

## Survivin is an essential mediator of arthritis interacting with urokinase signalling

Marcin Baran<sup>a, b</sup>, Linda Nilsson Möllers<sup>a</sup>, Sofia Andersson<sup>a</sup>, Ing-Marie Jonsson<sup>a</sup>, Anna-Karin Hultgård Ekwall<sup>a</sup>, Jan Bjersing<sup>a</sup>, Andrej Tarkowski<sup>a</sup>, Maria Bokarewa<sup>a, \*</sup>

<sup>a</sup> Department of Rheumatology and Inflammation Research, Sahlgrenska University Hospital, Gothenburg, Sweden

<sup>b</sup> Department of Microbiology, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, Krakow, Poland

Received: December 22, 2008; Accepted: February 17, 2009

### Abstract

Proto-oncogene survivin has recently been identified as a prognostic marker distinguishing patients with destructive rheumatoid arthritis (RA). In the present material of 132 RA patients and 82 controls, the levels of survivin correlated to urokinase (uPA) ( $r = 0.46$ ), a plasminogen activator over-expressed in inflamed joints and known to exhibit potent arthritogenic properties. Here we evaluate the functional relationship between these proteins using primary synovial fibroblasts and leucocytes of RA patients, human monocytic (THP-1) and fibroblast (MRC-5) cell lines. Using inhibitors of intracellular signalling, we show that uPA and survivin share common transduction pathways in synovial fibroblasts being dependent on the activity of tyrosine kinases, phosphatidylinositide 3 kinase and mitogen effector kinase. Moreover, uPA production is significantly reduced in fibroblasts if survivin synthesis has been silenced by siRNA. Importantly, silencing of survivin in fibroblasts prevented their invasive growth in knee joints of severe combined immune deficient mice. Interaction of uPA with receptor up-regulates survivin expression in leucocytes. In turn, survivin is required for the up-regulation of uPA receptor on the cell surface. These findings indicate that survivin is an essential mediator of arthritogenic properties of uPA regulating its synthesis in synovial fibroblasts and uPAR expression in leucocytes. Close correlation between survivin and uPA levels in patients with RA supports the importance of this connection for the pathogenesis of arthritis.

**Keywords:** survivin • arthritis • urokinase • oncogenes

### Introduction

Survivin (encoded by *BIRC5*) is the smallest member of the family of inhibitor of apoptosis proteins (IAPs). It consists of 142 amino acids comprising a 16.5-kD protein and may present in the form of different splice variants. Transcription of survivin is controlled by direct binding of oncogenic transcription factor STAT3, TCF-4 and tumour suppressor p53 to the promoter of *BIRC5* gene [1–3]. At the post-transcriptional level, survivin is stabilized by binding to molecular chaperones, including AIP and HSP90 [3–5]. Binding to chaperones facilitates translocation of survivin into mitochondria, where it accomplishes its cytoprotective effects through regulation of hepatitis B X-interacting protein, Smac/Diablo and X-linked IAP system of molecules [1, 6, 7].

Survivin displays a sharp cell cycle-dependent expression in the G2/M phase. It regulates cell cycle progression and mitosis by acting as a subunit in chromosomal passenger complex [8, 9]. Survivin interacts with a high number of proteins, regulators, transcriptional networks and modifiers, which establishes survivin as a key molecule expanding multiple parallel signalling in cellular homeostasis.

Expression of survivin is highly up-regulated in malignancy, being involved in the critical determinants of tumour progression such as cell proliferation, evasion of apoptosis, resistance to growth-inhibitory signals and angiogenesis [3]. Furthermore, levels of survivin in cancer cells correlates to poor prognosis and resistance to chemotherapeutic treatment [10, 11]. Fukuda and Pelus reviewed recent studies on a role for survivin in regulating function in normal cells under renewal and proliferation [12]. Studies on T cell development in mice using conventional knockout models showed that loss of survivin at early stages blocked transition of thymocytes from the double-negative to the double-positive stage, whereas survivin deletion at late stages decreased

\*Correspondence to: Maria BOKAREWA,  
Department of Rheumatology,  
Guldhedsgatan 10, S-413 46 Gothenburg, Sweden.  
Tel.: 46-31-3424692  
Fax: 46-31-823925  
E-mail: maria.bokarewa@rheuma.gu.se

a number of peripheral T cells without any effect on thymocyte development [13]. A requirement of survivin has been recently shown for the OX40-induced proliferation of effector T cells regulating G<sub>1</sub>-S transition in mature T cells [14]. Survivin expression in endothelial cells is recognized as an important mechanism of vascular remodelling. Indeed, disruption of survivin abrogates regeneration of endothelial cells and angiogenesis in response to vascular endothelial growth factor, basic fibroblast growth factor and angiogenic cytokines angiopoietin 1 and angiotensin II [15, 16]. In rheumatoid arthritis (RA), the hypertrophic synovial tissue forms pannus, which mimics the proliferative and invasive features of neoplasia invading and destroying periarticular cartilage and bone [17]. Deregulation of apoptosis machinery and p53-dependent cell damage control has been outlined among the major events responsible for synovial transformation [18–20]. We have recently shown that high levels of survivin in plasma are closely correlated with a destructive course of RA whereas the presence of antibodies against survivin was joint protective. Moreover, prospective evaluation of radiological changes in patients with early RA indicated high levels of survivin as an independent predictive factor for the development of erosions [21].

Urokinase (uPA) is a serine protease and an essential regulator of fibrinolysis converting plasminogen into its active form plasmin by a cleavage at Arg<sup>561</sup>-Val<sup>562</sup> [22]. Plasmin in turn degrades the proteins of the extracellular matrix, including fibrinogen, elastin, fibronectin and vitronectin [23, 24]. Independently of its serine protease activity, uPA modulates cell adhesion, migration and proliferation. These properties of uPA are related to its interaction and signalling through surface receptor (uPAR) [25]. Being a glycosylphosphatidylinositol-anchored receptor, uPAR lacks transmembrane and intracellular domains and involves lateral interactions with integrins, chemokine receptors and epidermal growth factor receptor for signal transduction [23, 26]. By interacting with the uPAR-integrin system, uPA triggers various intracellular signalling pathways, including tyrosine kinases, ERK and phosphatidylinositol 3 (PI3) kinases. It has been shown that when uPA binds to uPAR, several kinases are activated and invasion and migration are promoted [24]. Numerous studies have reported an increased uPA production in a whole variety of neoplasia. Elevated uPA expression is associated with clinical and pathological variables of aggressive disease and shorter survival in most studies [23, 27]. Furthermore, it has been shown that tumour dormancy is induced by down-regulation of uPAR in cancer cells involving integrin and MAPK signalling [28]. Activation of plasminogen in the inflamed RA joints is regulated by interleukin-1 [29, 30], potentially through its interaction with Toll/IL-1 receptor system of intracellular signalling. Rheumatoid synovial fibroblasts exhibit considerably increased uPA activity over the proliferating lining areas [31, 32]. Moreover, expression of uPA inside the joints has been shown to be both essential and sufficient for the development of arthritis [32–34].

