AUTHOR QUERY SHEET

Author(s): AI Dubikov, SG Kalinichenko
Article Title: Small molecules regulating apoptosis in the synovium in rheumatoid arthritis
Article No.: SRHE 474799

Dear Author,

Please check these proofs carefully. It is the responsibility of the corresponding author to check against the original manuscript and approve or amend these proofs. A second proof is not normally provided. Informa Healthcare cannot be held responsible for uncorrected errors, even if introduced during the composition process. The journal reserves the right to charge for excessive author alterations, or for changes requested after the proofing stage has concluded.

The following queries have arisen during the editing of your manuscript and are marked in the margins of the proofs. Unless advised otherwise, submit all corrections using the CATS online correction form. Once you have added all your corrections, please ensure you press the "Submit All Corrections" button.

Ref. no:	Query	Remarks
AQ1	Please give location of all manufacturers mentioned in this paper.	
AQ2	What does n-10 mean?	

Small molecules regulating apoptosis in the synovium in rheumatoid arthritis

AI Dubikov[1,2], SG Kalinichenko[3]

[1]Department of Internal Diseases, Vladivostok State Medical University, [2]Municipal Rheumatology Centre, and [3]Department of Histology, Vladivostok State Medical University, Vladivostok, Russia

Objectives: To study the rate of apoptosis and expression of pro- and anti-apoptotic molecules in the synovial membrane in early and late rheumatoid arthritis (RA).

Methods: Samples of the synovium, cartilage, synovial fluid, and blood serum were obtained from patients with seropositive RA. The localization of Bcl-2-, p53- and TUNEL-immunoreactive cells in the synovial membrane was studied. The level of the soluble Fas (sFas) receptor was determined in the blood serum and synovial fluid using an immunoassay.

Results: In early RA, p53-immunoreactive synovial cells of type A were found to form rare aggregates in the intima. In late RA, on the contrary, these cells increased in number and occurred predominantly in the synovial stroma. Bcl-2-immunoreactive synovial cells were observed in lymphocytic infiltrates in the intima. They were found mainly in early RA. In late RA, their number decreased. The apoptotic index determined from the proportion of TUNEL-reactive synovial cell nuclei reached a maximum in late RA. The temporal differences in the rate of apoptosis were correlated with the humoral level of sFas, which increased significantly in late RA. On the contrary, in the synovial fluid, the sFas level decreased monotonically from early to late RA.

Conclusion: In early RA, in the synovial membrane, the rate of apoptosis and p-53-immunostaining intensity were low, and Bcl-2-immunostaining intensity was high. The sFas level in synovial fluid was high. In late RA, the rate of apoptosis and p-53-immunostaining intensity increased, Bcl-2-immunostaining intensity decreased, as did the sFas level.

The concept of rheumatoid arthritis (RA) has evolved dramatically in the past 50 years. This evolution is to a large extent due to the accumulation of data on the so-called small molecules involved in intracellular signalling and the control of various events including apoptosis, or programmed cell death. Early RA stages are characterized by morphological changes such as synovial hyperplasia, neoangiogenesis, and infiltration with mononuclear cells (1–3). Macrophage- and fibroblast-like synovial cells play an important role in these processes. Fibroblast-like synovial cells exhibit pre-neoplastic properties, high infiltration potential, and proto-oncogene expression (2, 4). In late RA, the proliferation rate decreases and connective tissue often develops (5). One possible explanation for the proliferation of rheumatoid synovia is an imbalance between proliferation and apoptosis rates (6–8).

Alexander I. Dubikov, Department of Internal Diseases, Vladivostok State Medical University, 2, pr. Ostryakova, Vladivostok 690950, Russia.
E-mail: aihavlad@online.vladivostok.ru

Accepted 2 March 2010

The present study deals with apoptosis and expression of pro- and anti-apoptotic factors in the synovial membrane of the human knee joint in early and late RA.

Materials and methods

The material for the study was obtained from 78 patients with RA (70 women and eight men) with a mean age of 42.34 ± 1.49 years. The research was performed in strict compliance with the Helsinki Declaration. All patients gave written informed consent and the study was approved by the local ethics committee. All patients met the American College of Rheumatology (formerly, the American Rheumatism Association) 1987 revised criteria for RA (9). All patients suffered from typical seropositive RA persistent to non-steroidal anti-inflammatory drugs (NSAIDs). No spontaneous or drug-induced remissions were observed in the patients. The patients were selected as they were directed to a hospital. Prior to selection they were subjected to clinical, laboratory, and instrumental examination. During the examination period, the patients received only symptomatic NSAID medication. Two groups of patients were distinguished,

DOI: 10.3109/03009741003742771

those with early (2 months to 3 years) and late (> 3 years) RA (35 and 43 subjects, respectively).

