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Expression pattern of cell cycle-related gene products in synovial stroma and synovial lining in active and quiescent stages of rheumatoid arthritis

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Summary. Objective: To investigate the expression pattern of cell cycle related gene products in active and quiescent Rheumatoid arthritis (RA). Methods: Synovial tissue from 20 patients with active proliferative RA and 28 patients with RA in remission was immunohistochemically examined for expression of p53, p63, p21, p27, p16, cyclin D1, CDK4, RB, E2F, Ki-67 on tissue microarrays and by DNA flow cytometry for cell cycle phases. Results: Elevated expression of p53 and p27 was found in synovial lining and in stromal cells in proliferative active RA. In the remission stage this finding was confined to the synovial lining. Most of the cells were in the G0-phase. Ki-67 proliferation index was maximum 10% in synovial cells. Conclusion: The p53 pathway is activated in synovial cells in active RA as well as in quiescent stage of disease. Differences in the spatial expression pattern of proteins involved in the p53 pathway in RA in remission compared to actively proliferating RA reflect the phasic nature of the disease and support in our opinion the concept of adaptive role of p53 pathway in RA.

Key words: Cell cycle proteins, RA, Tissue array

Introduction

Rheumatoid arthritis (RA) is a complex systemic illness with an episodic course, characterized by involvement and progressive destruction of the joints.

Inflammatory and proliferative components are involved in the pathogenesis of the disease (Carson and Haneji, 1999). Any of these two components may dominate the respective current picture of affected joints (Fassbender, 2002). The dynamics of RA are reflected by the phasic nature of morphologic changes taking place in the synovial membrane of affected joints. The capacity of the synovial membrane to develop distinct exophytic surface hyperplasia as well as stromal fibrosis and neovascularisation leads to its ongoing remodelling.

Assessment of morphological changes in the synovial membrane can give information on duration and intensity of the pathological processes.

Morphologically one can distinguish different stages of the synovial changes in RA, which may be repeated after every exacerbation including a proliferation stage and a quiescent stage. The proliferative stage of RA is morphologically characterized by villous proliferation of the synovial membrane, which consists of the synovial lining and the synovial stroma. The synovial lining is predominantly multilayered; the normally flattened synovial lining cells are either cuboidal or high cylindrical in shape. The underlying synovial tissue ("synovial stroma", Fassbender, 2002) normally consists of loose fibrous tissue with few spindle-shaped synovial fibroblasts with small, dense nuclei. In the proliferative stage it is composed of fibrous tissue with neovascularisation, inflammatory infiltration as well a proliferation of transformed-appearing synovial fibroblasts, so called rheumatoid arthritis synovial fibroblasts- RAFS (Bradley et al., 2004). The cells become larger and rounded with an enlarged round to oval nucleus.

In the morphologically quiescent stage of RA the synovial stroma is moderately fibrosed and contains newly formed blood vessels. Only a few slender synovial fibroblasts are evident. The synovial lining layer in the quiescent stage of RA is predominantly composed of cuboidal synovial cells.

Clinical diagnosis of RA is based on criteria given by the American college of Rheumatology (ACR) in 1987. Clinical activity of RA can be assessed using a disease activity score based on the analysis of functional joint activity, joint inspection, and analysis of inflammatory blood parameter (Berger et al., 2004).

A growing number of investigations show that

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fibroblast-like synoviocytes play a critical role in joint destruction in RA independent from the inflammatory reaction (Bradley et al., 2004, Müller-Landner et al., 1996, Yamanishi and Firestein, 2001).

There is some evidence that alterations in the expression of cell cycle related proteins are involved in disease progression in RA. However, studies so far have mostly concentrated on p53 alterations.

Overexpression of p53in RA-synovial tissue has been reported in several studies (Firestein et al., 1997; Inazuka et al., 2000; Itoh et al., 2004). A possible role of p53 overexpression as a protective mechanism to arrest the cell cycle and cause apoptosis has been discussed (Inazuka et al., 2000; Seemayer et al., 2003; Yamanishi et al., 2002). Somatic mutations of p53 in a subset of RA patients and in experiments using animal models were reported in three different studies (Firestein et al., 1997; Reme et al., 1998). Another study indicates a possible repression of the p16 senescence program representing a general mechanism to sustain connective tissue hyperplasia including synovial hyperplasia in RA (Carson and Haneji, 1999). Expression patterns of cell cycle related gene products in synovial tissue of proliferative active and quiescent RA have not yet been systematically investigated.

