ERM and PCNA were purchased from Cell Signaling Technology (Danvers, MA, USA).

**Patients and controls**

Synovial tissues (STs) were obtained from patients with active RA (17 women and 1 man, aged 36–62 years) or OA (4 women and 2 men, aged 55–68 years), who were undergoing synovectomy or joint replacement. Non-arthritis (NA) STs were obtained by arthroscopic biopsy from three patients that had meniscal injuries or cruciate ligament rupture without history of acute or chronic arthritis. RA was diagnosed according to the 1987 revised criteria of the ACR [13], and OA was diagnosed according to the ACR criteria [14]. At the time of the study, with the exception of four RA patients, all patients were receiving therapy that included low-dose prednisone (≤10 mg/day) (*n* = 13), NSAIDs (*n* = 14) and non-biologic DMARDs (*n* = 11). The study was performed according to the recommendations of the Declaration of Helsinki and approved by the Medical Ethics Committee of the First Affiliated Hospital, Sun Yat-sen University, China. All patients gave informed consent to take part in the study.

**Cell culture**

STs were cut into small pieces and digested with collagenase in DMEM/F12 medium for 2 h at 37 °C to isolate synoviocytes. The synoviocytes were grown in DMEM/F12 medium containing 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin in a humidified incubator at 37 °C under 5% CO₂, 21% O₂ and 75% N₂. At confluence, the cells were trypsinized and passaged and used after 3–5 passages.

**Western blot analysis**

For each experiment, a total of 5 × 10⁵ cells were seeded, and at confluence (~70%), cells were made quiescent for 24 h in DMEM/F12 medium containing 0.5% FBS, and then treated with various agents. For detection of PCNA expression, nuclear extracts were prepared with NE-PER Nuclear and Cytoplasmic Extraction Reagents Kit (Pierce, Rockford, IL, USA) according to the manufacturer’s instructions. For detection of expression of signal proteins, cells were rinsed twice with ice-cold PBS and 0.5 ml of ice-cold lysis buffer [50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 100 μg/ml phenylmethylsulphonyl fluoride (PMSF), 0.1% SDS, 1% Nonidet P-40 (NP-40), 0.5% sodium deoxycholate, 10 μg/ml aprotinin, 2 μg/ml leupeptin, 10 mM EDTA] was added, incubated for 20 min on ice, and then were scraped and centrifuged. For protein extracts of ST, the tissues were rapidly frozen in liquid nitrogen and homogenized in ice-cold lysis buffer containing 20 mM HEPES–NaOH, 10 mM NaCl, 10 mM KCl, 5 mM MgCl₂, 1 mM dithiothreitol and Complete (Roche Molecular Biochemicals, 1 tablet/50 ml), and then the samples were centrifuged and the supernatant was stored at −80 °C until use. Protein concentrations were determined by the BCA protein assay (Pierce, Rockford, IL). Equal amounts of protein were solubilized in Laemmli buffer (62.5 mM Tris–HCl pH 6.8, 10% glycerol, 2% SDS, 5% β-mercaptoethanol and 0.00625% bromophenol blue), boiled for 5 min and then separated by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were probed with primary antibodies diluted 1:1000 for ERM and PCNA and 1:500 for phospho-ERM in TBS-T containing 5% non-fat milk at 4°C overnight. The membranes were incubated with the appropriate secondary antibodies for 1 h at room temperature. Immunoreactive bands were visualized by enhanced chemiluminescence (ECL; Amersham Pharmacia Biotech, Piscataway, NJ, USA) reaction. Each blot is a representative of at least three similar independent experiments.

**IF staining**

For detecting the distribution of P-ERM in RA FLS, cells were grown on glass coverslips. The cells were fixed with acetone and permeabilized with 0.1% Triton X-100 (Sigma Chemicals, St Louis, MO, USA) in PBS for 5 min at room temperature. The cells were incubated with anti-phospho-ERM antibody (diluted 1:100) for 1 h at room temperature, and then incubated with FITC-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). After washing in PBS, cells were incubated for 3 min with 0.25 mg/ml 4′6-diamino-2-phenylindole dihydrochloride (DAPI). For detection of cytoskeletal F-actin, the cells were incubated with AlexaFluor-546 phalloidin.
(Molecular Probes; Invitrogen, Eugene, OR, USA). The coverslips were mounted on glass slides with anti-fade mounting media and examined using a confocal fluorescence microscopy (Zeiss LSM510; Carl Zeiss Mocrolmaging, Thornwood, NY, USA).

