Cartilage Destruction Mediated by Synovial Fibroblasts Does Not Depend on Proliferation in Rheumatoid Arthritis

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The aim of the study was to investigate the relationship between invasion and proliferation in rheumatoid arthritis synovial fibroblasts (RASFs). *In vitro*, RASFs, normal synovial fibroblasts (NSFs), and RASFs transformed with SV40 T-antigen (RASFSV40) were analyzed for the expression of cell surface markers (Thy1, VCAM-1, ICAM-1, CD40, CD44) and their proliferation by flow cytometry. Furthermore, colony-forming unit assays were performed and the expression of matrix metalloproteinases (MMP)-14 and cathepsin K mRNA were determined by real-time polymerase chain reaction. *In vivo*, in the severe combined immunodeficiency (SCID) mouse co-implantation model, RASFs, NSFs, and RASFSV40 were tested for cartilage invasion, cellular density, and for their expression of the cell cycle-associated protein Ki67. In the SCID mouse co-implantation model, RASFs invaded significantly stronger into the cartilage than NSFs and RASFSV40. Of note, RASFSV40 cells formed tumor-like tissues, and the cellular density adjacent to the cartilage was significantly higher than in RASFs or NSFs. In turn, the proliferation marker Ki67 was strongly expressed in the SV40-transformed synoviocytes in SCID mice, but not in RASFs, and specifically not at sites of cartilage invasion. Using the colony-forming unit assay, RASFs and NSFs did not form colonies, whereas RASFSV40 lost contact inhibition. *In vitro*, the proliferative rate of RASFs was low (4.3% S phase) in contrast to RASFSV40 (24.4%). Expression of VCAM-1 was significantly higher, whereas of ICAM-1 was significantly lower, in RASFs than in RASFSV40. CD40 was significantly stronger expressed in RASFSV40, whereas CD44 and AS02 were present at the same degree in almost all synoviocytes. Expression of cathepsin K and matrix metalloproteinase-14 mRNA was significantly higher in RASFs than in the RASFSV40. Our data demonstrate clearly that invasion of cartilage is mediated by activated RASFs characterized by increased expression of adhesion molecules, matrix-degrading enzymes, but does not depend on cellular proliferation, suggesting the dissociation of invasion and proliferation in RASFs. (Am J Pathol 2003, 162:1549–1557)

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Rheumatoid arthritis (RA) is a common chronic arthropathy characterized by synovial hyperplasia and progressive joint destruction.¹ The pathogenesis of this disabling disease is not well understood, but activated RA synovial fibroblasts (RASFs) are thought to be key players in joint destruction.² Evidence for this is shown in the severe combined immunodeficiency (SCID) mouse co-implantation model of RA, because RASFs invade deeply and destroy human articular cartilage independent of T cells, macrophages, and inflammation.³ Moreover, specifically open questions are: 1) Does the invasive process of RASFs depend on proliferation? 2) Is the activation of RASFs similar to that of transformed cells?

In RA, the proliferation either in synovial tissues or in RASFs grown *in vitro* is considered to be low.^{4–6} However, some authors of recent publications reported a higher expression of cell cycle-associated proteins when investigating RA synovial tissues.^{7,8} In tumor cells it is well established that an enhanced proliferation correlates with increased invasiveness and metastasis. In turn, it could be shown that the expression of the cell cycle-associated protein Ki67 was an independent and significant prognostic factor in prostate cancer in terms of disease-specific survival. Correspondingly, in breast carcinoma Ki67 was also an independent prognostic factor for survival and tumor recurrence.⁹

The idea has been proposed that RASFs are transformed because RASFs express certain proto-onco-

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genes but lack certain tumor suppressor proteins,² and reveal a morphology resembling tumor cells,¹¹ but a direct proof for this is still lacking.

In the present study, we performed *in vitro* investigations using RASFs and SV40-transformed RASFs (RASFSV40) to compare the expression of cell surface markers, cell proliferation, the loss of contact inhibition and the expression of matrix-degrading enzymes. *In vivo*, we investigated, in the SCID mouse co-implantation model of RA, whether cartilage-invading cells proliferate, using the proliferation marker Ki67, and analyzed the effect of SV40 transformation with respect to invasion and cellular density.