The present study aims to evaluate alterations in the expression patterns of cell cycle related proteins in synovial lining and stromal cells in morphologically proliferative active and quiescent RA by examination of the expression patterns of proteins involved in cell cycle regulation, such as p16, CDK4, cyclin D1, RB, E2F, p53, p63, p21, p27, and by assessing the Ki-67 proliferation index.

Material and methods

Clinical data

The following clinical data were analyzed using a clinical questionnaire: age and sex of patients, affected joints, number of affected joints, duration of the symptoms, rheumatoid factor, clinical activity of disease. Activity of RA was determined clinically using a "disease activity score" (0-10) based on the functional joint activity, joint inspection and analysis of inflammatory blood parameters (leukocytosis, C-reactive protein). 0- no clinical signs; 1-3, slight clinical activity; 4-7, moderate activity; 8-10, severe clinical activity.

Tissue

Material retrieved by synovialectomy taken from 48 patients between 1998 and 2002 were selected from the tissue bank of the Centre of Rheuma-Pathology, WHO-collaborating Center, Mainz, Germany and from the tissue bank of the Institute of Pathology, University Heidelberg, Germany. Diagnosis of RA was established by clinical and histological criteria. 20 patients with a morphologically proliferative RA process and 28 patients with morphologically quiescent RA were included in the study. Ten probes with normal synovial tissue and 21 probes with osteoarthritis of the knee joint were taken as a control. All samples were formalin fixed and paraffin embedded via routine procedure.

Table 1. Antibodies used for immunohistochemistry.

MARKER	PRIMARY ANTIBODY	DILUTION	FRIMA/CLONE	SECONDARY ANTIBODY	DETECTION SYSTEM
P16	Mouse anti Human, IgG1,κ	1:250 over night (o.n.)	BD Biosciences, Heidelberg, Germany	Vectastasin Universal Quick Kit, Linaris, Wertheim, Germany	Peroxidase - AEC
P27	Mouse anti Human, IgG1,κ	1:250 o.n.	DAKO, Hamburg, Germany	Vectastasin Universal Quick Kit, Linaris, Wertheim, Germany	Peroxidase - AEC
P53	Mouse anti Human, IgG1,κ	1:250 o.n.	BD Biosciences	Vectastasin Universal Quick Kit, Linaris, Wertheim, Germany	Peroxidase - AEC
E2F	Mouse anti Human IgG2a	1:250 o.n.	Santa Cruz Biotechnology, Heidelberg, Germany	Vectastasin Universal Quick Kit, Linaris, Wertheim, Germany	Peroxidase - AEC
RB	Mouse anti Human, IgG1,κ	1:100 o.n.	BD Biosciences	Vectastasin Universal Quick Kit, Linaris, Wertheim, Germany	Peroxidase - AEC
P21	Mouse anti Human, IgG1,κ	1:250 o.n.	DAKO	Vectastasin Universal Quick Kit, Linaris, Wertheim, Germany	Peroxidase - AEC
Ki67 (MiB-1)	Mouse anti Human, IgG1,κ	1:100 o.n.	DAKO	Vectastasin Universal Quick Kit, Linaris, Wertheim, Germany	Peroxidase - AEC
CDK4	Mouse anti Human, IgG1	1:250 o.n.	SeroTec, UK	Vectastasin Universal Quick Kit, Linaris, Wertheim, Germany	Peroxidase - AEC
Cyclin D1	Mouse anti Human, IgG2a	1:250 o.n.	DPC Biermann	Vectastasin Universal Quick Kit, Linaris, Wertheim, Germany	Peroxidase – AEC
ER/PgR	Mouse antiHuman, IgG1κ	1:50	DAKO	Vectastasin Universal Quick Kit, Linaris, Wertheim, Germany	Peroxidase - AEC