Despite the co-expression of survivin and uPA in different neoplasia, no previous studies have been conducted on the functional relationship between the two proteins. Indeed, survivin and uPA have several common denominators, being in the crossroad of

tyrosine kinase and PI3-kinase intracellular signalling. Here, we present a study of the functional relationship between survivin and uPA with respect to (i) the role of survivin in the development of arthritis, (ii) the role of survivin for the uPA synthesis in synovial fibroblasts and for the expression of uPAR and (iii) the influence of uPA on survivin synthesis. Our results show that survivin is an essential mediator of arthritogenic properties of uPA, regulating its synthesis in synovial fibroblasts and uPAR expression in leucocytes. Silencing of survivin in fibroblasts prevented their invasive growth in mouse joint.

## Experimental procedures

### Material

Urokinase was a kind gift from Abbot laboratories (Chicago, IL). Dulbecco's Modified Eagle's Medium (DMEM)-Glutamax was from GIBCO (Paisley, UK). RPMI culture medium was from PAA: The Cell Culture Company (Pasching, Austria). Hepes, sodium pyruvate solutions and lipofectin transfection reagents were from Invitrogen (Paisley, UK). Lymphoprep was from Axis-Shield (Oslo, Norway). Foetal calf serum (FCS), gentamycin, bovine serum albumin (BSA), urea and Iscove's medium were from Sigma Aldrich (St. Louis, MO). Normocin was from Invivogen (San Diego, CA). Phycoerythrin (PE)-conjugated mouse anti-human CD87 and PE mouse IgG<sub>1k</sub> isotype control antibodies were from BD Pharmingen (San Diego, CA). The IL-8 antibody pair was from Endogen (Woburn, MA). Inhibitors of PI3-kinase (LY294002) and mitogen effector kinases (MEKs; PD90059) were from Biosource (Camarillo, CA). Inhibitors of NF- $\kappa$ B (parthenolide) and tyrosine kinases (genistein) were from Sigma (St. Louis, MO). [6-<sup>3</sup>H]-thymidine was from GE Healthcare (Piscataway, NJ). Survivin pre-designed siRNA and negative control RNA-1 were purchased from Ambion (Austin, TX). Protected siRNA targeting the *BIRC5* gene and *PLAU* gene as well as non-targeting control RNA were purchased from Dharmacon RNAi Technologies (Chicago, IL).

### Collection and preparation of synovial fluid and blood samples

Plasma and synovial fluid samples were collected from 132 RA patients (Rheumatology Clinic, Sahlgrenska University Hospital, Gothenburg, Sweden) and 82 matched controls. Clinical and demographic information on the cohorts is given in Table 1. The study was approved by the Ethical Committee of the University of Gothenburg. The informed consent was obtained from all patients enrolled in this study. Blood samples were obtained from healthy individuals ( $n = 66$ ) matched to the patients with RA by age and gender. Synovial fluid from 48 patients with non-inflammatory knee joint diseases (age 23–88 years, male 26, female 22) was used as control. Synovial fluid was obtained from knee joints by arthrocentesis, aseptically aspirated and transmitted into tubes containing sodium citrate (0.129 mol/l; pH 7.4). Blood samples were simultaneously obtained from the cubital vein and directly transferred into sodium citrate medium. Collected blood and synovial fluid samples were centrifuged at  $800 \times g$  for 15 min., aliquoted and stored frozen at  $-20^{\circ}\text{C}$  until use.

**Table 1** Clinical and demographic characteristics of patients with rheumatoid arthritis

	Erosive RA	Non-erosive RA	P-value*	Controls
	<i>n</i> = 70	<i>n</i> = 62		<i>n</i> = 82
Gender, f/m	50/20	45/16	n.s.	69/29
Age, years	61.5 ± 2.0	54.2 ± 1.3	<i>P</i> < 0.05	58.4 ± 1.4
Rheumatoid factor, +/-	51/18	14/45	<i>P</i> < 0.001	Not assessed
Disease duration, years	12.1 ± 1.3	7.8 ± 1.3	<i>P</i> < 0.001	0
Treatment with DMARDs**	51	18	<i>P</i> < 0.03	0
Methotrexate	34 (48.5%)	12 (19%)		
Other***	17 (24%)	6 (10%)		
Oral corticosteroids	17 (24%)	20 (32%)		
Non-treated	19 (27%)	43 (69%)	<i>P</i> < 0.05	
C-reactive protein, mg/l	42 ± 8	49 ± 9	n.s.	Not assessed
WBC count, ×10 <sup>9</sup> /l				
Blood	8.5 ± 0.4	7.8 ± 0.2	n.s.	Not assessed
Synovial fluid	15.7 ± 2.5	14.2 ± 2.5	n.s.	
uPA, ng/ml				
Blood	13.9 ± 6.3	7.5 ± 3.6	<i>P</i> = 0.02	1.94 ± 1.3
Synovial fluid	18.2 ± 8.9	4.7 ± 1.8	<i>P</i> = 0.001	3.82 ± 2.0
Survivin, ng/ml				
Blood	37.1 ± 16.1	3.2 ± 0.7	<i>P</i> < 0.001	0.22 ± 0.07
Synovial fluid	44.1 ± 22.0	3.0 ± 0.4	<i>P</i> < 0.001	0.12 ± 0.09

n.s., not significant.

\**P*-values are referred to a comparison between the groups with erosive and non-erosive RA.

\*\*DMARDs, disease-modifying anti-rheumatic drugs.

\*\*\*Other, sulfasalazine 5, gold salts 4, leflunomide 1, cyclosporine A 5 (in combination with methotrexate 2, with azathioprine 1, with leflunomide 1, with sulfasalazine 1).

## Cell culture preparation and stimulation conditions

Peripheral blood mononuclear cells (PBMCs) were prepared from heparinized blood of healthy individuals by separation on a Lymphoprep density gradient, then resuspended to  $1 \times 10^6$ /ml in Iscove's complete medium and cultured at 37°C in 5% CO<sub>2</sub>. For extracellular release of cytokines, supernatants were collected following 48 hrs of stimulation. Cell stimulation was performed in 2–5 independent experiments using PBMC from 6 to 15 healthy individuals. The results obtained in all the experiments were pooled.

Synovial tissue was obtained from knee joint synovial tissues of patients subjected to prosthesis surgery (Sahlgrenska University Hospital). Primary synovial fibroblasts were isolated from minced synovial tissue using collagenase/dispase. Cells were cultured, at 37°C in 5% CO<sub>2</sub>, in 10-ml culture flasks suspended in DMEM-Glutamax supplemented with 0.25% gentamycin, 0.2% normocin and 10% FCS. Synovial fibroblast cultures from passage 3 were homogenous containing >98% of CD90<sup>+</sup> and <1% of CD68<sup>+</sup> cells. Cells in passages 3–5 were used for the experiments.

Human monocytic cell line (THP-1) originally obtained from (American Type Culture Collection, Manassas, VA) was cultured in RPMI 1640 supplemented with 2.5 mM HEPES, 100 μM sodium pyruvate, 50 μg/ml gentamycin and 10% FBS. Human fibroblast cell line (MRC-5), originally obtained from EUACC, was cultured in DMEM-Glutamax medium supplemented with 50 μg/ml gentamycin and 10% FCS.

For stimulation, cells were cultured to confluence in complete medium and distributed on 96-well plates. Adherent cells were left overnight to attach to the plate surface before stimulation. Inhibitors of intracellular signalling were introduced to the cell medium 1 hr before stimulation. Levels of uPA and IL-8 were measured in supernatants following 24, 48 and 72 hrs of stimulation. Survivin levels were measured in cell lysates prepared by freezing the cells in -20°C overnight in 6M urea.

