Fibroblast-like synoviocytes from patients with rheumatoid arthritis are more sensitive to apoptosis induced by the viral protein, apoptin, than fibroblast-like synoviocytes from trauma patients

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Abstract

Objective

Fibroblast-like synoviocytes (FLS) from patients with rheumatoid arthritis (RA) show characteristics of transformation. Because the chicken anemia virus protein, apoptin, induces apoptosis solely in transformed cells, it was investigated whether FLS from patients were more sensitive to apoptin-induced apoptosis than FLS from normal joints obtained from trauma patients.

Methods

FLS were transduced with maltose-binding protein (MBP)-apoptin recombinant protein or MBP as a control protein by microinjection. After 24 hours, cells were fixed and stained with immunofluorescence to detect apoptin or MBP and the number of dead cells was assessed. Furthermore, phosphorylation of apoptin was analysed in FLS from patients with RA and from trauma patients by in vitro *kinase assay.*

Results

FLS from patients with RA were significantly more sensitive to apoptin-induced apoptosis than FLS from trauma patients (p = 0.0263). Furthermore, MBP-apoptin induced more apoptosis than MBP in RA FLS (p = 0.004). No phosphorylation of apoptin was observed in FLS from patients with RA.

Discussion

FLS from patients with RA are more sensitive to apoptin-induced apoptosis than normal FLS, which is consistent with a transformed phenotype of these cells. However, given the lack of phosphorylation of apoptin in RA FLS the mechanism of action of apoptin seems to differ between tumour cells and RA FLS.
This study indicates that apoptin may help to identify a new therapeutic pathway against hyperplasia of the synovium and joint destruction in RA.

Key words

Rheumatoid arthritis, synoviocytes, apoptosis, synovial tissue, pathogenesis, synovitis.

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Introduction

A normal synovial joint like a knee or a hip is composed of two opposing bones, connected by the capsule. The inside of this capsule comprises the synovial membrane, which is composed of a lining layer of 1 or 2 cell layers and a sublining stroma, with stromal cells and blood vessels.

The most common disease of the synovium, rheumatoid arthritis (RA) is a chronic inflammatory disease characterised by synovitis, hyperplasia of the synovial lining and degradation of cartilage and bone. Degradation of cartilage is thought to be mediated by fibroblast-like synoviocytes (FLS) of the lining layer and many data point to the fact that these cells show a transformed phenotype. FLS from RA joints exhibit an anchorage independent growth pattern in soft agarose (1) and show invasive behaviour *in vitro* and *in vivo* (2, 3). Furthermore, these FLS (but not their normal counterparts) express oncogenes (4), like Ras and c-myc and matrix metalloproteinases for cartilage destruction (3).

Because hyperplasia is caused by an imbalance between proliferation and apoptosis, much effort has been put on delineating the mechanism underlying these features in RA FLS. However, at this moment it is not clear whether the cells show increased proliferation or decreased apoptosis or both. Some studies show an increase in the rate of proliferation of FLS in RA as compared to Osteoarthritis (OA) or normal controls (5), but other studies find no difference in the rate of proliferation in either RA or normal FLS (6). Similar controversies hold true for apoptosis. Some studies report an increased rate of apoptosis in RA synovium (7-9), and that RA FLS are susceptible to Fas-mediated apoptosis (10). However, these cells are mainly found in the sublining layer of the synovium (8). In contrast, other studies report that the lining layer cells are resistant to Fas-induced apoptosis (11) and show up-regulation of the protein, Bcl-2, which inhibits one of the apoptosis regulating pathways (12).

Given the fact that inflammation leads to pain and to loss of function of joints and is easy to measure, therapies for RA are primarily focused on the inflammation of the joint rather than on maintaining its normal structure. Degradation of cartilage by FLS leads to loss of structure in the joint and can also continue in the absence of detectable inflammation. Thus, therapies targeting the hyperplasia of FLS and the subsequent formation of pannus tissue leading to anatomical malformation may present a welcome expansion of the therapeutic options in destructive joint diseases.

Apoptin, a chicken anemia virus protein, has been shown to induce apoptosis specifically in transformed cells and tumours but not in normal cells (13). Over-expression of apoptin in human tumour cell lines leads to p53 independent cell death (14). Moreover, expression of Bcl-2, which normally inhibits apoptosis, has been shown to enhance apoptin-induced apoptosis (15).