Synovium samples were obtained in the course of arthroscopy and synovectomy performed in accordance with indications (continuously recurrent knee-joint synovitis for at least 2 months). Informed consent was obtained from all patients. Control samples of synovial membrane and cartilage were obtained from seven healthy subjects (men 21–50 years) who died from a combined trauma. The control samples were taken within 2 h after death. Samples of synovial fluid were obtained from 10 patients (four men and six women aged 25–55 years) at exploratory arthrotomy of their knee joints indicated by alleged meniscus injury. Samples of blood serum and synovial fluid were frozen at −70°C immediately after sampling.

Apoptosis was determined by TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling) staining. The method is based on detection of fragmented DNA chains. Samples of synovial membrane and cartilage were fixed in 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS; pH 7.4) for 3–4 h at 4°C. They were then washed in 7–8 changes of 0.1 M PBS (pH 7.4) for 24 h and placed in 15% buffered sucrose solution. Sections (20 μm thick) were obtained using a freezing microtome. The sections were postfixed in ethyl acetate solution for 5 min at −20°C and rinsed twice in PBS. Each section was incubated with 75 μL of equilibration buffer for at least 10 s at room temperature. TUNEL-positive structures were revealed using the ApopTag® Fluorescein Direct In Situ Apoptosis Detection Kit (Chemicon). The sections were processed following the standard protocol. They were incubated in a humidified chamber for 1 h at 37°C, rinsed in stop-buffer for 10 min, and incubated in a solution of Fab fragment of pig anti-rabbit immunoglobulin (Ig) secondary antibodies conjugated with fluorescein isothiocyanate (FITC; Dako) diluted 1:100. The sections were then rinsed in PBS and mounted on slides in glycerol. The sections were observed using a Polyvar light microscope with an FITC filter (B1 450–490 nm).

The localization of Bcl-2 and p-53 was studied in sections of the knee-joint synovium. The sections were incubated in a solution of rabbit anti-Bcl-2 primary polyclonal antibodies (Chemicon) or mouse anti-p-53 primary monoclonal antibodies (Chemicon) diluted 1:200 in PBS with normal goat serum for 9 h, washed in PBS, incubated in a solution of biotinylated goat secondary antibodies diluted 1:100 (Vector Laboratories) for 1 h, avidin–biotin–peroxidase complex (Vectastain ABC Kit, Vector Laboratories BA-2000) for 1 h, and 0.03% diaminobenzidine and 0.01% hydrogen peroxide solution in PBS for 10–20 min, dehydrated in an ethanol series, and mounted in DEPEX.

The level of soluble sFas in blood serum (sFas-ser) and synovial fluid (sFas-syn) was determined by solid-phase immunoassay using the Bender MedSystems kit and expressed in pg/mL.

The number of TUNEL-positive nuclei was estimated in 10 000 μm^2 fields of sections of the synovium or cartilage using an eyepiece graticule. The apoptotic index was calculated as the ratio of the total number of TUNEL-positive nuclei to the number of cells stained with toluidine blue and having prominent non-pycnotic nuclei. The number of Bcl-2- and p-53-immunoreactive cells was counted using the Magiscan GM 3 system (Joyce-Loeble) and their proportion calculated relative to the total number of haematoxylin–eosin-stained cells.

All statistical tests were performed using the Statistica 6.0 (Statsoft) package.

Results

In both early and late RA, intensely fluorescent TUNEL-positive apoptotic nuclei were observed. The number of TUNEL-positive cells differed in early and late RA. Control samples contained no TUNEL-positive nuclei.