## Transfection with siRNA

Cells were seeded at  $2.5 \times 10^4$  cells/well on a 96-well plate in antibiotic-free DMEM-Glutamax media overnight. On the second day, cells were

transfected with siRNA targeting survivin (*BIRC5*, target sequence 5'-GGAAAGGAGAUCAACAUUU-3' [ON-TARGETplus]) or a cocktail of four siRNA sequences targeting uPA (PLAU, ON-TARGETplus SMARTpool) using Dharmafect2 transfection reagent according to the manufacturer's instructions. siSTABLE non-targeting RNA was used as a negative control. Transfection was performed using 25, 50 and 100 nM of oligonucleotides and evaluated for cell viability and efficacy. The final concentration of 50 nM was chosen for all the experiments. The transfection was stopped after 4 hrs by adding antibiotics and cells were allowed to grow for an additional 48–72 hrs. At that time-point cells were evaluated for viability and expression of survivin, uPA and IL-8.

## Urokinase-induced models of arthritis

Naval Medical Research Institute (NMRI) mice and severe combined immune deficient (SCID) mice were purchased from Charles River Laboratories (Uppsala, Sweden). All mice were housed at the animal facility of the Department of Rheumatology & Inflammation Research. NMRI mice were kept under standard conditions of temperature and light and fed laboratory chow and water *ad libitum*. SCID mice were kept in the chambers supplied with continuous airflow and fed with autoclaved laboratory chow and water *ad libitum*. The study was approved by the Ethical Committee of Gothenburg University and the requirements of National Board for Laboratory Animals were followed.

Urokinase (300 UI/knee) was instilled intra-articularly in the knee joints of healthy mice in a volume of 20  $\mu$ l as described [33] to study if siRNA targeting survivin (2  $\mu$ g/knee, *BIRC5*, target sequence 5'-GGAAAGGAGAUCAACAUUU-3') prevents development of uPA-induced arthritis. The siRNA preparation protected from RNase degradation (siSTABLE) [35] was injected intra-articularly simultaneously with uPA. The control group obtained an identical amount of non-targeting RNA sequence. Following 3 days, the injected knees were excised and subjected to morphological evaluation.

Human fibroblast cells (MRC-5) were injected in the knee joint of SCID mice. In the preliminary experiments two cell doses were used:  $10^5$  cells/knee,  $n = 5$ , and  $10^6$  cells/knee,  $n = 5$ . The intensity of synovitis was in direct relation to the cell number injected in the knee joint. Further experiments were performed injecting  $10^6$  MRC-5 cells/knee. To study if survivin is essential for the arthritogenic properties of fibroblasts, MRC-5 cells transfected with siRNA targeting survivin or non-targeting RNA sequence (see above) were instilled in the knee joints. The injected knees were excised 3 days later for histological evaluation.

## Histological examination

Mouse knee joints collected for the histological examination were stored for 3 days in 4% (vol/vol) buffered formaldehyde. Following EDTA decalcification and paraffin embedding, tissue sections of the knee joints were cut and stained with haematoxylin/eosin. All the slides were coded and evaluated blindly by two investigators regarding synovitis and erosion of bone/cartilage. Synovial hypertrophy was defined as a membrane thickness of more than two cell layers [33]. The histological scoring system was used as follows: 1, mild; 2, moderate and 3, severe synovitis and joint damage.

Human synovial tissue excised during a joint replacement surgery was submitted to routine fixation and paraffin embedding and cut into 4- $\mu$ m-thin sections. Tissue sections were deparaffinized. Antigens were retrieved for 20 min. using Diva Decloaker and HotRinse (HistoLab Products AB, Gothenburg, Sweden) in an electric pressure cooker. Endogenous peroxi-

dase was depleted by incubating with glucose oxidase followed by an overnight incubation with a titrated amount of polyclonal rabbit anti-human survivin (AF886, R&D Systems, Minneapolis, MN) or polyclonal rabbit anti-human uPA antibodies (Abam plc, Cambridge, UK). Following washing, the sections were incubated with biotinylated sheep anti-rabbit IgG antibodies (Sigma). Colour reaction was completed with Vectastain Elit ABC kit (Vector Laboratories, Burlingame, CA) and 3-amino-9-ethyl-carbazole containing  $H_2O_2$ . Sections were counterstained with haematoxylin.

## Determination of uPA and survivin levels

uPA levels in patient samples and cell supernatants were determined by a sandwich ELISA (Technoclone GmbH, Vienna, Austria). Survivin levels were measured by a sandwich ELISA using a pair of matched antibodies (R&D Systems). Values of circulating survivin above 300 pg/ml, corresponding to mean + 3 S.D. of a healthy control group, were defined as high [21].

## Determination of cytokine levels

Levels of IL-8 were measured by a sandwich ELISA using a pair of mouse anti-IL8 antibodies. Tested samples were related to the results obtained by serial dilution of human recombinant IL-8 and expressed in picograms per millilitre.

## Flow cytometry

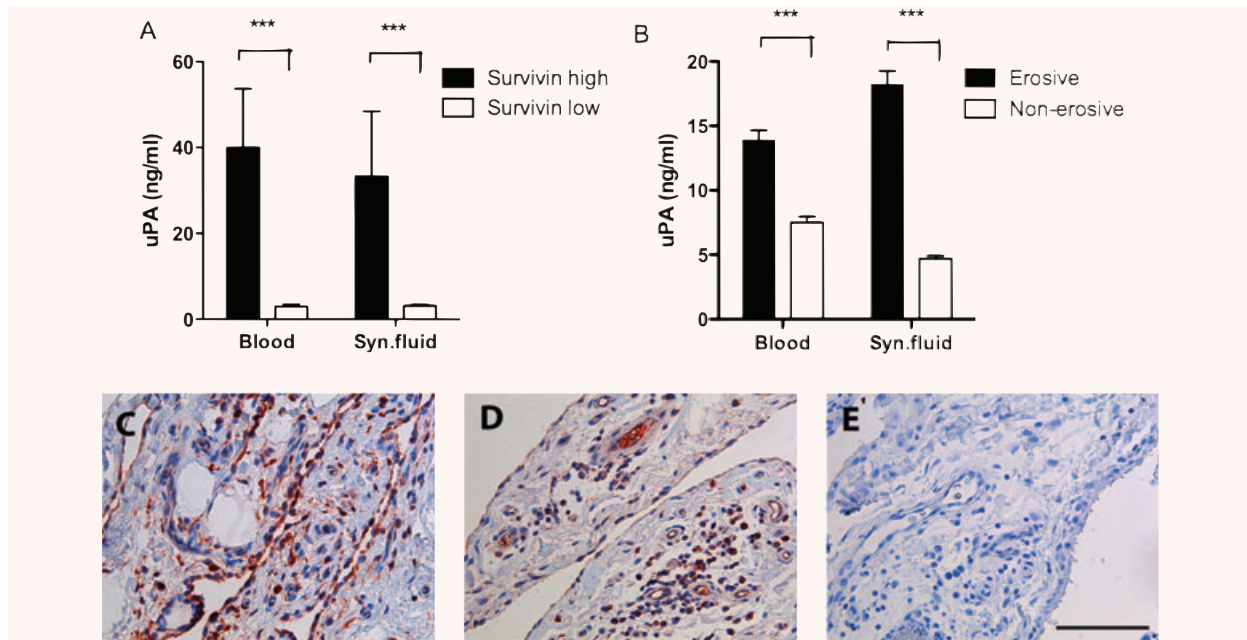
Cells were stimulated as above, washed, blocked with 1% mouse serum and incubated with optimal concentrations of phycoerythrin (PE)-conjugated mouse anti-CD87 (uPA receptor) monoclonal antibodies and IgG<sub>1k</sub> isotype matched controls. Following 1 hr of incubation on ice, staining was discontinued by washing and the cell pellet was re-suspended in PBS buffer containing 1% BSA, 0.5 mM EDTA and 0.1% NaN<sub>3</sub>. Finally, 10,000 to 50,000 cells were analyzed with a FACS Calibur equipped with FlowJo software. The results are presented as mean fluorescence intensity (MFI).