Materials and Methods

Tissue Preparations

Synovial tissue specimens were obtained from 13 patients with RA and 1 with osteoarthritis undergoing synovectomy or joint replacement. Samples were fixed in 4% neutral buffered formalin for 6 to 8 hours, dehydrated in increasing concentrations of ethanol, and then embedded in paraffin using an automated tissue processor. All RA patients used in the present study fulfilled the American College of Rheumatology (formerly the American Rheumatism Association) criteria for the diagnosis of RA.¹²

Cell Culture

Synovial fibroblasts were obtained by enzymatic digestion of synovial tissues from 11 RA patients and 2 normal synovial tissues. Briefly, the tissues were minced and incubated with 1.5% dispase (Roche, Rotkreuz, Switzerland) in phosphate-buffered saline (PBS) for 1 hour at 37°C under continuous agitation. The cells were pelleted, resuspended, and cultured in Dulbecco's modified Eagle's medium/F12 (Gibco Life Technologies, Basel, Switzerland) containing 10% fetal calf serum, glutamine (0.2 mmol/L), HEPES buffer (10 mmol/L), penicillin-streptomycin (50 IU/ml), and amphotericin B (2.5 µg/ml) (all Gibco Life Technologies).

Transformation of Synoviocytes with DNA Encoding for SV40 T-Antigen

RASFs were transfected with plasmids encoding SV40 DNA containing the origin of replication, the transcription start, and the small and large T-antigen, as described earlier by Haas and colleagues.¹³ After cellular crisis stable transformation was confirmed by immunofluorescence investigating the expression of SV40 T-antigen and p53. SV40-transformed cells and control cells were fixed on chamber slides (Nalge Nunc International, Naperville, IL) and incubated with either anti-SV40 T-antigen antibodies (DPO2, 0.4 µg/ml; Oncogene Research Products, San Diego, CA) or with anti-p53 antibodies (0.4 µg/ml; DAKO, Glostrup, Denmark) for 30 minutes at room

temperature. Mouse isotype-matched IgGs were used as negative controls. Cy3-conjugated sheep anti-mouse sera (0.14 µg/ml; Dianova, Hamburg, Germany) were applied as secondary antibodies for 30 minutes at room temperature. Rinsed in PBS, the slides were finally mounted with fluorescent mounting medium (DAKO) and analyzed with a Zeiss immunofluorescence microscope at a wavelength of 515 to 545 nm.

Characterization of RASFs, Normal Synovial Fibroblasts (NSFs), and RASFSV40 by Flow Cytometry

Cultured synovial fibroblasts were detached using 2.5 mmol/L of ethylenediaminetetraacetic acid and washed once with PBS. Cells were then stained with the indicated antibodies. The following antibodies, which were either fluorescein isothiocyanate- or phycoerythrin- conjugated, were used to determine the expression of surface molecules: anti-CD40, anti-VCAM-1 (CD106), anti-HLA-DR, anti-ICAM-1 (CD54) (all BD Pharmingen, Basel, Switzerland), anti-CD44 (Immunotech, Marseille, France), and anti-Thy1 (clone ASO2, Dianova). Cells were analyzed on a FACS Calibur flow cytometer and data were processed using CellQuest software (Becton Dickinson, San Jose, CA).

Determination of Proliferation by Flow Cytometry

Nuclear isolation and DNA staining with propidium iodide was performed with the Cycle Test Plus DNA reagent kit (Becton Dickinson, Basel, Switzerland) according to manufacturer's instructions from four different RASFs (passages 3 to 6), RASFSV40, and U937 cells. Analysis of propidium iodide-stained nuclei were performed on a Becton Dickinson FACS Calibur flow cytometer and DNA histograms were analyzed by determining the percentage of cells in G₀/G₁, S phase, and G₂ phase. The generation time of the cells was calculated according to the following formula: doubling time = 6 hours × 100/S phase. Cells were passaged and grown at least for 48 hours before measuring.