Tissue microarrays

Sections were cut from each paraffin block and stained with hematoxylin and eosin (HE). As recommended in previous studies (Griffin et al., 2003), representative regions of synovial tissue were selected. From regions including synovial lining and stroma cells two tissue cylinders (1,5 mm in diameter) were obtained from the corresponding areas on the paraffin block. The cylinders were arrayed into a recipient paraffin block using a tissue chip microarrayer (Beecher Instruments, Micro-Array-Technology). Subsequent 3 µm sections were cut from the recipient block and mounted on sialinised glass slides to support adhesion of the tissue samples.

Immunohistochemistry

Expression of the investigated cell cycle related proteins was determined immunohistochemically using standard protocols for staining procedures. Immunostaining was performed using an automated platform-Dako autostainer (Dako, Denmark). Primary antibodies were diluted in commercially available antibody Diluent (Dako ChemMate-TM) and detection was carried out using the Dako ChemMate-TM kit. Non-binding monoclonal mouse IgG1 was used as negative control. The sections were finally counterstained with haematoxylin and mounted.

The antibodies used in the study are summarized in table 1.

Quantitative evaluation

Proliferating synovial stroma cells and synovial lining cells were evaluated separately for nuclear staining in cases with morphologically proliferative active RA and quiescent RA. Inflammatory infiltrations were not analyzed. Quantitative evaluation was performed independently by two pathologists using a semiquantitative score from 0 – 3, as shown in table 2. For p16, p21, p27, p63, Rb, CDK4, E2F and Ki67 any synovial area of uniform nuclear staining was considered positive, as recommended in previous studies (Kang et al., 2002). For p53 and cyclin D1, nuclear staining in 10% or more of the cells (score 2 and 3) is considered as an overexpression.

Table 2. Semiquantitative evaluation score for immunohistochemical staining in synovial cells.

SCORE	PERCENTAGE POSITIVY SYNOVIAL CELLS
0	0
1	1-10%
2	11-50%
3	> 50%

DNA flow cytometry

Additionally, ten cases of proliferative active RA and ten cases of RA remission were investigated by DNA flow cytometry for analysis of cell cycle phases. Preparation of single cell suspension from paraffin-embedded material was done according to the Hedley technique with modifications.

50 µm tissue sections were dewaxed using two changes of xylene, rehydrated and washed twice in distilled water. The sections were digested in 0.9% NaCl (pH 1.5) containing 0.5% pepsin (Serva, Heidelberg, Germany) for 30 min at 37 °C with intermittent vortex mixing. The single cell suspensions (absolute cell number: 30.000-110.000) were stained in 1 µg/ml 4',6-diamidino-2-phenylindole (DAPi) (Fa. Parec, Münster, Germany) in TRIS-buffer (pH 7.8) according to the method of Otto.

A preparation of normal human lymphocytes was used to calibrate the G0/G1 diploid peak.

For flow cytometry the DNA content was measured in a PAS II flow cytometer (Partec, Münster, Germany) with a 100 W mercury vapor lamp. The following filters were used: KG 1, BG 38 and UG 1 for excitation; TK 420 as dichroic mirror; and GG 435 as barrier filter. The fractions of cells in cell cycle phases G0/G1, S and G2/M phases were calculated by a computerised mathematical model.

Statistical analysis of data

Exploratory analysis of the data was performed using R 1.7.1 (http://www.r-project.org). Continuous data was evaluated using the Wilcoxon rank test. For count data, Fisher's exact test was used.

Statistic evaluation of the flow cytometry was performed by means of Sigma Plot. Significance was determined by the t-test.

Results

Analysis of clinical data

In the present study of 48 cases of RA females (34) were affected markedly more often than males (14).

All patients showed an oligo- or polyarticular joint destruction. The biopsies were taken from the radio ulnar joint in 47.5% (n=18) of the cases, from the knee joint in 20.8 % (n=10), from the hip in 6.2% (n=3), from the MTP joins in 6.2% (n=3) and from the elbow joint in 19.3% (n=14) of the investigated cases.