## Laboratory parameters of disease activity

Serum levels of C-reactive protein (CRP) were measured with a standard nephelometric assay with an established normal range of 0–5 mg/l. The erythrocyte sedimentation rate was measured by the Westergren method having normal range 0–20 mm/hr. White blood cell counts (WBC) in blood and in synovial fluid were performed using microcellular counter F300 (Sysmex, Toa, Japan).

## Statistical analysis

The level of uPA and survivin in the blood and synovial fluid samples were expressed as mean  $\pm$  S.E.M. Comparison in paired blood and synovial fluid was analyzed by the paired t-test. Differences in uPA and survivin levels between the patients and the controls were calculated by the Mann–Whitney U-test. For all the statistical evaluation, *P* values below 0.05 were considered significant.



**Fig. 1** Levels of uPA in blood and synovial fluid in patients with rheumatoid arthritis stratified according to levels of survivin and presence of joint destructions. Levels of uPA and survivin were measured in the paired blood and synovial fluid samples of 132 patients and exhibited significant correlation ( $r = 0.46$ ,  $P = 0.0015$ ). **(A)** The patients having joint destruction (erosive group,  $n = 70$ ) had significantly higher uPA levels as compared with those without destruction (non-erosive,  $n = 62$ ). **(B)** The levels of uPA were significantly higher in blood and synovial fluid of the patients having high survivin levels. **(C)** Immunohistochemical staining of human synovial tissue for the expression of survivin (reddish-brown staining) in the lining layer of synovial fibroblast-like cells. **(D)** Expression of uPA (reddish-brown staining) in the lining areas of synovia and in the inflammatory cell infiltrate. **(E)** Immunohistochemical staining of human synovial tissue using mouse IgG (negative control). Bar size 100  $\mu\text{m}$ . Nuclei are counterstained with haematoxylin.

## Results

### Clinical correlation between survivin and uPA in patients with RA

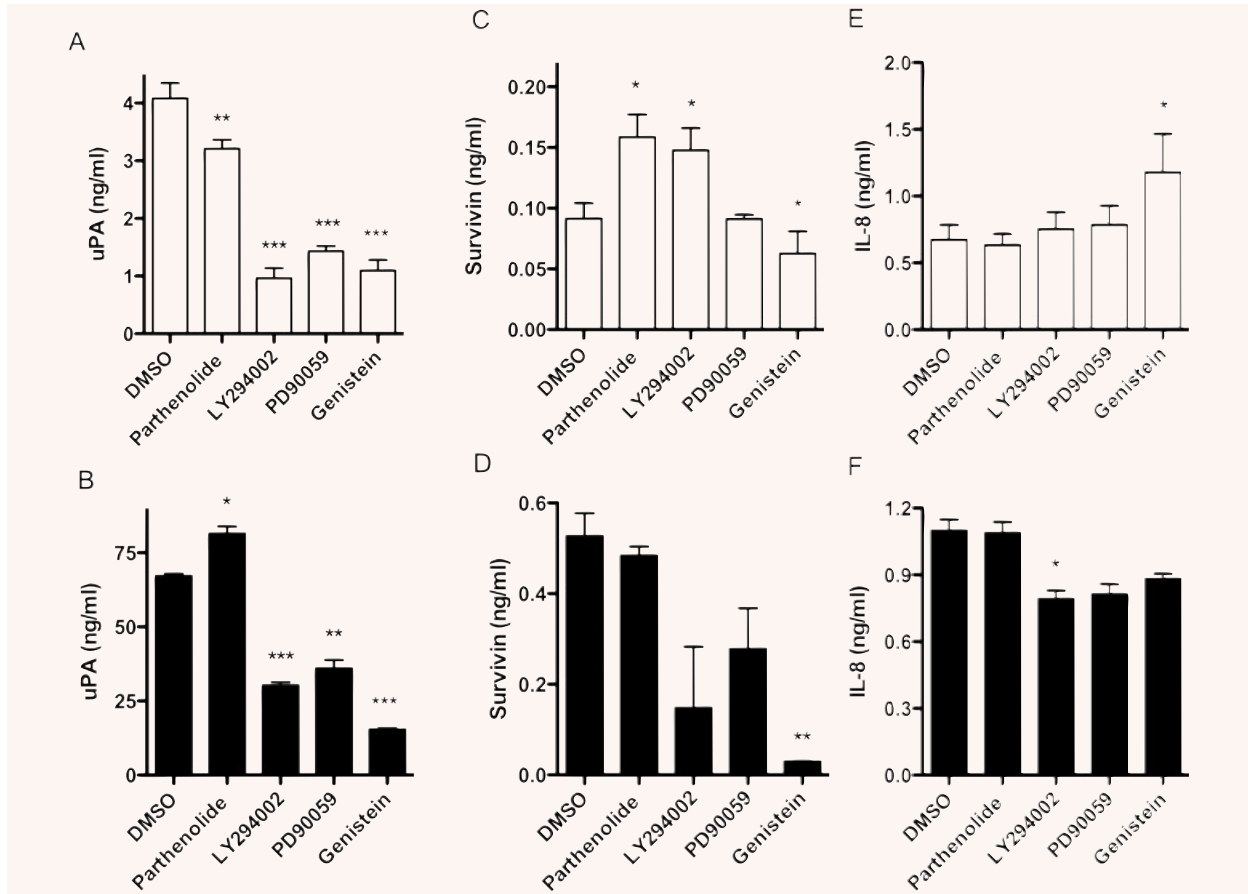
Levels of uPA and survivin were measured in the blood and synovial fluid of 132 patients with RA and compared to a control group of 82 individuals (Table 1). Synovial fluid samples from patients with RA had significantly higher levels of survivin ( $5.17 \pm 22.0$  versus  $0.12 \pm 0.09$  ng/ml,  $P = 0.001$ ) and uPA ( $11.6 \pm 3.5$  versus  $3.8 \pm 2.0$  ng/ml,  $P = 0.025$ ) as compared with the controls. Stratification of the patient material with respect to the presence of joint destruction at radiological examination (erosivity) indicated that RA patients with high levels of survivin were accumulated in the erosive group (erosive: 25/70 versus non-erosive: 5/62, chi-square 4.91,  $P = 0.003$ ). High levels of uPA were associated with the presence of survivin as well as with erosive RA (Fig. 1A and B). The levels of uPA and survivin strongly correlated to each other ( $r = 0.46$ ,  $P = 0.0015$ ). Immunological evaluation of human synovia visualized the presence of survivin in the intimal lining layer (Fig. 1C), whereas uPA expression was observed both in the lining layer and in leucocytes infiltrating synovia (Fig. 1D).