For evaluating the expression of P-ERM in ST, ST specimens were fixed in 4% paraformaldehyde. The samples were stained with rabbit anti-phospho-ERM antibody and FITC-conjugated secondary antibody according to the manufacturer’s instructions. The stained samples were examined by confocal fluorescence microscopy and the representative sections were photographed.

**Treatment with ERM small interfering RNA**

Small interfering RNA (siRNA) targeting ERM or control siRNA was designed and obtained as a pool of four or more siRNA duplexes from Shanghai GenePharma (Shanghai, China). RA FLS were cultured in 12-well plates. A transfection mixture of 100 nM siRNA and 10 μg/ml lipofectin in serum-free medium was added to medium-aspirated FLS for 4 h. The FLS were then incubated with complete DMEM/F12 containing 10% FCS for 48 h before experiments. At the end of culture, the effect of these siRNAs on protein expression of total ERM or P-ERM was analyzed using western blot.

**FLS proliferation assays**

RA FLS were cultured for 24 h at a density of 1 × 10^4/well in 96-well plates in serum-free medium. After starving, the cells were incubated with TNF-α (10 ng/ml) or IL-1β (4 ng/ml) for 72 h, and then incubated with 10 μM bromodeoxyuridine (BrdU) for 1 h. BrdU incorporation was assessed in triplicate, using a cell proliferation ELISA kit (Roche, Shanghai, China) according to the manufacturer’s instructions.

**Statistical analysis**

Data are expressed as mean (±SEM). Student’s t-test or one-way analysis of variance (ANOVA) was used analysis the differences between groups. P ≤ 0.05 was considered to be statistically significant.

**Results**

**Increased expression of P-ERM proteins in STs and FLS from RA patients**

The expression of P-ERM proteins in ST was measured by western blot analysis. As shown in Fig. 1A, expression of P-ERM proteins was higher than in OA ST or in NA samples. In agreement with these findings, immunohistochemical analysis revealed that expression of the P-ERM proteins was prominent in RA ST and mostly localized in the synovial lining and sublining cells, whereas expression was much less prominent in OA ST and in NA ST (Fig. 1B). These results indicated that P-ERM protein expression was increased in RA ST. We also examined the expression of P-ERM in isolated and cultured FLS from RA, OA and NA individuals. As shown in Fig. 1C, P-ERM protein expression was increased in RA FLS as compared with FLS from OA patients and NA controls.

**Induction of P-ERM in RA FLS by cytokines**

Since pro-inflammatory cytokines, especially TNF-α and IL-1β, play a critical role in the pathogenesis of RA, we examined the effect of the cytokines on the threonine phosphorylation of ERM in confluent RA FLS. As shown in Fig. 2A, treatment with TNF-α (10 ng/ml) induced an increase in P-ERM expression in a time-dependent manner; however, there was no detectable change in the protein expression of total ERM.

We further evaluated the effect of IL-1β in phosphorylation of ERM in RA FLS. Consistent with the TNF-α stimulation, IL-1β treatment induced a significant increase in P-ERM expression in a time- and dose-dependent manner (Fig. 2B). We also demonstrated the induction of PDGF in ERM phosphorylation by RA FLS (data not shown).

The distribution of P-ERM in RA FLS was examined by IF staining. As shown in Fig. 2E, before treatment with TNF-α, P-ERM proteins were localized at the cell periphery as well as in the cytoplasm. TNF-α stimulation induced an increase in the amount of P-ERM proteins, consistent with the immunoblotting analysis. The P-ERM proteins were present primarily at the cell periphery at 12 h. TNF-α stimulation did not alter the distribution and amount of ezrin within RA FLS.

**The signalling mechanisms of phosphorylation of ERM in TNF-α-induced RA FLS**

To investigate the signalling mechanisms leading to phosphorylation of ERM in TNF-α-induced RA FLS, we used known inhibitors of several signalling pathways, including SB203580 (an inhibitor of p38 MAPK) and Y27632 (an inhibitor of ROK), and we documented that these chemical inhibitors exhibited no cytotoxicity at the concentration used in our experiments (data not shown). TNF-α stimulation induced rapid activation of p38 MAPK (Fig. 3A), and pre-treatment with 10 μM SB203580 completely prevented ERM phosphorylation at 12 h (Fig. 3B). Pre-treatment with Y27632 (10 μM) also prevented ERM phosphorylation. These results indicate that p38 MAPK and ROK pathways might be involved in TNF-α-induced ERM phosphorylation in RA FLS.