Colony-Forming Unit Assay

Colony-forming unit assays were performed in two modifications according to McBride and colleagues.¹⁴ 1) RASFSV40 and RASFs (*n* = 4, passages 4 to 7) and NSFs were seeded in serial dilutions reaching from 312 to 5000 cells per flask and kept growing for 15 to 17 days. As positive control HT1080 fibrosarcoma cells were used. 2) Alternatively, 100 to 150 cells of synovial fibroblasts and control cells were placed on confluent skin fibroblast layers for 25 to 28 days. Finally, cells were fixed with 4% buffered formalin and stained with hematoxylin and eosin and analyzed for the formation of colonies or their overgrowth on the confluent fibroblast layers.

Quantification of Matrix Metalloproteinase (MMP)-3, -9, -13, and -14 and Cathepsin K mRNA by Real-Time Reverse Transcriptase-Polymerase Chain Reaction (PCR)

RNA was extracted from RASFs ($n = 11$, passages 3 to 6) and RASF^{SV40} ($n = 1$) by the reverse transcriptase-PCR RNA Miniprep Kit (Stratagene) including DNase treatment. RNA (300 ng) was reverse-transcribed with RT kit (PE Applied Biosystems) to cDNA (15 ng/ μ l). One μ l (15 ng) of cDNA was used for one quantitative real-time PCR reaction. Specific primers and TAMRA/FAM-labeled probe were used for the specific and quantitative detection of MMP-14, also called MT-1 MMP, and cathepsin K mRNA. Primers and probes were checked for specificity by GenBank analysis. The following primers and probes were used for the specific and quantitative detection of MMP-3, -9, and -14, and cathepsin K mRNA: MMP-3: forward primer, 5'-GGG CCA TCA GAG GAA ATG AG-3'; reverse primer, 5'-CAC GGT TGG AGG GAA ACC TA 3'; probe, 5'-AGC TGG ATA CCC AAG AGG CAT CCA CAC-3'; MMP-9: forward primer, 5'-GGC CAC TAC TGT GCC TTT GAG-3', reverse primer, 5'-GAT GGC GTC GAA GAT GTT CAC-3', probe, 5'-TTG CAG GCA TCG TCC ACC GG 3'; MMP-13: forward primer, 5'-TCC TAC AAA TCT CGC GGG AAT-3'; reverse primer, 5'-GCA TTT CTC GGA GCC TCT CA-3'; probe, 5'-CAT GGA GCT TGC TGC ATT CTC CTT CAG-3'; MMP-14: forward primer, 5'-TGG AGG AGA CAC CCA CTT TGA-3'; reverse primer, 5'-GCC ACC AGG AAG ATG TCA TTT C-3'; probe, 5'-CCT GAC AGT CCA AGG CTC GGC AGA-3'; cathepsin K: forward primer, 5'-CTT TGC TCT GTA CCC TGA GGA GAT-3'; reverse primer, 5'-TGT TAT ATT GCT TCC TGT GGG TCT T-3'; probe, 5'-TTC CAT AGC TCC CAG TGG GTG TCC A-3'. Expression of 18S rRNA was used as internal standard using predeveloped primer/probes (PE Applied Biosystems). DNA contamination of all samples was evaluated by using nonreverse transcriptase control RNA as reaction template.

SCID Mouse Co-Implantation Model Experiments

SCID mice were obtained from Charles Rivers GmbH (Sulzfeld, Germany) and kept permanently under sterile conditions. Implantation of RASFs ($n = 5$, passages 3 to 4), NSF ($n = 1$, passage 6), and RASF^{SV40} ($n = 1$) together with normal human cartilage was executed as described previously.¹⁵ Two independent experiments were performed with a total of 34 SCID mice. Briefly, the cells were trypsinized and dissolved in an inert type I collagen sponge (Gelfoam; Pharmacia, Upjohn, Dbendorf, Switzerland). Sponges were co-implanted with pieces of human articular cartilage of 1-mm³ size under the renal capsule of SCID mice. Mice were anesthetized by intraperitoneal injection of xylocaine (lidocaine hydrochloride, 0.014 mg/g of body weight; Astra Pharmaceutica, Dietikon, Switzerland) and Ketalar (ketamine hydrochloride, 0.09 mg/g of body weight; Parke-Davis, Baar,

Switzerland) in an isotonic solution. An incision was made on the left flank and the left kidney was exteriorized. Using forceps the renal capsule was carefully incised and sponge and cartilage were co-engrafted adjacent to the kidney. Peritoneum and skin were closed using 5-0 Prolene suture material. After 60 days, mice were sacrificed, and the implants were fixed in 4% buffered formalin and embedded in paraffin according to standard procedures.