### Synovial fibroblasts are a source of uPA in synovial fluid

Synovial fibroblasts of RA patients produced spontaneously sufficient levels of uPA ( $n = 5$ ,  $10.24 \pm 2.70$  ng/ml). Flow cytometric analysis detected no expression of uPA receptor (uPAR) either in primary synovial fibroblasts or in synovial fibroblasts stimulated with uPA. Analogous spontaneous production of uPA and an absence of uPAR were observed in a MRC-5 fibroblast cell line (Fig. 2). Leucocytes from synovial fluid and peripheral blood produced no detectable levels of uPA, whereas flow cytometry revealed expression of uPAR on human peripheral blood leucocytes.

### Common intracellular pathways regulate synthesis of uPA and survivin

Primary synovial fibroblasts and MRC-5 cells were cultured in the presence of specific inhibitors targeting tyrosine kinases (genistein), MEK (PD98059), PI3-kinase (LY294002) and NF- $\kappa$ B (parthenolide) signalling pathways and their effects on synthesis of survivin, uPA and IL-8 were evaluated. Synthesis of uPA in



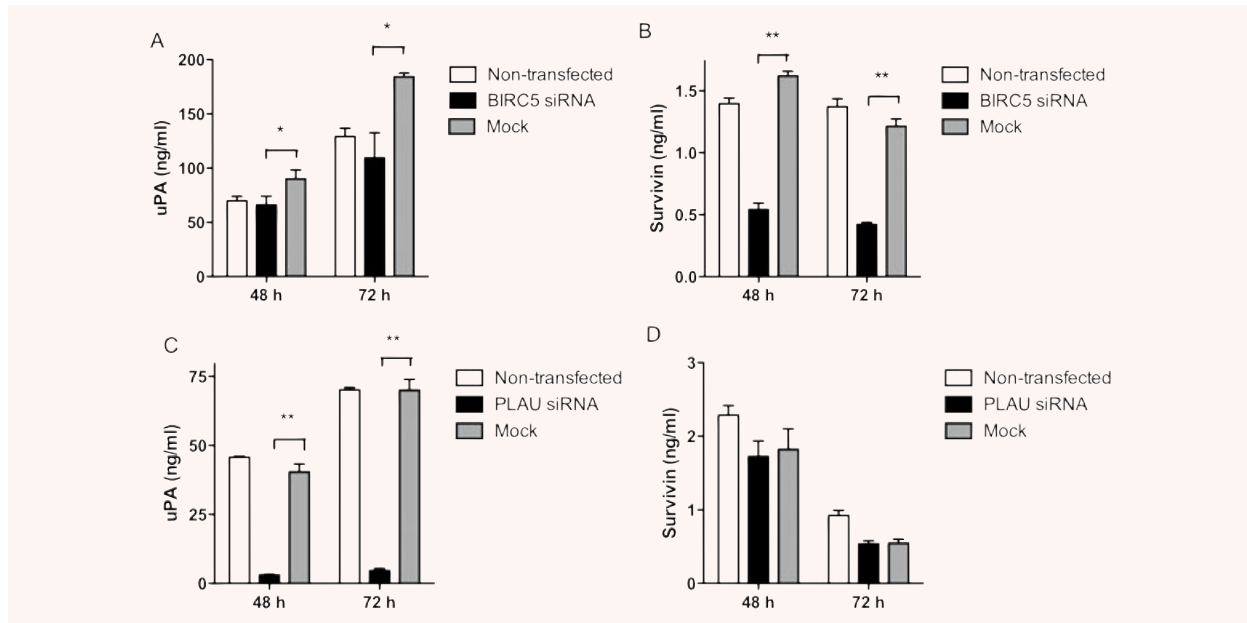
**Fig. 2** Intracellular regulation of uPA and survivin production in primary synovial fibroblasts and MRC-5 fibroblast cell line. Primary synovial fibroblasts were obtained from synovial tissue of RA patients ( $n = 3$ ). Cells in passage 3–5 were used in the experiments (open bars). MRC-5 was used in passage 15–20 (black-filled bars). Cells ( $2 \times 10^5$ /ml) were cultured in the presence of specific inhibitors of tyrosine kinases (genistein), PI3-kinase (LY294002), MEK 1 (PD98059) and NF- $\kappa$ B (parthenolide), all in a final concentration of 50  $\mu$ M. The uPA levels were measured in the supernatants following 48 hrs (A, B). The levels of survivin were measured in cell lysates (C, D). To reassure the viability of cell cultures, supernatants were also evaluated for IL-8 (E, F). Differences in comparison to cultures stimulated with DMSO with  $P < 0.05$  are marked with asterisks.

primary synovial fibroblasts as well as in MRC-5 cells was dependent on the activity of tyrosine kinases, PI3-kinase and MEK (Fig. 2A and B). In contrast, blocking of NF- $\kappa$ B pathway with parthenolide did not significantly change uPA synthesis in synovial fibroblasts. Inhibition of tyrosine kinases, PI3-kinase and MEK significantly decreased intracellular expression of survivin (Fig. 2C and D). In synovial fibroblasts, the expression of survivin exhibited the same overall trend as uPA, with the exception of the introduction of PI3-kinase inhibitor that had no inhibitory effect on survivin in synovial fibroblasts. The expression of survivin was not dependent on the activity of NF- $\kappa$ B either in primary synovial fibroblast cultures or in MRC-5 cells. To exclude the non-specific suppressive effect of intracellular inhibitors on viability and function of cell cultures, the levels of IL-8 were measured in the supernatants of the primary synovial fibroblast cultures as well as of

MRC-5. The inhibitors of tyrosine kinases, PI3-kinase, NF- $\kappa$ B and MEK had no effect on the production of IL-8 in the tested cell cultures (Fig. 2E and F).

### uPA expression in human fibroblasts is survivin dependent

To evaluate whether uPA production is dependent on survivin, MRC-5 cells were transfected with siRNA targeting survivin (50 and 100 nM) or a control RNA sequence as described in the experimental procedures. The levels of uPA were measured in the supernatants of the cell cultures 48 and 72 hrs after transfection. A significant reduction of uPA production (by 27% after 48 hrs and by 50% after 72 hrs) was observed (Fig. 3A). Simultaneously,



**Fig. 3** The production of uPA in human fibroblasts is survivin dependent. MRC-5 cells were seeded overnight in antibiotic-free medium in a concentration of  $2.5 \times 10^5$ /ml. Cells were transfected with siRNA targeting survivin gene (50 and 100 nM) and non-targeting RNA (Mock) for 4 hrs. Transfection was stopped by addition of antibiotics. The levels of uPA were measured in the supernatants following 48 and 72 hrs of incubation (A). A significant time-dependent decrease of uPA was observed in the cultures where survivin transcription was down-regulated. Successful transfection with siRNA was proved by a decrease of intracellular survivin levels measured in the lysates of the same cells (B). To evaluate if survivin synthesis was dependent on uPA, MRC-5 cells ( $2.5 \times 10^5$ /ml) were transfected with siRNA targeting *uPA* gene (25, 50 and 100 nM). The levels of uPA and survivin were measured following 48 and 72 hrs. Transfection with siRNA successfully down-regulated uPA synthesis (C), whereas the intracellular levels of survivin were only minimally affected (D).

a successful silencing of survivin was proved in the lysates of MRC-5 cells (Fig. 3B). To further evaluate if survivin production is dependent on uPA, MRC-5 cells were transfected with siRNA targeting uPA (25, 50 and 100 nM). As a result of the transfection, a significant down-regulation of uPA production was observed in the supernatants 48 and 72 hrs following transfection (Fig. 3C). In contrast, only a minor reduction of survivin levels was observed in the MRC-5 cultures transfected with the highest amount of siRNA (Fig. 3D). This inhibitory effect was also seen in the cells transfected with a non-targeting RNA sequence, suggesting a non-specific inhibitory effect.