The mean patient age was 43 years, ranging from 24 to 68 years.

Duration of disease was less than 5 years in 16.6% (n=8) of cases, duration of disease was 6-10 years in 50% (n=24) of cases and more than 10 years in 33.4% (n=16).

Rheumatoid factor was positive in 40% (n=19) of cases (seropositive RA), 29 cases represent seronegative

RA.

When reviewing all the RA cases with the morphological feature of proliferative active RA (20 cases), 4 patients were in the severe clinical activity group, 9 patients were in the moderate clinical activity group, and 7 patients were in the slight clinical activity group.

Cases with morphological features of quiescent RA (28 cases) included 13 patients with slight clinical activity of RA, 6 patients with moderate clinical activity

Table 3. Data of evaluation of expression of the investigated cell cycle related proteins and proliferations index in synovial lining and synovial stromal cells in proliferative stage of RA (20 cases).

ANTIBODY	SCORE	LINING CELL LAYER (N/20)	LINING CELL LAYER (%)	STROMAL CELLS (N/20)	STROMAL CELLS (%)
P53	0	13	65	12	60
	1	6	30	5	25
	2	1	5	3	15
	3	0	0	0	0
P27	0	12	60	11	55
	1	6	30	5	25
	2	2	10	4	20
	3	0	0	0	0
P21	0	15	75	15	75
	1	5	25	5	25
	2	0	0	0	0
	3	0	0	0	0
Ki-67	0	0	0	0	0
	1	20	100	20	100
	2	0	0	0	0
	3	0	0	0	0
P16, CD4, RB, E2F, CyclinD1	0	17	85	15	75
	1	3	15	5	25
	2	0	0	0	0
	3	0	0	0	0

Table 4. Data of the semiquantitative evaluation of expression pattern of the investigated cell cycle related gene products in synovial lining and synovial stromal cells in quiescent stage of RA (28 cases).

ANTIBODY	SCORE	LINING CELL LAYER (N/20)	LINING CELL LAYER (%)	STROMAL CELLS (N/20)	STROMAL CELLS (%)
P53	0	24	85.7	28	100
	1	4	14.3	0	0
	2	0	0	0	0
	3	0	0	0	0
P27	0	24	85.7	28	100
	1	4	14.3	0	0
	2	0	0	0	0
	3	0	0	0	0
P21	0	26	92.9	28	100
	1	2	7.1	0	0
	2	0	0	0	0
	3	0	0	0	0
Ki-67	0	19	67.9	28	100
	1	9	32.1	0	0
	2	0	0	0	0
	3	0	0	0	0
P16, CD4, RB, E2F, CyclinD1	0	27	96.4	28	100
	1	1	3.6	0	0
	2	0	0	0	0
	3	0	0	0	0

and 9 patients had no clinical activity.

Analysis of immunohistochemistry and flow cytometry was performed according to the histomorphological features of proliferative active versus quiescent RA.

Analysis of immunohistochemical stainings of cell cycle related proteins and Ki-67

The results of the quantitative evaluation of the expression of investigated cell cycle related gene products and Ki-67 in proliferative active RA and RA in remission stage of the disease are summarized in tables 3 and 4.

Expression patterns of the investigated cell cycle related gene products in proliferative active synovium

Morphological characteristics of the synovial membrane in proliferative active RA are shown in figure 1a. Expression of p53 was found in both, synovial lining cells and synovial fibroblasts (slight to moderate reactivity) in 7 cases. In one case expression of p53 was seen only in the synovial fibroblasts.

Proliferating synovial fibroblasts showed an elevated expression of p53 in more than 10% of cells (score 2) in 3 cases. In five cases an expression of p53 in less than 10% of cells (score 1) was found. In these cases a positive nuclear staining for p53 was observed in less than 10% of synovial lining cells (Fig. 1c).

Positive staining for p27 was found in 9 cases (slight to high reactivity). All these cases showed p27 expression in synovial fibroblasts (Fig. 1e), whereas synovial lining cells showed slight expression of p27 in 8 cases (Table 3).

Slight positive nuclear staining for p21 was found in both synovial lining cells and synovial fibroblasts in less than 10% of cells in 5 cases.