Invasion into the cartilage was quantified according to a semiquantitative score ranging from 0 to IV referring to the number of invading cell layers and the number of affected cartilage sites: 0, no invasion; 0.5, invasion of one to two cell layers; I, invasion of three to five cell layers; I.5, invasion of three to five cell layers at three independent sites of the cartilage; II, invasion of 6 to 10 cell layers; II.5, invasion of 6 to 10 cell layers at three independent sites; III, invasion of >10 cell layers; III.5, invasion of >10 cell layers at two independent sites; IV, invasion of >10 cell layer at three or more sites of the cartilage. Cellular density was assessed on sections with invasion and adjacent to the cartilage in three high-power fields at $\times 400$ magnification by counting.

Immunohistochemistry on Paraffin-Embedded SCID Mouse Sections and Synovial Tissues

Tissue specimens obtained from the SCID mouse experiments and in addition from 13 RA and 1 osteoarthritis patients were sectioned, mounted on 5-aminopropyltriethoxysilane-coated slides, and dried at 50°C for at least 2 hours. Sections were dewaxed in xylol, rehydrated in decreasing concentrations of ethanol, and pretreated with microwave heating in citrate buffer (0.01 mol/L, pH 6.0) and kept at 70°C for 30 minutes in a heat incubator for antigen retrieval.

If not mentioned otherwise, all following procedures were performed at room temperature. The slides were incubated with blocking solutions A and B (Vector Laboratories, Burlingame, CA), each for 15 minutes. To decrease unspecific background reactions, slides were blocked with 2% horse serum in 4% nonfat milk in Tris-HCl (0.1 mol/L, pH 7.6) for 30 minutes. Primary anti-Ki67 antibodies (DAKO) were diluted 1:100 (concentration, 1 μ g/ml) and incubated overnight at 4°C. Mouse isotype-matched IgG sera in adapted concentrations served as negative controls. As secondary antibodies, goat anti-mouse antibodies labeled with biotin (diluted 1:200, concentration 5 μ g/ml; Jackson ImmunoResearch) were incubated for 30 minutes. Then, alkaline phosphatase-conjugated streptavidin (DAKO) was applied, also for 30 minutes, in a dilution of 1:50 (concentration 5.4 μ g/ml). Finally, staining was developed with BCIP/NBT solution (Boehringer Mannheim, Mannheim, Germany) for 20 to 30 minutes. Between all reaction steps extensive washing was performed with Tris-HCl (0.1 mol/L, pH 7.6). Before the application of the developing solution the slides were washed with Tris-HCl (0.1 mol/L) at pH 9.7. Tissue sections from a p53-positive neurological tumor (glioblastoma multiforme) served as positive controls. Moreover, SCID mouse sections derived from the RASF^{SV40} experi-

Figure 1. Expression of surface marker molecules. Almost all investigated synovial cells were positive for the fibroblast marker ASO2, CD44, and negative for MHC II (HLA-DR). RASF^{SV40} when compared to RASFs revealed a significant lower expression of VCAM-1 (CD106) and a significant higher expression of ICAM-1 (CD54) and of CD40. Also, NSF demonstrated a lower expression of VCAM-1 and a higher expression of ICAM-1.

ments were stained with anti-p53 antibodies (clone DO7, final concentration 4 μ g/ml, incubation for 40 minutes at room temperature; DAKO) using the same streptavidin-biotin detection system. The percentage of positive cells was determined by counting all cells and the number of the positive cells at $\times 400$ magnification in at least 10 high-power fields. All immunohistochemical reactions were repeated at least twice.

Statistical Analysis

For statistical analysis, the Mann-Whitney *U*-test was used. *P* values of less than 0.05 were considered to be significant. Data were expressed as means $\pm$ SEM.