TUNEL-positive synovial cell nuclei exhibited signs of DNA fragmentation and appeared as fluorescent points (apoptotic bodies) merging with each other to form rings, semicircles, and continuous homogeneous aggregations (Figure 1). They were situated at the site of a former nucleus, distributed evenly in the cytoplasm, displaced to the plasma membrane, or clustered at one of the somatic poles. Apoptotic cells observed in the sections of the synovium and cartilage had no signs of necrosis and vice versa. In early RA, TUNEL-positive cells occurred mainly in the perivascular space coinciding with inflammatory infiltrates (Figure 1A). The walls of blood vessels were TUNEL-negative. In the intima, individual fluorescent fragmented nuclei of synovial cells were observed. Occasionally, they formed aggregations.

These results indicate a low rate of apoptosis in early RA (Table 1). The level of apoptosis was correlated with the duration and intensity of the inflammatory infiltration of the synovium. In late RA, the level of apoptosis increased dramatically. The number and proportion of stromal elements in the apoptotic structures of the hypertrophied synovium increased (Figure 1B). The number of TUNEL-positive cells in perivascular infiltrates also increased. However, these cells were mainly localized in the subintima. The apoptotic index of chondroblasts was remarkably high. Chondroblasts were mainly limited to the superficial layers of the cartilage, where they formed groups containing each 1–3 apoptotic bodies. In the deeper layers of the cartilage characterized by lower proliferation rate, TUNEL-positive nuclei occurred rarely.

The localization of the p53- and Bcl-2-immunoreactivity in the synovial membrane depended on the RA stage. In early RA, p53-immunoreactive synovial cells appeared as rare aggregations in the intima (Figure 2A). They were rounded or oval in shape and were probably

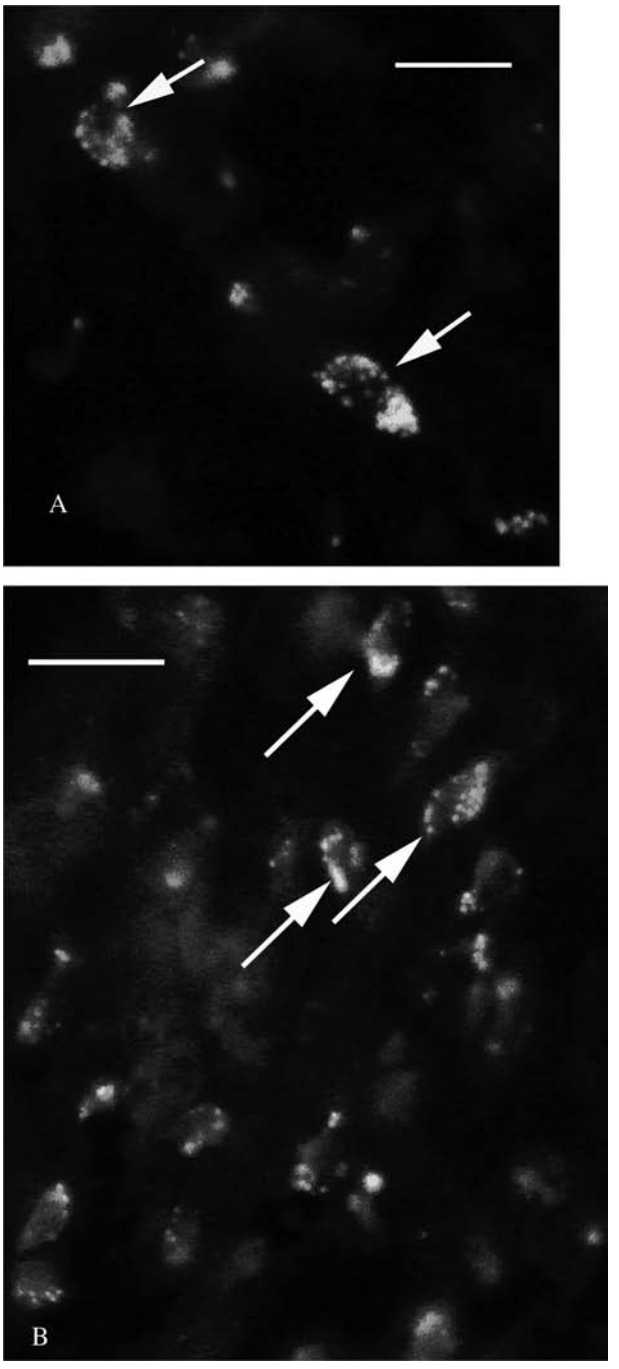

Figure 1. TUNEL-positive nuclei in the knee-joint synovial membrane in rheumatoid arthritis (RA). Apoptotic nuclei appear as intensely fluorescent spots (arrows) occasionally merging to form homogeneous ring-shaped aggregations lying at the nuclear periphery. (A) Apoptosis in synovial cells in the focus of a perivascular infiltrate in early RA. (B) Mass apoptosis of fibroblast-like synovial cells in the synovial stroma in late RA. Scalebar: 25 μm.

infiltrating cells or type A synovial cells. In late RA, p53-immunoreative cells occurred more frequently. They were mainly localized in the synovial stroma (Table 2).