In 4 out of 8 patients with morphologically proliferative RA expression of p53 was associated with expression of p27 and p21. Of those patients three had moderate clinical activity and 1 had a severe clinical activity of RA. All patients were female. Duration of disease ranged between one and 10 years.

Slight positive nuclear staining for p16, CDK4, cyclin D1 (Fig. 1g), RB (fFg. 1h) and E2F was found in singular synovial lining and stromal cells in 3 cases.

Statistical significant correlations between expressions level of investigated cell cycle related gene products and clinical activity of RA (p=0.64), sex (p=1.0), age of patients (p=1.0), duration of symptoms (p=0.1.0), or affected joints (p=0.80) were not found in our study.

Expression patterns of the investigated cell cycle related gene products in remission phase of RA

In the remission phase of RA the synovial stroma shows a scant celluraity with a low number of spindle-shaped synovial fibroblasts (Fig. 1b). An expression of any of the investigated proteins was not detected in these cells.

As described for lesions from proliferative active RA the synovial lining layer in the remission phase of RA was still predominantly composed of multi-layered cuboidal synovial cells with enclosed fragments of necrotic joint cartilage and bone.

Slight to moderate nuclear staining for p53 (Fig. 1d) and p27 in less than 10% of synovial lining cells (each score 1) was found in 4 cases, expression of cyclin D1, RB and E2F (score 1) was detected in one case.

Hyperplastic synovial tissue in osteoarthrosis patients showed in 4 of 21 cases joint destruction. No expression of p53 in the lining layer was detected.

Expression patterns of the investigated cell cycle related gene products in normal synovium

Normal synovial membrane did not show any expression of any of the investigated proteins.

Analysis of cell cycle phases by flow cytometry

Proliferative active synovium

Analysis of cell cycle phases showed most of the cells (92.4±1.5%) to be in the G0/1-phase. S-phase was detected in 5.3±1.76% of cells. G2+M-phase was detected in 2.2±0.7% of investigated cells (proliferation index: 7.3±1.5).

Remission phase of RA

Analysis of cell cycle phases showed most of the cells (96.03±1.8%) in the G0/1-phase. S-phase was detected in 4.9±1.1% of cells. G2+M-phase was detected in 1.02±0.7% of investigated cells (proliferation index: 5.92±1.5).

No statistically significant differences in comparison with proliferative active stage of RA were found (p=1.0).

Discussion

In contrast to normal synovial tissue used as control, where no expression of any investigated cell cycle related protein was detected, an elevated expression of several cell cycle related proteins was found in RA, especially in the proliferative active stage, but also in remission of disease.

In the proliferative stage of RA, an elevated expression of some of the investigated proteins was detected both in the synovial lining layer and in synovial fibroblasts, whereas in the group of cases with quiescent RA expression of proteins was confined to the synovial lining cells.

The most striking finding was an elevated expression of p53 and p27. The tumor suppressor gene TP53 has a well-established role in cell cycle control and