Results

Characterization of RASFs, NSFs, and RASF^{SV40} in Vitro

As shown in Figure 1 several cell surface markers were investigated by flow cytometry. More than 98% of RASFs, RASF^{SV40}, and NSFs expressed the fibroblast marker ASO2. Along this line, the expression of HLA (DR) class II was restricted to less than 0.5% of the cells. Cells, 70.5% (± 5.7) of cultured RASFs, stained positive for the vascular cellular adhesion molecule (VCAM-1 or CD106) and this was significantly higher than in RASF^{SV40} ($16.6 \pm 4.6\%$, $P < 0.001$). Similarly low was the expression in NSFs with 19.7% (± 16.8) VCAM-1-positive cells. In contrast, the intracellular adhesion molecule (ICAM-1 or CD54) was significantly higher in RASF^{SV40} ($98.7 \pm 0.3\%$,

Figure 2. Expression of SV40 and p53 in SV40 T-antigen transformed cells. SV40-transformed RASF^{SV40} (a) accumulated high levels of SV40 T-antigen and p53 (b) in the nucleus. The IgG controls of the SV40-transformed cells were negative (c and d). Original magnifications, $\times 400$.

$P = 0.04$) and higher in NSFs ($81.7 \pm 1.8\%$) than in RASFs ($23.2 \pm 9.9\%$). CD40 was expressed in 79.4% (± 8.1) of RASF^{SV40} and this rate of expression was significantly higher than in RASFs ($4.5 \pm 2.5\%$, $P = 0.004$) and higher than in NSFs ($0.2 \pm 0.15\%$).

To prove that RASF^{SV40} were stably transfected with SV40 DNA the expression of the SV40 T-antigen and p53 was determined by immunofluorescence (Figure 2). RASF^{SV40} revealed in 100% of the cells a nuclear staining for the SV40 T-antigen and p53, whereas RASFs and control fibroblasts did not express SV40 T-antigen or p53 in more than 5% of the cells (not shown). Slides of RASF^{SV40} incubated with IgG control sera were negative.

As demonstrated in Table 1, cell-cycle analysis of cultured synovial fibroblasts was performed after nuclear isolation and propidium iodide staining. In RASFs S phase indexes were low, on average 4.3% (± 0.6) corresponding to a generation time of 140 hours (± 19.5). On the other hand, RASF^{SV40} demonstrated S phase indexes of 24.4% (± 3.3) corresponding to a cell doubling time of ~ 25 hours (± 3.3). U937 monocytic cells served as positive control with a generation time of 22 hours (± 4.3).

Intriguingly, in the colony-forming unit assay (Figure 3), RASF^{SV40} formed colonies, whereas RASFs and NSFs did not. In addition, SV40-transformed synoviocytes, but not untransformed synoviocytes, could overgrow feeder layers of confluent skin fibroblasts and RASFs, even when seeded in high dilutions. These results indicate that SV40-transformed synoviocytes reveal an autonomous growth potential and have lost contact inhibition in contrast to the untransformed synovial fibroblasts.

Table 1. Determination of the Proliferation of RASFs, RASF^{SV40}, and U937 Cells

Cell cycle phases	G ₀ /G ₁ (%)	S phase (%)	G ₂ (%)	Generation time (hours)*
RASF (<i>n</i> = 4)	88.1 $\pm$ 1.4	4.3 $\pm$ 0.6	7.7 $\pm$ 0.8	139.5 $\pm$ 19.5
RASF ^{SV40}	45.4 $\pm$ 2.1	24.4 $\pm$ 3.3	30.6 $\pm$ 1.4	24.6 $\pm$ 3.3
U937	56.3 $\pm$ 2.9	27.5 $\pm$ 5.4	16.8 $\pm$ 2.5	21.8 $\pm$ 4.3

Proliferation was determined by flow cytometry after nuclear isolation and propidium iodide staining. In RASF S phase indexes in average were 4.3% resulting in a generation time of 140 hours. On the other hand, RASF^{SV40} revealed an S phase index in mean of 24.4% corresponding to a generation time of 25 hours.