Since apoptin induces apoptosis specifically in transformed cell lines and since FLS exhibit features of a transformed phenotype, the effect of intracellular injection of apoptin in RA FLS compared to the effect of intracellular injection of normal FLS was tested.

Materials and methods

Patients and synovial tissue

Synovial tissue was obtained from patients with RA during joint replacement surgery. All patients met the criteria of the American college of Rheumatology. As a control, synovial tissue from patients without inflammatory joint disease was obtained during joint replacement surgery after femoral fracture. Tissue was harvested by an orthopaedic surgeon and collected in sterile phosphate buffered saline (PBS). Connective tissue and fat were removed and tissue was digested with collagenase IA (1 mg/ml; Sigma, St Louis, MO, USA) for at least two hours at 37ºC. Cells were separated from the tissue using a 200 μm filter (NPBI, Emmer-Compascuum, The Netherlands) and cultured in 75 cm² culture flasks (Cellstar, Greiner, Alphen aan de Rijn, The Netherlands) with Iscove's modified Dulbecco's medium (IMDM; Biowitthaker, Verviers, Belgium) sup-

plemented with glutamax (GibcoBRL, Paisley, UK), penicillin and streptomycin (Boehringer Mannheim, Germany), and 10% fetal calf serum (FCS; GibcoBRL, Paisley, UK) at 37ºC and 5% CO_2.

After the cells reached confluence they were detached with 0.25% trypsin and split in a 1:3 ratio. FLS of passage 4 or 6 were used for all experiments. Light microscopy and Giemsa staining indicated that more than 95% of cells were FLS.

Expression and purification of proteins
The cloning, expression, and purification of recombinant maltose-binding protein (MBP)-Apoptin protein, its control protein MBP and recombinant His-tagged Apoptin protein have been described previously(16; 17). In short, the construct pMalTB-VP3 encodes MBP-Apoptin, an N-terminal fusion of MBP to Apoptin, separated by a flexible linker encoding 10 asparagine residues. The construct pMalTB encodes MBP alone. Both proteins were expressed in Escherichia coli and purified by standard methods. The purity of the proteins were generally > 95%. In the case of His-Apoptin purification was performed on NiNTA columns as described (17). In parallel experiments, we have identified the location and function of MBP-Apoptin and MBP protein in normal versus tumorigenic mammalian cells (18).

Transduction of cells with apoptin protein
On the day before transduction, the cells were plated on uncoated 35-mm glass bottom microwell dishes (Mattek, Ashland, USA) at a density of 100,000 cells. During microinjection, cells were incubated in RPMI 1640 medium (25 mM HEPES, pH 7.2, 5% fetal calf serum, penicillin, and streptomycin) at 37ºC. Cells were returned to IMDM medium immediately after microinjection. Microinjection was performed with 0.5-µm microneedles (sterile femtotips II; Eppendorf) under an inverted microscope (Axiovert 135 TV; Zeiss), equipped with a programmable microinjector (IM 300; Narishige Co.) and a joystick hydraulic micromanipulator

(MMO-202; Narishige Co.). MBP-Apoptin protein (3mg/ml in PBS) or MBP protein (3 mg/ml in PBS) was co-microinjected with lysine-fixable rhodamine-dextran (Molecular Probes) that was used as a marker to trace injected cells. Directly prior to injection, protein samples were filtered over 0.22 µm microfilter-tube (millipore) or at 15,000 x g centrifugation for 15 min. Microinjection was preformed under the condition of injection pressure of 0.5 to 1.0 pounds/square inch, and an injection time of 0.2 to 0.5 sec. For each experiment, 100 cells were injected per dish (18). After 24 hours of incubation at 37ºC and 5% CO_2 the cells were washed with PBS, fixed with 80% acetone (in H_2O) for 10 minutes and dried in air. The fixed cells were kept at -20 oC until staining.