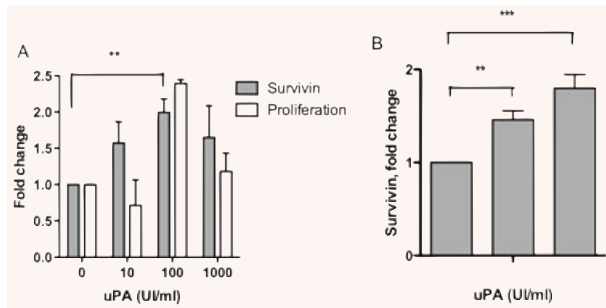
### uPA up-regulates production of survivin in human leucocytes

To investigate the functional relationship between uPA and survivin in human leucocytes, PBMCs from healthy individuals ( $n = 5$ ) were stimulated with uPA (0, 10, 100, 1000 UI/ml). The levels of survivin were evaluated in cell lysates 48 hrs following stimulation. A dose-dependent intracellular up-regulation of survivin production was observed (Fig. 4A). A relation of survivin expression

to the proliferative capacity of uPA was measured by [ $^3$ H]-thymidine intake of the uPA-stimulated PBMCs (Fig. 4A). This induction of survivin level in PBMCs was only partly related to proliferation of cells. The uPA stimulation had a similar effect on THP-1 cells, a human monocytic cell line. In concordance with the effect on PBMCs, THP-1 cells dose-dependently increased survivin production in response to uPA as compared with non-stimulated cells (in fold increase,  $1.80 \pm 0.15$ ,  $P = 0.0002$ ) (Fig. 4B).

### Survivin production is essential for the uPA-induced up-regulation of uPAR

Flow cytometric evaluation of THP-1 cells revealed expression of uPAR. uPAR was further up-regulated on the cell surface following uPA stimulation (300 UI/ml) (Fig. 5A). THP-1 cells were transfected with siRNA targeting survivin (50 nM) and non-targeting RNA sequence. Successful inhibition of survivin was proved in the THP-1 lysates (not shown). Flow cytometric evaluation of the transfected cells showed a significant reduction of uPAR expression on the cell surface as a result of survivin targeting transfection (Fig. 5B).



**Fig. 4** uPA up-regulates production of survivin in human leucocytes. Cells were seeded in concentration of  $1 \times 10^6$ /ml in 96-well plates and incubated for 48 hrs with uPA at final concentrations of 0-10-100-1000 UI/ml (PBMC,  $n = 5$ , **A**), and 0-30-300 UI/ml (THP-1,  $n = 13$ , **B**). Following 48 hrs, survivin levels were evaluated in the cell lysates by an ELISA. Results are presented as the increase in the ratio of survivin levels in uPA-stimulated cells in comparison to non-stimulated cell cultures. In addition, the proliferation rates in uPA-stimulated PBMC cultures are presented (**A**, open columns).

### Essential role of uPA receptor for survivin production

To study a requirement of uPAR for uPA-induced expression of survivin, antibodies against uPAR (10  $\mu$ g/ml) were introduced to THP-1 cultures 1 hr prior to uPA stimulation. Introduction of anti-uPAR antibodies significantly reduced uPA-induced up-regulation of survivin ( $P < 0.02$ , Fig. 6A). In contrast, treatment with irrelevant immunoglobulins had no influence on survivin expression following stimulation with uPA. In contrast, production of IL-8 in the cells incubated with anti-uPAR antibodies remained unchanged (Fig. 6B).

### The role of PI3-kinase in uPA-induced survivin synthesis

To determine if the PI3-kinase pathway is of importance for the uPA-induced survivin production, THP-1 cells were incubated with a PI3-kinase inhibitor (LY294002) at final concentrations of 12.5, 50 and 100  $\mu$ M 1 hr prior to uPA stimulation. Inhibition of PI3-kinase activity sufficiently decreased intracellular levels of survivin by 62% ( $P < 0.002$ ) as compared with untreated cells (Fig. 6C). In addition, inhibition of PI3-kinase did not change IL-8 production in THP-1 cells (Fig. 6D).

### The role of survivin in experimental arthritis

MRC-5 cells transfected with siRNA targeting survivin (see above) and grown for 48 hrs were injected in knee joints of SCID mice ( $10^6$  cells/knee,  $n = 5$ ). Control mice ( $n = 5$ ) were injected with

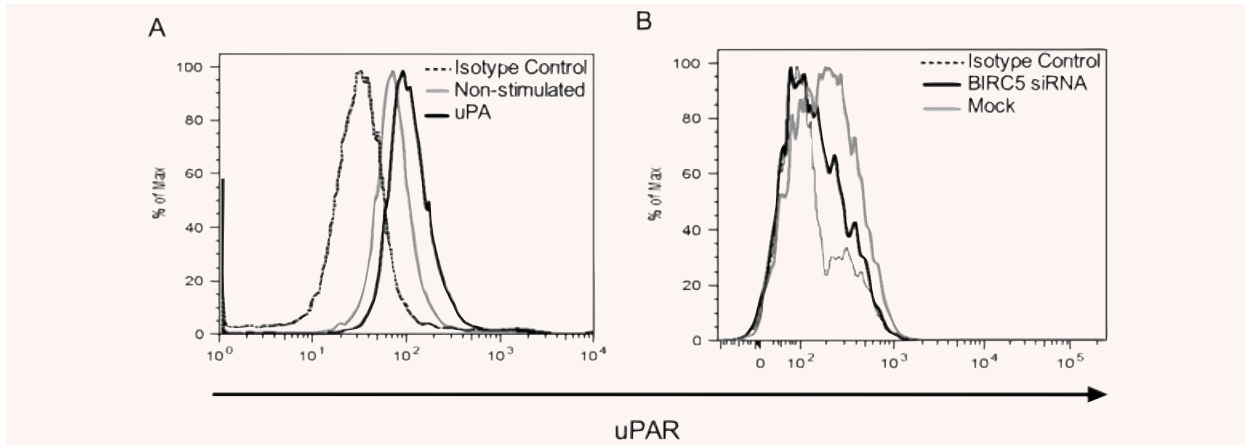
identical amount of MRC-5 cells transfected with non-targeting RNA. Morphological evaluation of knee joints 3 days after the cell instillation found signs of synovitis and cartilage destruction in all 5 joints injected with  $10^6$  MRC-5 cells transfected with non-targeting RNA (Fig. 7A). In contrast, only two out of five joints injected with MRC-5 cells where survivin was silenced had signs of mild synovitis. No cartilage destruction was found in the joints injected with MRC-5 cells lacking survivin (Fig. 7B). To further evaluate if instillation of siRNA targeting survivin could prevent uPA-induced arthritis, siRNA targeting survivin and protected from RNase destruction was instilled in the knee joint of healthy mice simultaneously with uPA (300 UI/knee,  $n = 10$ ). Histological evaluation of the injected knee joints showed signs of synovitis in 9 out of 10 joints injected with siRNA targeting survivin and in 7 out of 10 joints injected with non-targeting RNA sequence.