As distinct from p53-immunoreactive cells, the number of Bcl-2-immunoreactive synovial cells was a maximum in early RA. At this stage, Bcl-2-immunoreactive cells occurred in almost all sections studied (Figure 2B). In late RA, their number decreased (Table 2). Bcl-2 immunoreactivity was mainly observed in the lymphocytic infiltrates, intima, and smooth muscle cells of the synovial vessels. In late RA, the localization of Bcl-2 immunoreactivity was essentially the same, but its intensity decreased considerably. In late RA, the levels of sFas-ser increased significantly compared to early RA (Table 1). The level of sFas-syn exhibited the opposite trend, decreasing steadily from early till late RA.

Discussion

In this study we have shown that the level of apoptosis in synovial cells was low in early RA and high in late RA. These results are consistent with the dynamics of Bcl-2, p53, and sFas-ser. The pathological changes in the human synovial membrane in RA coincide with a decrease in the density of chondroblasts, which undergo apoptosis in long-term RA. It is possible that these effects inhibit growth and regeneration of cartilage resulting in secondary osteoarthrosis.

The morphological heterogeneity of TUNEL-immunoreactive cells reflects differences in the mechanism of their programmed death, suggesting alternative apoptosis pathways (8, 10). The mitochondrial pathway is induced by the transcription factor p53, whose deficiency leads to tumour-like proliferation of synovial cells (11). An alternative pathway occurs in lymphocytes and is blocked by Bcl-2 (12).

The expression of Bcl-2 in synovial tissues changed monotonically from hyperexpression in early RA to hypoexpression in late RA. It is possible that Bcl-2 expression in early RA limits the rate of apoptosis and facilitates proliferation, survival, and active functioning of respective inflammatory elements. This is consistent with our previous data. Thus, smooth muscle cells of synovial vessels obtained from patients with RA exhibited high levels of Bcl-2 expression (13). In another study, synovial tissues of patients with RA exhibited higher levels of immune ribonucleic acid (iRNA) compared to patients with osteoarthrosis (14).

As the main target of the Bcl-2 family are the mitochondria, it is possible that apoptosis occurs mainly via the mitochondrial pathway in RA. In this pathway, p53 plays an important role in mitochondrial membrane permeabilization and apoptosis (15–17). The mitochondrial membrane contains p53AIP1 protein mediating p53-dependent apoptosis (18).

According to our data, in early RA and acute adjuvant-induced arthritis, the levels of Bcl-2 were high, while the levels of p53 and apoptotic index were low (7). In late RA and chronic adjuvant-induced arthritis, the expression of p53 increased dramatically, and that of Bcl-2 decreased, resulting in an increase in the rate of apoptosis in the tissues of the joint. Earlier, some authors argued for the ability of p53 to repress the Bcl-2

Table 1. Apoptotic index of cells of the cartilage and synovial membrane of the human knee joint and sFas levels in synovial fluid in rheumatoid arthritis (RA).

	Control	Early RA	Late RA
Chondroblasts	–	0.07 ± 0.05	0.4 ± 0.09*
Chondrocytes	–	–	0.01 ± 0.03
Synovial lymphocytes	–	–	0.03 ± 0.01
Synovial cells	–	2.1 ± 0.5	5.8 ± 0.2*
sFas-syn (n-10) (pg/mL)	210.42 ± 55.90	2665.21 ± 262.27*	1381.00 ± 16.52*

Values given as mean ± SEM.
*Differences between the control and non-control groups significant at $p < 0.05$.

promoter and for the ability of Bcl-2 to suppress the proapoptotic activity of p53 (12, 19). Thus, in RA pathogenesis, the level of apoptosis in the synovial tissues may be determined by the Bcl-2/p53 balance functioning as a 'molecular rheostat'.