*The generation time (GT) of the cells was calculated according to the following formula: GT = 6 h $\times$ 100/S phase.

Figure 3. Testing the loss of contact inhibition in the colony-forming unit assay. Colony-forming unit assay performed in two modifications: either synovial cells were seeded in empty flasks in low cell numbers (serial dilutions from 312 to 5000 cells, **a–c**) or 100 to 150 cells were placed on confluent skin fibroblast layers (**d–f**). After 2 to 4 weeks, cells were analyzed for the formation of colonies. SV40-transformed RASFSV40 formed clearly colonies under both conditions (**a** and **d**), whereas untransformed synoviocytes could neither grow on the confluent skin fibroblast layer nor form colonies when seeded in high dilutions. Only some cells attached to the flask and grew slowly, but without forming colonies (**b** and **c**). These data indicate that the RASFSV40, but not the untransformed synoviocytes have lost contact inhibition. Original magnifications, $\times 50$.

Using real-time PCR the quantitative mRNA levels of cathepsin K and MMP-14 in cultured RASFs and RASFSV40 were determined (Table 2). Cathepsin K mRNA was significantly stronger expressed in RASFs than in RASFSV40 ($P = 0.026$) and the expression of cathepsin K mRNA was on average 180 times higher than in the SV40-transformed synoviocytes. In addition, MMP-14 mRNA was also significantly higher in RASFs than in RASFSV40 ($P = 0.026$) with a 3.5-fold increase. Moreover, expression of MMP-3, MMP-9, and MMP-13 were also investigated by real-time PCR, however without any significant differences in the expression levels between RASFs and RASFSV40.

Testing Invasion, Cellular Density, and Proliferation of Synovial Fibroblasts in SCID Mice

RASFs invaded significantly more strongly than NSFs, as demonstrated previously,³ and most interestingly also than RASFSV40 ($P < 0.01$) into the co-implanted cartilage (Figure 4 and Table 3). No statistical differences were found in terms of the invasiveness between NSFs and SV40-transformed synoviocytes. Of interest, RASFSV40 revealed a significantly higher cellular density adjacent to the cartilage than RASFs and NSFs ($P < 0.02$) (Figure 5).

Table 2. Quantitative Comparison of the Expression of mRNA of Cathepsin K and MMP14 between Cultured RASFs and RASFSV40

	* Δ Ct cathepsin K	x-fold increase	* Δ Ct MMP-14	x-fold increase
RASF ($n = 11$)	12.4 ± 0.3	$2^{7.5} = 181x$	11.5 ± 0.14	$2^{1.8} = 3.5x$
RASFSV40	19.9 ± 1.0	1x	13.3 ± 0.84	1x

The expression of cathepsin K and MMP-14 mRNA was determined by quantitative real-time PCR and found 181- and 3.5-fold more elevated, respectively, in nontransformed RASF ($P < 0.05$ for both).

* Δ Ct represents the difference between the cycle number of 18S and the cycle number of the measured gene expression. The lower the Δ Ct cycle number, the higher is the expression of the specific gene, which is presented in logarithmic scale to the base of 2. X-fold increase is referred to the basal expression of RASFSV40.

Figure 4. Cartilage invasion of RASFs in the SCID mouse co-implantation model. NSFs attached to the cartilage, but revealed only a very limited invasion, whereas RASFs invaded deeply into the co-implanted cartilage and destroyed it almost completely. In contrast, the SV40-transformed synovial cells attached to the cartilage and formed proliferative tumor-like tissues, but without invading the cartilage. Original magnifications, $\times 400$.

To confirm the stable SV40 transformation *in vivo* and that the cells adjacent to the cartilage are of human origin, paraffin sections derived from the RASF^{SV40} SCID mice experiments were investigated for the expression of human p53. All engrafted cells expressed high levels of p53 and no staining occurred with IgG isotype-specific control sera (data not shown).

To investigate the role of proliferation in cartilage invasion, paraffin sections from the SCID mice experiments of RASFs, NSFs, and RASF^{SV40} were stained for the cell cycle-associated protein Ki67. Particularly, sections from RASFs with strong invasion into the co-implanted cartilage were included.