## Discussion

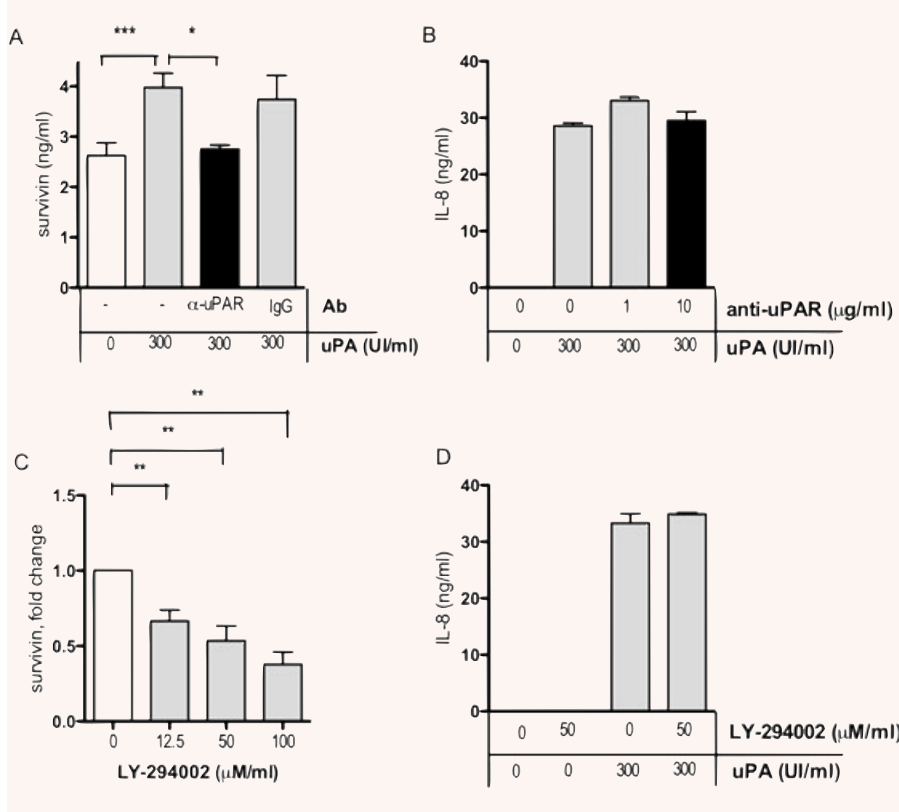
In the available material from RA patients we observed that survivin and uPA are accumulated in circulation and inside the joints of a distinct patient group. This group of RA patients is characterized by an active joint inflammation having cartilage and bone destruction as a consequence of that inflammatory process. It has been shown previously that intra-articular fibrinolytic system is represented by over-expression of uPA, predominantly by synovial fibroblasts localized in the proliferative lining area of synovia [31, 32]. Expression of uPAR-mediating proliferative properties of uPA may be found in leucocyte infiltrates gathered in the vicinity of fibroblasts. We have previously shown that uPA has efficient arthritogenic properties when instilled inside joints [33]. In addition to correlation of uPA and survivin levels in blood and synovial fluid, these proteins showed similar distribution in human synovial tissue on immunochemical evaluation. Indeed, the expression of survivin and uPA were most pronounced in the lining layer of human synovial tissue consisted of fibroblast-like and macrophage-like cells. Analogously, correlations in the protein levels and expression patterns of uPA and survivin were reported in malignant tissues in breast cancer [36].

We address a question if this co-expression is a coincidence or if mutual/common mechanisms regulate expression of uPA and survivin. Several steps were undertaken to test this concept (Fig. 8). To prove a concept of a link between uPA and survivin, we employed siRNA-induced silencing of these proteins. We observed that survivin expression was essential for an adequate uPA release from human fibroblasts. In contrast, survivin was successfully expressed even in the cells where uPA was down-regulated. In addition, the invasive potential of human fibroblasts was intimately connected to survivin production. Silencing survivin in MRC-5 fibroblasts clearly prevented articular cartilage destruction induced by these cells following their intra-articular instillation. One may argue that cell death and/or apoptosis due to intracellular down-regulation of survivin could be a reason for low uPA production.

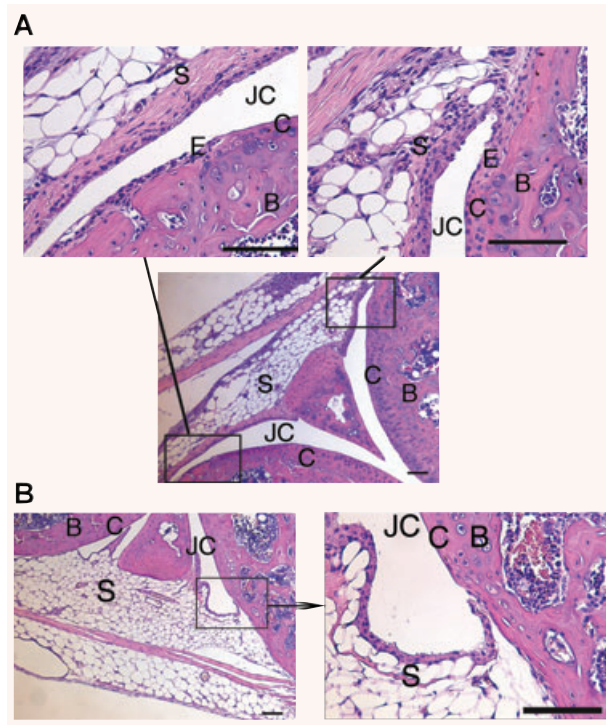




**Fig. 5** Survivin production is essential for the uPA-induced up-regulation of uPAR. **(A)** THP-1 cells were seeded in concentrations of  $5 \times 10^5$ /ml and stimulated with uPA (300 UI/ml). Following 24 hrs of stimulation, cells were collected for flow cytometric analysis. Non-specific binding was blocked with 1% mouse serum, and cells were stained with anti-uPAR antibodies PE-conjugated. Isotype identical mouse IgG PE-conjugated were used as a control. Stimulation with uPA increased expression of uPAR (black bold line) as compared with non-stimulated THP-1 cells (grey bold line). Isotype control is shown in dash line. **(B)** THP-1 cells were seeded in concentration of  $2.5 \times 10^5$ /ml and transfected with siRNA targeting survivin gene (175 nM) or non-targeting sequence (Mock) in serum and antibiotic-free medium for 4 hrs. Transfection was stopped by addition of FCS and antibiotics. Cells were stimulated with uPA (300 UI/ml) for additional 48 hrs and submitted to flow cytometry. The cell cultures with inhibited survivin production showed a significantly lower expression of uPAR (black bold line) as compared with the cells with unaffected production of survivin (grey bold line). Staining with isotype identical IgG<sub>1k</sub>-PE is shown by a dashed line. Sufficient suppression of survivin following transfection was proved in cell lysates.