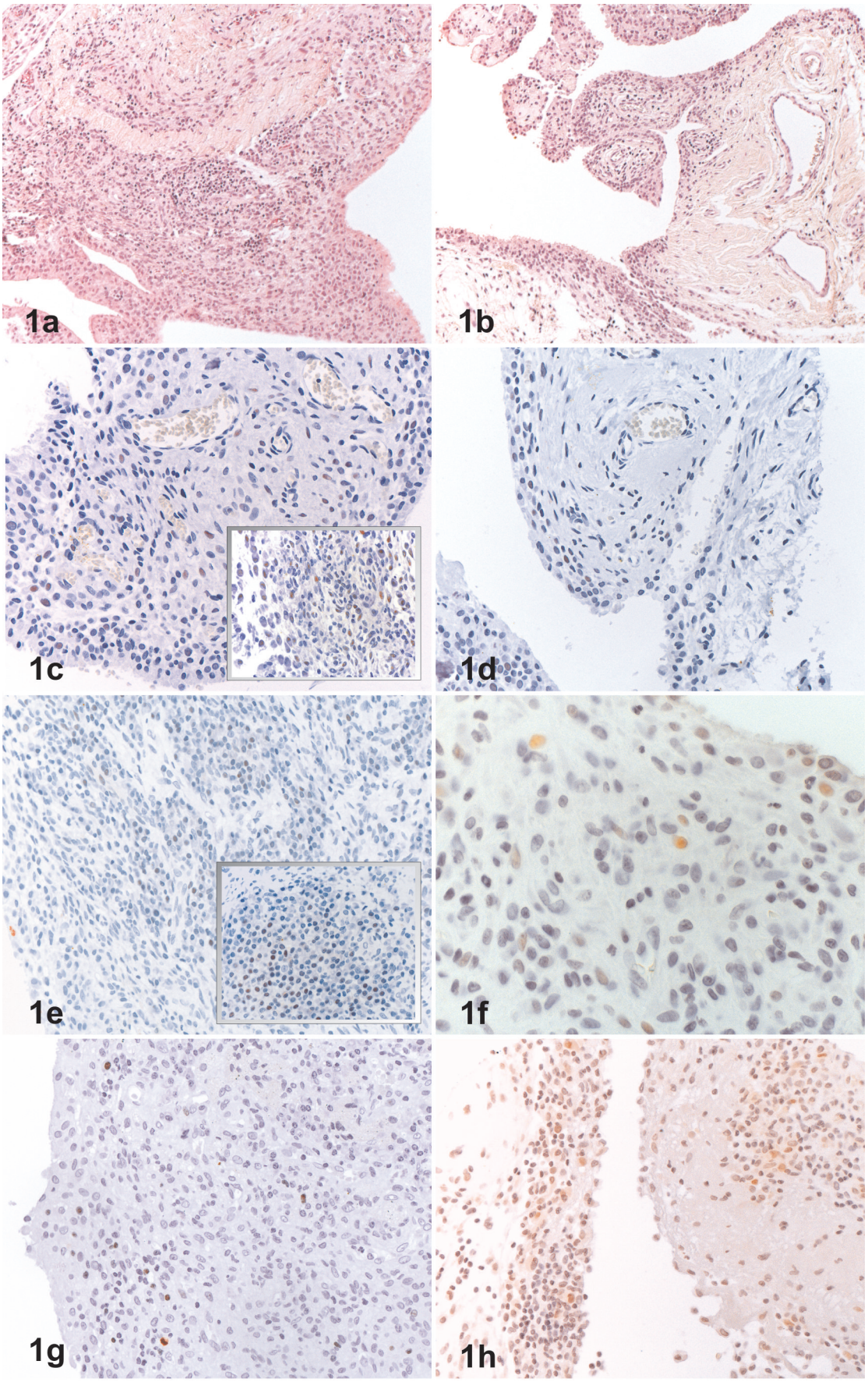

Fig. 1. a. Hyperplastic synovial membrane in the proliferative active stage of RA with proliferation of synovial lining and stromal cells. HE, x 7.2. **b.** Synovial membrane in quiescent stage of RA: no proliferation of synovial fibroblasts is seen. Synovial lining is mulitilayered. HE, x 7.2. **c.** Immunostaining for p53 in proliferative active RA showed a positive nuclear staining in numerous synovial lining cells as well as synovial fibroblasts. x 13, insert x 26. **d.** Immunostaining for p53 in the synovial membrane in RA in remission showed a positive nuclear staining in only few synovial lining cells, while no positive nuclear staining is seen in synovial stroma. x 13. **e.** Immunostaining for p27 in proliferative active RA a positive nuclear staining in numerous synovial lining cells as well as synovial fibroblasts. x 26, insert x 31.2. **f.** Immunostaining for cyclin D1 in proliferative active RA: positive nuclear staining in singular synovial fibroblasts and lining cells. x 62.4. **g.** Immunostaining for Ki-67 in proliferative active RA: positive nuclear staining in less than 10% of synovial cells (score 1). x 26. **h.** Immonostaining for RB in proliferative active RA showed positive nuclear staining in less than 10% of synovial fibroblasts. x 26

apoptosis. The critical role of p53 in pathogenesis of RA was demonstrated in many previous studies on synovial tissue in vivo and vitro as well as in experiments with SCID mice (Sun and Cheung, 2002; Itoh et al., 2003; Leech et al., 2003; Seemayer et al., 2003). P53 was demonstrated to be expressed in synovial fibroblasts and synovial lining cells, and its overexpression was postulated a characteristic feature of RA.