RASF^{SV40} revealed strong Ki67 signals specifically in the tumor-like formations. In contrast, RASFs and NSFs demonstrated only negligible Ki67 staining. Of note, the sites of invasion were negative for the expression of Ki67, indicating that low proliferating RASFs were capable to invade the co-implanted human cartilage (Figure 6).

Moreover, tissue specimens from 13 RA and 1 osteoarthritis patients were investigated for the expression of Ki67. In RA samples 7.2% (± 0.6) of the cells expressed Ki67 in the nucleus, whereas in 1 osteoarthritis sample

Table 3. Quantification of the Invasiveness in the SCID Mouse Co-Implantation Model of RA

Cell type	Passage	Number of animals	Invasion score $\pm$ SEM
RASF75	4	3	2.8 ± 0.17
RASF76	3	3	2.7 ± 0.67
RASF77	3	6	2.8 ± 0.31
RASF78	4	4	3.1 ± 0.52
RASF80	3	4	3.5 ± 0.35
Mean RASF		20	3.0 ± 0.18
NSFs 53	6	9	1.2 ± 0.19
RASF ^{SV40}	—	5	1.3 ± 0.44

In the SCID mouse experiments, RASFs invaded significantly stronger into the co-implanted cartilage than NSFs and RASF^{SV40}, ($P < 0.01$). No statistical differences in terms of invasiveness were found between NSF and RASF^{SV40}.

Figure 5. Cellular density of cells adjacent to the cartilage in SCID mice. Three high-power fields (hpf) of cells adjacent to the cartilage were counted to assess the cellular density of those cells potentially invading the co-implanted cartilage. SV40-transformed RASFs demonstrated a significantly higher cellular density than RASFs and NSFs ($P < 0.02$).

Figure 6. Expression of the cell cycle-associated protein Ki67 in the SCID mouse co-implantation model of RA. Ki67 was not found to be expressed in RASFs at sites of cartilage invasion, as shown here for RASFs 76 to 78. In contrast, RASF^{SV40} revealed a strong Ki67 staining, specifically in the tumor-like formations. Original magnifications, $\times 400$.

4.6% stained positive for this marker (data not shown). However, the expression of Ki67 varied from sample to sample and even within one sample from area to area. In 8 of 13 RA samples local proliferation in one region of synovial hyperplasia ranged from 13 to 21%.

Discussion

The present study shows clearly that cartilage invading RASFs are low proliferative cells. In contrast, highly proliferating SV40-transformed RASFs revealed minimal cartilage invasion. Differences in the cartilage invasive potential between RASFs and transformed synoviocytes might be attributed to a distinctive activation of intracellular signaling pathways, altered attachment because of the different expression of adhesion molecules such as VCAM-1 and ICAM-1, or changed expression of matrix-degrading enzymes such as MMP-14 or cathepsin K. In view of our *in vitro* results that RASFs are activated, but low proliferative and do not loose contact inhibition, we conclude that the activation pathways of RASFs differ from truly transformed cells.

The proposed concept of the dissociation of invasion and proliferation in RASFs is of interest because recently Jung and co-workers¹⁶ demonstrated in human colorectal adenocarcinomas that cells at the invasion front revealed low proliferation. Accordingly, the study from

Khoshyomn and colleagues,¹⁷ using *in vitro* the co-culture of tumor spheroids and rat fetal brain, presented evidence that the tumor invasion of gliomas and meningiomas did not correlate with the proliferation marker Ki67. The authors point out that migration and proliferation seem to be mutually exclusive for a given cell at a certain time point, supporting the dichotomous process of proliferation and invasion in gliomas.