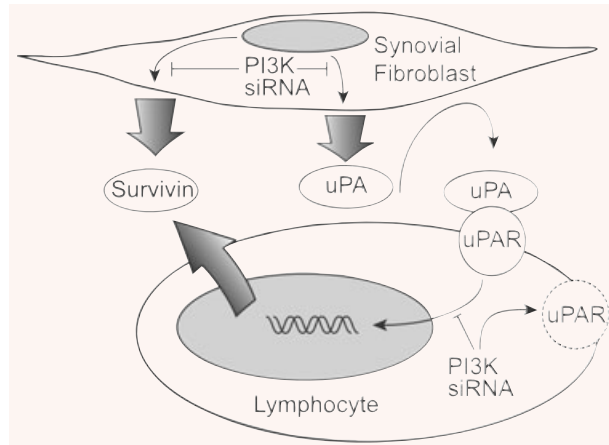
**Fig. 6** Essential role of uPA receptor for survivin production. THP-1 cells ( $n = 3$ ) were seeded in a concentration of  $5 \times 10^5$ /ml. Anti-human uPAR antibodies (a-uPAR, 10  $\mu$ g/ml) and non-specific IgG were introduced into the media 1 hr prior to uPA stimulation (300 UI/ml). Following 48 hrs of stimulation, supernatants were collected for IL-8 analysis. Survivin levels were measured in the cell lysates by an ELISA. Blocking uPA stimulation on the uPAR level significantly diminished survivin production in THP-1 cells **(A)**. In contrast, production of IL-8 in the same cell cultures remained unchanged **(B)**. The role of PI3-kinase in uPA-induced survivin synthesis was evaluated by culturing THP-1 cells ( $5 \times 10^5$ /ml) in the presence of PI3-kinase inhibitor (LY294002) at final concentrations of 12.5, 50 and 100  $\mu$ M for 48 hrs. Survivin levels were evaluated in the cell lysates using a sandwich ELISA **(C)**. IL-8 levels were measured in supernatants **(D)**. Results are shown as the ratio of survivin level between LY294002-treated cells and non-treated cell cultures.



**Fig. 7** Survivin is an essential mediator of fibroblast invasion into the cartilage. MRC-5 cells transfected with siRNA targeting survivin or with non-targeting RNA sequence (mock) were injected into the knee joints of healthy SCID mice ( $10^6$  cells/knee). Silencing of survivin was proved in cell lysates. Morphological evaluation of knee joints was performed 3 days later. (A) All knee joints injected with MRC-5 cells transfected with mock sequence and producing survivin had infiltration of mice synovia and cartilage destruction (slide in the middle). Photos above show erosive changes of the joint cartilage (right slide) and synovial infiltration with fibroblasts (left slide). (B) In contrast, instillation of MRC-5 with inhibited survivin expression caused only mild inflammation, and no cartilage destruction was seen.

However, high levels of IL-8 release in the same cell cultures prove sufficient cell function of these cultures irrespective of survivin levels. These observations showed that survivin played a key role in the regulation of the invasive properties of fibroblasts and provided experimental support for clinical association between high levels of survivin and destructive joint disease in RA [21].

Further on, specific inhibitors of intracellular signalling were employed to visualize the pathways essential for expression of uPA and survivin. We observed that functional tyrosine kinases, mitogen-activated protein kinases and PI3-kinase were prerequisite for uPA and survivin production by synovial fibroblasts. Activation of these pathways has been recently shown to be essential for the development of erosive arthritis in mice [37, 38]. In contrast, disruption of PI3-kinase using a selective PI3-kinase gamma inhibitor ameliorated development of the collagen type II-induced arthritis [39–41].



**Fig. 8** Survivin is an essential regulator of uPA–uPAR signalling in the inflamed joints. Synovial fibroblasts are a source of uPA in the joint cavity. Expression of uPA is required and sufficient for development of arthritis. Silencing of survivin using siRNA leads to an abrogation of uPA production in synovial fibroblasts. Leucocytes express uPA receptor (uPAR), providing a basis for the intracellular effects of uPA. In leucocytes, survivin regulates uPA effects by mediating expression of uPAR on the surface of human leucocytes. These observations provide experimental support for clinical association between uPA and survivin in patients with destructive rheumatoid arthritis.

Evaluation of uPA signalling in human leucocytes enlightened a different relationship between uPA and survivin expression in these cells (Fig. 8). On the one side, stimulation of human leucocytes with uPA resulted in a dose-dependent up-regulation of survivin. This up-regulation was mediated through the uPA receptor because blocking uPAR with antibodies abrogated survivin synthesis. Analogously to fibroblasts, survivin expression in leucocytes was dependent on PI3-kinase activity. Because uPAR mediated its effects through complex formation with multiple receptors, evaluation of uPAR partner specific for survivin production is awaited. It has been recently demonstrated that activation of chemokine receptor resulted in stable increase in survivin production [42]. On the other side, survivin regulates uPA-induced expression of uPA receptor in human leucocytes. This second aspect of survivin up-regulation may be an important feed-back loop in the uPA-induced intra-articular matrix degradation. Our findings show that survivin is an inducible factor possibly supporting prolonged existence and accumulation of highly differentiated leucocytes in the inflamed synovia. However, we could not prevent uPA-induced joint inflammation by silencing survivin simultaneously with uPA injection, suggesting production of uPA as a primary effector event of uPA–uPAR signalling. One of the possible explanations to this inefficiency is mobilization of pre-existent proteins in response to uPA injection rather than initiation of *de novo* synthesis.

To summarize, we present survivin as a new mechanism regulating the uPA–uPAR system in the proliferating lining layer of synovial cells. Survivin regulates the production and release

of uPA by fibroblasts as well as the expression of uPA receptor on the surface of mononuclear leucocytes potentiating uPA signalling. This study shows for the first time that intracellularly expressed survivin has the ability to modulate processes outside the cell membrane potentially participating in tissue remodelling. Similar mechanisms might take place in modified cell lines, suggesting its implication for neoplastic tissues. Survivin targeting approach has been evaluated in experimental treatments of several malignancies both for direct anti-tumoral effects and for sensitizing cancer cells to subsequent therapeutic interventions [43, 44]. Survivin antagonists tested in clinical settings of anti-tumour drugs include an anti-sense molecule, transcriptional repressors, but also compounds perturbing survivin expression (siRNA and shRNA) and phosphorylation. Notably, inhibition of cyclin-dependent kinase responsible for stabilization of cytoplasmic survivin by phosphorylation at the T34 site has been recently shown to be an efficient tool in the

treatment of RA in animal model [45]. The results of our study in combination with the results of Sekine *et al.* [45] show that survivin targeting strategies may present a new direction in the treatment of arthritis.

## Acknowledgements

Marcin Baran was supported by an ERASMUS scholarship during his stay at the University of Gothenburg. This study was supported by grants from the Medical Society of Gothenburg, the Swedish Association Against Rheumatism, the King Gustaf V's Foundation, the Swedish Medical Research Council, the Inflammation Network, the Nanna Swartz' Foundation, AME Wolff Foundation, Rune and Ulla Amlövs Trust, Swedish Foundation for Strategic Research, the Pharmacist Hedberg foundation and the University of Gothenburg.

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