Immunofluorescent staining of cells
After transduction and fixation, cells were stained for detection of MBP or apoptin (VP3c; 16). Cells were washed three times with PBS containing 0.05% Tween-20 (Sigma, Steinheim, Germany) and incubated with rabbit anti-MBP (Santa Cruz, Heerhugowaard, The Netherlands) or with rabbit anti-VP3c in PBS with 5% normal goat serum for 1 hour. And subsequently incubated with the second antibody FITC-labeled goat anti-rabbit (Santa Cruz, Heerhugowaard, The Netherlands) for 30 minutes. The nucleus of the immunofluorescent stained cells was visualised with DAPI/DABCO/-Glycerol solution containing 100 mM Tris-HCL, 2.3% 1,4-diazabicyclo-(2,2,2)-octane (Dabco) and 10 µg/ml 4',6-Diamidino-2-pheyindole (DAPI) (Sigma, St Lois, USA). DAPI cause a regular staining in intact nuclei, but an irregular and/or weak staining in apoptotic cells (18). Injected cells were counted by fluorescence microscopy and cell-death was assessed by cellular and/or nuclear morphology.

In vitro *kinase assay*
The assay was performed essentially as described (17). Briefly, 10-cm dishes containing FLS were cultured in IMDM medium and cells harvested at 80% density by scraping. Pellets were

rinsed 1x in PBS and then snap-frozen in liquid N2 for later use.

Pellets were lysed on ice with in vitro-kinase buffer (IVKB) (20 mM Hepes 7.4, 20 mM $MgCl_2$, 150 mM NaCl, trypsin inhibitor, pepstatin, leupeptin, aprotinin, PMSF1, β-glycerol phosphate, sodium orthovanadate, and sodium fluoride, at standard concentrations, as well as the phosphatase inhibitor cocktails 1 and 2 [Sigma]). Protein concentrations of cell lysates were determined by Bradford assay and 15 µg protein was incubated with 2 µg recombinant His-tagged apoptin and ATP. The labelled protein was precipitated using NiNTA-agarose. The precipitate was run on an SDS-PAGE gel followed by Western blotting. A sample of the lysate alone was also put on gel to determine "total" kinase activity. Western blotting was performed using Bio-Rad equipment as recommended by the manufacturer. Phopshorylated Apoptin was detected using a specific polyclonal anti-phospho-antibody specifically recognising phosphorylated apoptin (17). Equal loading was controlled using a monoclonal anti-apoptin antibody.

Statistical analysis
Statistical analysis was performed with SPSS version 9.5. Statistical differences in apoptosis between RA and normal FLS and between transduction with MBP-apoptin and MBP was determined by a Student's t-test.

Results
Induction of apoptosis by apoptin protein in FLS
FLS of 5 patients with RA and 5 trauma patients were tested for their ability to undergo apoptosis induced by apoptin. Patient characteristics are shown in Table I. In each case, 100 cells were transduced with MBP-apoptin protein or MBP control protein by means of microinjection. 24 hours later cells were fixed, stained and subsequently analysed for apoptosis by immunofluorescent staining. After 24 hours, 80-90% of the transduced cells were still adherent and were evaluated for apoptosis. As shown in Figures 1A-D, apoptin induced apotosis in 29% ± 5.8

Table I. Patient characteristics. RA means patient with RA fullfilling the ACR classification criteria. FR means otherwise healthy patient with a fracture.