**The role of P-ERM in modulating proliferation of RA FLS**

Since expression of P-ERM proteins is increased in RA FLS, we explored whether this overexpression is required for optimal proliferation of RA FLS. The role of ERM was examined using siRNA targeting of ERM. RA FLS were incubated with control siRNA or siRNA for ERM. As shown in Fig. 4A, these siRNAs treatment efficiently and specifically suppressed their respective target. Treatment with three siRNAs combined inhibited the expression of total ERM proteins. Similarly, treatment with ERM siRNA,
either singly or in combination, also prevented TNF-α-stimulated expression of P-ERM proteins (Fig. 4B).

To explore the effect of ERM modulation by TNF-α on RA FLS proliferation, transfected cells were treated or not treated with TNF-α and BrdU (10 μM). As shown in Fig. 4C, in control siRNA-treated cells, TNF-α substantially increased FLS proliferation as measured by BrdU incorporation. However, this increase was completely reduced by treatment with ERM siRNA. Furthermore, PCNA expression, assessed by western blot analysis, was also reduced as compared with control siRNA-treated cells (Fig. 4C). Similarly, we also found that ERM siRNA treatment inhibited IL-1β-induced increase in BrdU incorporation and PCNA expression in RA FLS (Fig. 4D). Taken together, these results provide novel evidence that ERM phosphorylation mediates cytokine-induced proliferation in RA FLS and that suppression of ERM phosphorylation by RNA interference technique may alter RA FLS function.

Since ERM phosphorylation was inhibited by the p38 inhibitor SB203580, or the ROK inhibitor Y27632, the effect of these inhibitors on TNF-α-induced proliferation increases was also evaluated. As expected, these inhibitors suppressed TNF-α-induced proliferation in RA FLS (data not shown).

The effect of ERM protein inhibition on cytoskeleton alteration of RA FLS

Since ERM proteins have been considered as regulators of cytoskeleton formation in some cell lines, we assessed whether F-actin reorganization was affected in response
**Fig. 2** Induction of ERM phosphorylation in RA FLS by cytokines. Confluent FLS were treated with TNF-α or IL-1β at the indicated dose for the indicated times, and P-ERM and total ERM were measured by western blot analysis or IF staining. **(A)** Dose-dependent changes in threonine phosphorylation of ERM induced by TNF-α for 12 h. **(B)** Time-dependent changes in phosphorylation of ERM induced by TNF-α (10 ng/ml). **(C)** Dose-dependent changes in threonine phosphorylation of ERM induced by IL-1β for 6 h. **(D)** Time-dependent changes in phosphorylation of ERM induced by IL-1β (4 ng/ml). Data are expressed as mean (S.E.M.) of densitometrical quantification (right panel) from at least three independent experiments and presented as fold changes over controls after normalization by total ERM levels. Vs vehicle-treated controls: *P < 0.05, **P < 0.01. **(E)** Threonine-P-ERM proteins predominantly localized on the periphery of FLS after treatment with TNF-α (10 ng/ml) for 12 h. P-ERM or ezrin (green) and nuclei (blue) were examined using IF staining and confocal microscopy. The pictures represent three independent experiments with similar observations. Original magnification ×400.
Fig. 3 The signals involved in TNF-α-induced phosphorylation of ERM in RA FLS. Pre-treatment with a specific p38 inhibitor, SB203580, or ROK inhibitor, Y27632, suppressed TNF-α-induced phosphorylation of ERM. RA FLS were pre-treated with or without 20 μM SB203580 or 10 μM Y27632 for 1 h before they were stimulated with buffer or 10 ng/ml TNF-α for 12 h. Vs Basal: * P < 0.05; vs TNF-α treatment: # P < 0.05.

to ERM inhibition in RA FLS, and F-actin was stained with phalloidin. As expected, TNF-α stimulation led to an increase in the condensation of actin stress fibre formation, which was markedly prevented in ERM siRNA-transfected cells (Fig. 5).

**Discussion**

Our study showed that P-ERM protein expression was increased in ST and FLS derived from RA patients. We also found that expression of P-ERM was upregulated by cytokines TNF-α, IL-1β and PDGF. ROK and p38 MAPK signal-mediated TNF-α induced ERM phosphorylation. Importantly, knockdown of the expression of ERM proteins by RNA interference techniques prevented phosphorylation of ERM, and thereby inhibited TNF-α or IL-1β-induced RA FLS proliferation and TNF-α-induced cytoskeleton formation. These data suggest that ERM proteins are of relevance for proliferation of RA FLS and may contribute to synovial hyperplasia in RA.