Overexpression of p53 can be induced by DNA damage in RA, probably as a consequence of reactive oxygen and nitric oxide produced during inflammation (Firestein et al., 1997). TP53 mutations in synovial membrane taken from RA patients were also demonstrated (Sun and Cheung, 2002). The p53 antibody used in this study has been reported to detect both, mutant and wt p53 (BD bioscience, Clone Pub 1801). While immunohistochemically detectable overexpression of p53 in more than 50% of tumour cells has been reported to be associated with overexpression of a non functioning gene product of mutated p53, for example in DCIS of the breast (Done et al., 2001), the degree of p53 expression detected in our study is not likely to be caused by p53 mutations.

Another aspect of TP53 function in RA is the adaptive cell cycle regulation.

In their study on the regulation of joint destruction and inflammation by p53 in inflamed synovium of p53 -/- DBA1 mice, Yamanashi et al. (2002) suggested that p53 is induced in the rheumatoid joint and other sites of inflammation as a protective mechanism to suppress expression of metalloproteinases, alter the cytokine network, cause apoptosis and arrest the cell cycle.

This data corresponds to our findings that the elevation of p53 expression was accompanied by a relatively low proliferative activity of synovial cells, detected simultaneously by Ki-67 and by analysis of cell cycle phases, showing most of the cells in the G0/1 phase.

As an important factor in cell cycle regulation, acting as checkpoint regulator, p53 functions by upregulation of p27 and p21, which are known as inhibitors of cycline dependent kinases (CDK). We could show an elevated expression of p27 and p21 in synovial lining cells and synovial fibroblasts in proliferative active RA, corresponding to low expression levels of cyclin dependent kinases (CDK4, Cyclin D1). This was an important finding, which had not been reported previously. Although p21 was shown to be a major regulator of cell cycle progression, it is now clear that p21 subserves multiple functions including pro- and anti apoptotic process (Dong et al, 2004). A possible role of p21 in apoptotic process in RA should be clarified in further studies.

Matching to the low levels of CDKs, we also detected low expression levels of retinoblastoma tumor supressor gene (RB) and E2F. These findings can be explained by the fact that CDKs phosphorylate RB, which acts as a gatekeeper and is needed for leaving the G1 phase. Phosphorylation of RB leads to the release of RB-bound E2F, which promotes the cell into the S-phase. Low expression levels of these cell cycle related gene products and the low level of p16 expression in absence of aberrant p16 expression are reflected by low mitotic activity of synovial lining and stromal cells found by analysis of the cell cycle and can give a possible explanation for our finding, that most cells were detected within the G0/G1 phases of cell cycle.

Another interesting finding in our study was the demonstration of p53 in synoviocytes of the lining layer in RA in the stage of remission, but not in normal synovium. P53 expression in the synovial lining cells seems to occur independent from proliferation of synovial fibroblasts. To address the question whether p53 expression of synovial lining cells could be a result of close contact with fragments of damaged joint cartilage and bone we additionally investigated p53 expression in synovial tissue of OA patients. However we did not detect expression of p53 in any of the investigated OA cases. It appears that the synovial lining cells are more susceptible to the permanent genotoxic environment of RA joints compared to synovial stromal cells. Our results indicate that the role of p53 in proliferation of synovial lining cells in RA is independent from the grade of proliferative activity of the disease.

Conclusion

An elevated expression of cell cycle related proteins could be demonstrated in the synovial membrane of proliferative active RA as well as in RA in remission phase.

The phasic nature of the morphological changes in RA is reflected by differences in the spatial expression pattern of proteins involved in the p53 pathway in RA in remission compared to actively proliferating RA. These findings support in our opinion the concept that the activation of p53 represents an adaptive process in RA.

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