Patient nr	Male/ Female	Age	Joint	Rheumatoid Factor
RA053	F	73	Elbow	+
RA055	F	37	Elbow	
RA077	F	60	Knee	+
RA088	M	60	Ankle	+
RA103	F	29	Elbow	+
FR007	F	85	Hip	
FR008	F	66	Hip	
FR013	M	53	Hip	
FR051	F	72	Hip	
FR054	M	46	Hip	
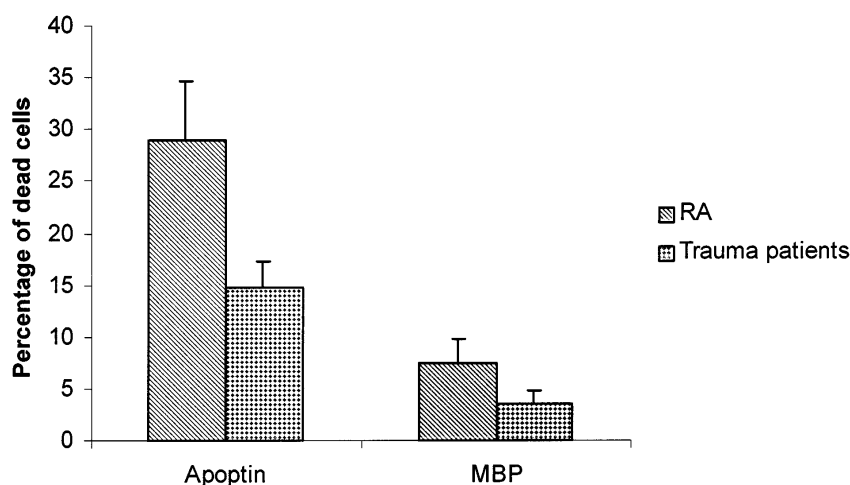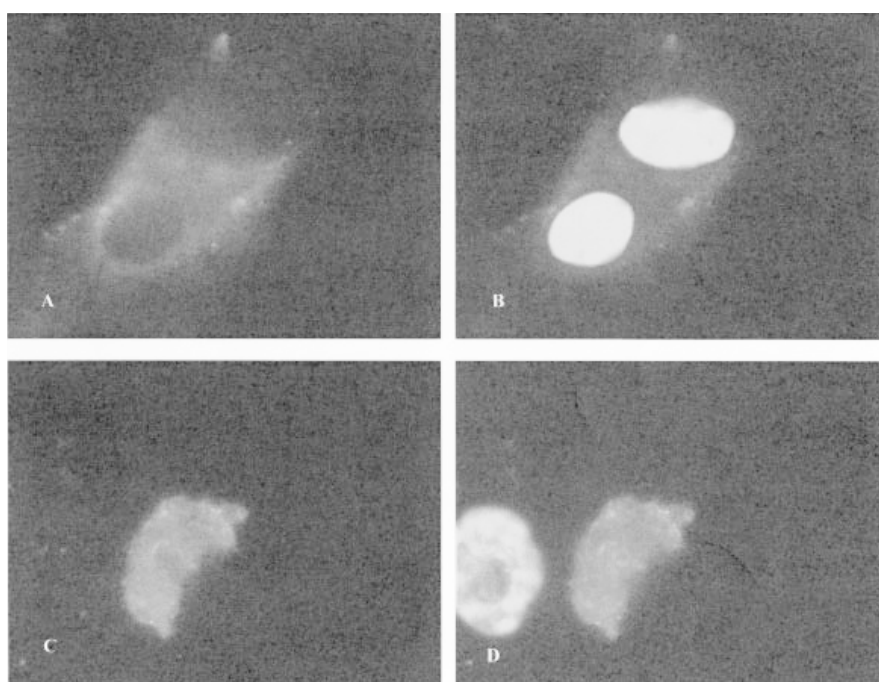
Fig. 1. Examples of a live (**A** and **B**) and apoptotic (**C** and **D**) cell microinjected with MBP-apoptin protein. MBP-apoptin protein is shown in green (**A** and **C**) and **B** and **D** shows a merge picture of apoptin and DAPI, staining nuclear intact DNA strongly (**B**) or apoptotic DNA weakly (**D**). E: Mean percentage of apoptotic cells 24 hours after injection of MBP-apoptin protein or MBP protein. Striped bars show the percentage of dead cells for FLS from patients with RA, blocked bars ahow the percentage of dead cells for FLS from trauma patients.

(mean ± SEM) in the injected cells from patients with RA, while only 14.8% ± 2.4 of the injected FLS from traumapatients died (p = 0.0263). RA-cells injected with the control protein MBP (7.4% ± 2.46) did undergo apoptosis significantly much less than those transduced with MBP-Apoptin (p = 0.009; Figure 1E).

*Phosphorylation of apoptin protein in FLS*
Because in tumour cell lines apoptin is phosphorylated at a specific position, namely threonine at position 108 and this phosphorylation was shown to be important for its mode of action in these cells (17), we also investigated the phosphorylation status of apoptin in RA FLS vs trauma FLS. However, despite containing considerable kinase activity as determined by autophosphorylation (data not shown) in neither the RA- nor the trauma FLS preparations could apoptin phosphorylation ever be observed (see example in Figure 2). This suggests that apoptin induced apoptosis in RA FLS is mediated by a different mechanism than apoptosis induced in tumour cell lines.