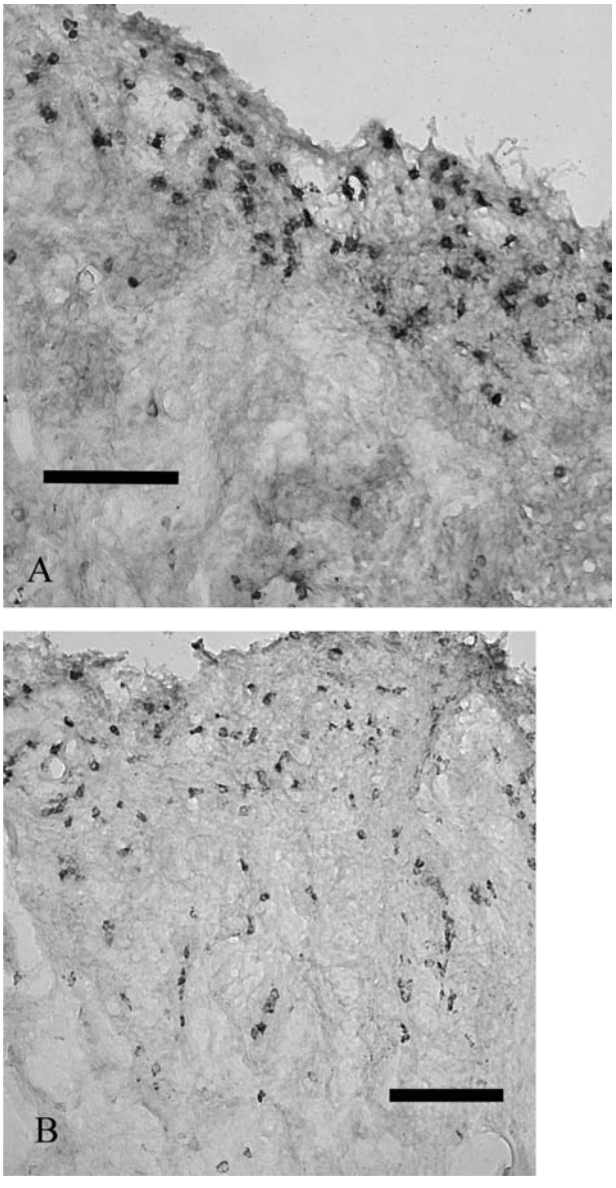

Figure 2. Expression of p53 and Bcl-2 in knee-joint synovial cells in different RA stages. (A) p53-immunoreactive cells in the intima in early RA. (B) Localization of Bcl-2 in the intima and subintima in early RA. Scalebar: 150 μm.

Bcl-2 expression has been examined in studies of many diseases. High levels of Bcl-2 expression have been associated with poor prognosis in urinary bladder cancer, Hodgkin's disease, and prostate cancer (20–22). In addition, the Bcl-2/Bax ratio proved to be an efficient prognostic marker in chemotherapy of acute myeloid leukaemia and gastric cancer (23, 24). However, very little is known about the clinical role of Bcl-2 in RA.

According to our data, an increase in Bcl-2 expression is significantly correlated with the number of inflamed joints ($r = 0.67$, $p < 0.05$) and the functional class of the patients ($r = 0.71$, $p < 0.05$). This suggests that Bcl-2 is a valuable prognostic marker and a new target in RA therapy.

sFas is known to activate the sFas-dependent apoptosis pathway by binding to the sFas ligand. The dynamics of the sFas level correlated with that of p53 and Bcl-2 and with the rate of apoptosis. In late RA, an increase in the apoptotic index was accompanied by a decrease in the sFas-syn level. The sFas level was significantly correlated with RA activity ($r = 0.62$, $p < 0.05$), the functional class of the patients ($r = 0.83$, $p < 0.05$), and the apoptotic index ($r = -0.74$, $p < 0.05$). Thus, sFas can be used as an integrative indicator of the extent and characteristics of apoptosis in the synovial membrane.

The present results suggest an important role for apoptosis in RA. Studying the mechanisms and suppression of apoptosis may result in new approaches to stage-dependent RA therapy.