Initially, ERM proteins were considered as the crosslinkers between the actin cytoskeleton and plasma membrane. Recent studies, however, have implied that ERM proteins may play important roles in cell proliferation and apoptosis and in signal transduction involved in a wide variety of cellular functions [7, 15]. For instance, it has been shown that ezrin participates in regulation of cell motility, adhesion and morphogenesis, cell survival and cellular proliferation [7, 9, 15]. In endothelial cell lines, phosphorylation of ERM proteins modulates permeability [12, 16]. Ezrin and moesin have been indicated in regulation of Fas-mediated apoptosis [17–19]. ERM proteins also control migration of some cell lines [20–22].

Increasing evidence implies an important role of ERM proteins in cell proliferation. For example, in RS-4 mouse fibroblasts, microinjection of the purified IgG of anti-ezrin antiserum into the cytoplasm prevented their entry into S phase, suggesting a proliferative function of ezrin [23]. In a recent study, stem cell factor stimulation-induced phosphorylation of ERM proteins is shown to mediate melanocyte proliferation and migration through the PI3K pathway [24]. Several studies from tumour cell lines also show a direct relationship between ERM protein expression and cell proliferation [25–27].

In the present study, we first showed that the increased expression of P-ERM proteins in ST and fibroblast-like synoviocytes from RA patients is involved in modulation of TNF-α- or IL-1β-induced proliferation of RA FLS. Since RA synovial hyperplasia was considered to arise from increased FLS proliferation and/or decreased FLS apoptosis [1], our results indicate that increased phosphorylation of ERM in FLS may contribute to RA synovial hyperplasia. Our data thus identify a novel function of actin-binding proteins ERM in RA FLS.

Indeed, it has also been shown that depletion of gelso- lin, another actin-binding protein known to modulate actin depolymerization by preventing F-actin formation, from a mouse model with RA, resulted in aggravation of synovial hyperplasia and inflammation [28, 29]. This suggests an important role for some actin-binding proteins such as ERM in the progression of RA.

As a linker between membrane and cytoskeletal binding sites, ERM proteins need to be conformationally activated/phosphorylated to fulfill their biological functions. Although the mechanisms of phosphorylation of ERM proteins in RA FLS have not yet been described in other cell lines, such as endothelial cells and epithelial cells, the phosphorylation of ERM is regulated by many stimuli including TNF-α and IL-1β [8, 25, 30]. In our study, we found that in RA FLS ERM proteins are activated in a dose- and time-dependent manner by TNF-α, IL-1β or PDGF, critical cytokines in the pathogenesis of RA, suggesting that phosphorylation of ERM may mediate a variety of cytokine-induced signal events in RA FLS.

Previous studies reported that some signalling pathways, such as p38 MAPK and Rho, are required for ERM phosphorylation in several cell lines. In human pulmonary microvascular endothelial cells, it has been demonstrated that the p38 MAPK signal mediates TNF-α-induced phosphorylation of ERM proteins [12]. Another study also shows the modulation of RhoA/ROK signal on ERM phosphorylation in the nucleus accumbens [31]. Consistent with previous studies, in the present study, we demonstrated that activation of p38 and
Fig. 4 Inhibition of P-ERM proteins by siRNA decreased proliferation of RA FLS. (A) RA FLS were either untreated (U) or treated with control siRNA (C) or siRNA targeting ezrin (E), radixin (R) or moesin (M), either singly or in combination, at the dose of 100 nM. The effect of these siRNAs on the expression of total ERM protein was assessed using western blot analysis. (B) The effect of treatment with siRNA targeting ezrin (E), radixin (R) or moesin (M), either singly or in combination, on the TNF-α-induced phosphorylation of ERM. Data are presented as mean (s.e.m.) of densitometric quantification (right panel) from at least three independent experiments and expressed relative to the controls. (C and D) The effect of treatment with siRNA targeting ezrin (E), radixin (R) or moesin (M) in combination on TNF-α- (C) or IL-1β- (D) induced proliferation, measured by BrdU incorporation into cell DNA (left panel) and by PCNA immunoblotting (right panel). Data represent mean (s.e.m.) of five independent experiments from five different RA patients. Vs control siRNA: *P < 0.05; vs control siRNA with TNF-α or IL-1β treatment: #P < 0.05.
Rho kinases is required for TNF-α-induced ERM phosphorylation and cell proliferation, as evaluated by the inhibitory effect of p38 inhibitor SB203580 and ROK inhibitor Y27632, suggesting that ERM proteins may be downstream targets of the p38 and ROK signal essential in modulating RA FLS proliferation, providing a novel therapeutic target that could be exploited to regulate pathological synovial hyperplasia in RA.

**Rheumatology key messages**

- Phosphorylation of ERM proteins is increased in RA ST and FLS.
- Increased ERM phosphorylation may contribute to proliferation of RA FLS.
- Inhibition of elevated ERM phosphorylation may be a new therapeutic approach in RA.

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