In RA, it is suggested that synovial hyperplasia is primarily dependent on the effect of tumor necrosis factor (TNF)- α mediated by TNF receptors and nuclear factor- κ B. *In vitro*, it could be shown that TNF- α as well as interleukin-1 β enhanced proliferation of RASFs, but not of normal synoviocytes.¹⁸ TNF- α reduced the Fas-dependent cell death in RASFs, indicating that this might also contribute to synovial hyperplasia.¹⁹ On the other hand, the treatment with therapies directed against TNF- α are only effective in 60 to 80% of patients and the disease recurs when the anti-TNF- α treatment is ceased, sustaining that it is disease suppressive, but not curative.²⁰ It is unclear, why 20 to 40% of the patients with RA do not respond. Of interest, in another SCID mouse model engrafting RA tissues as well as cartilage and bone (SCID-HuRag) the administration of TNF- α enhanced inflammation, but did not lead to bone and articular cartilage damage, indicating that other pathways independent of TNF- α may contribute to joint destruction in RA.²¹

In our study, 5 to 10% of all cells in RA tissues stained positive for the cell cycle-associated protein Ki67. However, in some areas a considerably higher expression of Ki67 with 13 to 21% of the local cells was detected. Assuming that 60 to 70% of the cells are nonproliferating CD68-positive macrophages, this shows that locally a high number of synovial fibroblasts could proliferate and contribute to synovial hyperplasia.^{7,8,22} However, the expression of anti-apoptotic molecules such as Flice inhibitory protein (FLIP)²³ and sentrin-1 (SUMO-1)²⁴ in RA synovium, specifically in synovial fibroblasts, underlines also the notion that inhibition of apoptosis contributes to synovial hyperplasia.

Because RASFs neither revealed a loss of contact inhibition nor a strong cell proliferation, we conclude from our *in vitro* investigations that RASFs do not appear to be transformed. Along this line, it is noteworthy that neither the expression of proto-oncogenes nor of tumor suppressor proteins is sufficient to generate a transformed phenotype.²⁵ On the other hand, RASF^{SV40} were highly replicative, but only minimally cartilage invasive, which is not surprising in light of the fact that SV40 transformation also results in a reduced expression of matrix-degrading enzymes.

Because the experiments from Lafyatis and colleagues¹⁰ showed that not only RASFs grew anchorage independently in soft agar, but most importantly, also normal synoviocytes, these data suggest that both cell types are not transformed. Similar to our results, the SV40-transformed RASFs obtained by Lemaire and colleagues²⁶ showed a high level of growth factor-independent proliferation, grew anchorage independently and formed tumors in immunodeficient mice. However, the invasive behavior of these cells was not examined.

Most interestingly, Logan and co-workers²⁷ have shown that SV40 T-antigen inhibited the expression of metalloproteinases and invasion of human placental cells. Accordingly, our studies revealed a significantly higher level of cathepsin K and MMP-14 mRNA in RASFs compared to RASF^{SV40}. Just recently, cathepsin K was shown to play a critical role in cartilage degradation by RASFs^{28,29} and MMP-14 (MT1-MMP) is thought also to be important in the matrix degradation of RA.^{30,31}

SV40-transformed RASFs have been considered for a long time to be an interesting tool for standardizing studies in particular in the pharmaceutical industry. This notion was based on *in vitro* experiments investigating the expression and stimulation of cytokines and/or cellular surface markers.^{13,32–34} The draw-back of all these studies was that functional assays, specifically invasion assays, were not performed. However, the current study detected striking differences between RASF^{SV40} and RASFs in terms of their invasive behavior. In contrast, human papilloma virus E6 protein-transfected synovial fibroblasts revealed increased proliferation and invasiveness,³⁵ suggesting that the transformation mechanisms of the SV40 T-antigen and the E6 protein might be different.

Moreover, the present study also obtained differences in the expression of surface markers between RASFs and SV40-transformed synoviocytes. The cell adhesion molecule VCAM-1 (CD106) was more expressed in RASFs

than in RASF^{SV40}. VCAM-1 was previously shown to be strongly expressed in RASFs at sites of invasion in SCID mice.³ Worthwhile to mention, CD40 was stronger expressed on RASF^{SV40} suggesting that CD40 might not be directly involved in the invasive process.

In summary, our data indicate that invasion of cartilage is mediated with an activated phenotype of synovial fibroblasts characterized by a strong expression of adhesion molecules, matrix-degrading enzymes, and inhibitors of apoptosis,^{23,24} but not dependent on cell proliferation, suggesting a dissociation of invasion and proliferation in the pathogenic process.

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