Discussion
The results in this study show that FLS from patients with RA were significantly more sensitive to apoptin-induced apoptosis than FLS from trauma patients. Furthermore, MBP-Apoptin induced about 4 times more apoptosis in RA cells than control MBP protein did, which shows that Apoptin is a potent apoptosis inducer in RA-derived FLS. Because in previous reports it has been demonstrated that apoptin induces apoptosis only in tumour and other transformed cell lines (13-21) (see Table II), these data support the transformed nature of FLS in patients with RA. However, the mechanism of action of apoptin in FLS seems to be different from its mechanism of action in tumour cell lines, given the fact that apoptin is not phosphorylated at amino acid residue 108 in RA FLS as is the case in tumour cell lines. Strikingly, Danen-van Oorschot *et al.* (22) have reported that Apoptin contains two different domains that have the ability to induce

Table II. Apoptin sensitivity of several human cell lines For references see text.

Cell line	Apoptin sensitive	Cell type
VH10	-	Fibroblast
VH10-SV40LT	+	Transformed fibroblast
FSK	-	Keratinocyte
SVK14	+	Transformed keratinocyte
HaCaT	+	Transformed epithelial cell
Saos-2	+	Osteosarcoma
U2OS	+	Osteosarcoma
NW-18	+	Transformed fibroblast
SCC-15	+	Transformed epithelial cell
Hep3B	+	Hepatoma
G401	+	Kidney rhabdoid tumor
HepG2	+	Hepatoma
DOHH-2	+	B lymphoma
Jobo-0	+	Epstein-Barr-virus immortalized B cell
K562	+	Acute myeloid leukaemia
KG1	+	Acute myeloid leukaemia
HSMC	-	Smooth muscle cell
HUVEC	-	Endothelial cell
T cells	-	Lymphocyte
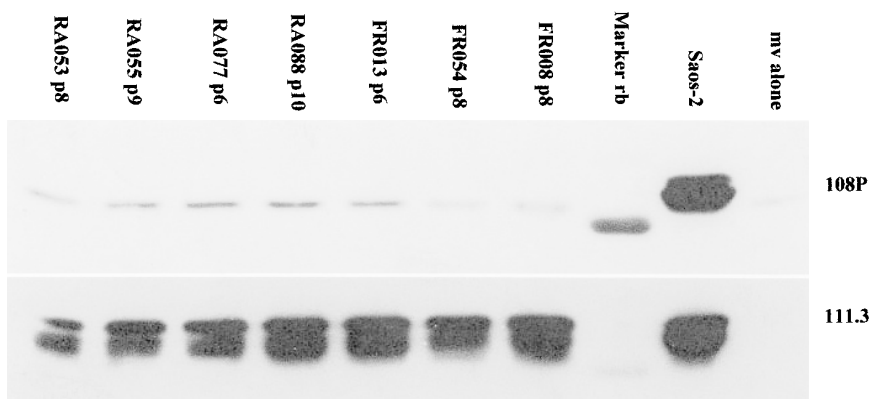
Fig. 2. *In vitro* kinase assay. The ability of cell lysates from different patients to phosphorylate apoptin at amino acid residue 108 was measured as described in materials and methods. Saos-2 cells were tested as a positive control. mv alone: MBP-apoptin alone. Upper panel shows a Western blot of phosphorylated apoptin, the lower panel shows a loading control with MBP-apoptin.

apoptosis independently. Only one domain seems to be regulated by phosphorylation. Therefore, it seems likely that only one of the Apoptin apoptotic domains is activated in RA FLS. A possibility for further research may be to explore the mechanism by investigating whether e.g. caspases and death effector domain-associated factor (DEDAF) (which are involved in apoptin-induced apoptosis) (23, 24) are also involved in RA FLS.

In this study, cells were transduced with apoptin using microinjection. This is a difficult and laborious technique, but the amount of injected cells is known precisely. Another technique frequently used to transduce cells is via adenoviral vectors. However, with this technique it is difficult to determine exactly how many cells are transduced and furthermore when cells die it is very difficult to quantify whether these cells died from the procedure or from apoptin expression. In this study 100 cells were injected and after 24 hours the number of remaining live and dead cells were counted. The disadvantage of this method is, that only adherent apoptotic cells can be analysed and not apoptotic cells detaching from the culture surface. Thus, if less than 100 cells are retrieved after 24 hours the effect of apoptin may be underestimated.

In conclusion, although few patients were tested so far, the chicken anaemia virus derived protein, apoptin, induces a significant degree of apoptosis in RA FLS, but not in normal FLS. Thus, further work using apoptin may help to identify (a) novel RA-specific pathway(s) which can be used as a therapeutic target. Such a novel RA-FLS targeted therapy could potentially be used to preserve the normal structure of the joint.

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