Conclusion

We have demonstrated that, in early RA, in the synovial membrane, the rate of apoptosis and p-53-immunostaining intensity were low, and Bcl-2-immunostaining intensity was high. The sFas level in synovial fluid was high. In late RA, the rate of apoptosis and p-53-immunostaining intensity increased, and Bcl-2-immunostaining intensity decreased, as did the sFas level. Hence, disease progression in early RA may be related to low rates of apoptosis and p53 expression in synovial cells. Apoptosis-related molecules are promising targets for early RA therapy.

Table 2. The percentage of p53- and Bcl-2-immunoreactive cells in synovial tissues in rheumatoid arthritis (RA).

	p53-immunoreactive cells			Bcl-2-immunoreactive cells		
Cell elements	Control	Early RA	Late RA	Control	Early RA	Late RA
Lymphocytes	–	2.7 ± 0.5	5.1 ± 0.3	–	7.6 ± 1.1	0.8 ± 0.1*
Synovial cells	–	4.8 ± 1.5	12.3 ± 0.5	1.21 ± 0.10	10.4 ± 0.5	1.1 ± 0.2*

Values given as mean ± SEM.
*Between-group differences significant at $p < 0.05$.

References

1. Dubikov AI, Cherepovsky AV, Belogolovyh LA, Medved EE. Effects of the anti-TNF-antibody infliximab on nitric oxide (NO) synthesis by synovial membrane and cartilage in adjuvant arthritis. Ann Rheum Dis 2004;63(Suppl 1):P163.
2. Firestein GS. Invasive fibroblast-like synoviocytes in rheumatoid arthritis. Passive responders or transformed aggressors? Arthritis Rheum 1996;39:1781–90.
3. Bramlage CP, Kaps C, Ungethüm U, Bramlage P, Koziolek M, Wessels J, et al. Modulatory effects of inflammation and therapy on GDF-5 expression in rheumatoid arthritis synovium. Scand J Rheumatol 2008;37:401–9.
4. Raychaudhuri SP, Raychaudhuri SK. The regulatory role of nerve growth factor and its receptor system in fibroblast-like synovial cells. Scand J Rheumatol 2009;38:207–15.
5. Dubikov AI, Belogolovykh LA, Medved EE. Apoptosis as a mechanism of autoimmune inflammation in human knee joint. Bull Exp Biol Med 2004;138:568–70.
6. Catrina AI, Ulfgren AK, Lindblad S, Grondal L, Klareskog L. Low levels of apoptosis and high FLIP expression in early rheumatoid arthritis synovium. Ann Rheum Dis 2002;61:934–36.
7. Dubikov AI. p53 and bcl-2 molecules as regulators of apoptosis in rheumatoid arthritis (RA) synovial tissues. Ann Rheum Dis 2005;64(Suppl 3):P89.
8. Firestein GS, Yeo M, Zvaifler NJ. Apoptosis in rheumatoid arthritis synovium. J Clin Invest 1995;96:1631–8.
9. Arnett FC, Edworthy SM, Bloch DA, McShane DJ, Fries JF, Cooper NS, et al. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum 1988;31:315–24.
10. Yao Q, Wang S, Gambotto A, Glorioso JC, Evans CH, Robbins PD, et al. Intra-articular adenoviral-mediated gene transfer of trail induces apoptosis of arthritic rabbit synovium. Gene Ther 2003;10:1055–60.
11. Itoh K, Hase H, Kojima H, Saotome K, Nishioka K, Kobata T. Central role of mitochondria and p53 in Fas-mediated apoptosis of rheumatoid synovial fibroblasts. Rheumatology 2004;43:277–85.
12. Smith MD, Walker JG. Apoptosis a relevant therapeutic target in rheumatoid arthritis? Rheumatology 2004;43:405–7.
13. Perlman H, Georganas C, Pagliari LG, Koch AE, Haines K 3rd, Pope RM. Bcl-2 expression in synovial fibroblasts is essential for maintaining mitochondrial homeostasis and cell viability. J Immunol 2000;164:5227–35.
14. Liang HY, Jiang M, Chen HM, Wang YC. Overexpression of proto-oncogene bcl-2 in rheumatoid synovium. Br J Rheumatol 1996;35:803–4.
15. Rich T, Allen RL, Wyllie AH. Defying death after DNA damage. Nature 2000;407:777–83.
16. Vousden KH. p53: death star. Cell 2000;103:691–4.
17. Solau-Gervais E, Zerimech F, Lemaire R, Fontaine C, Huet G, Flipo RM. Cysteine and serine proteases of synovial tissue in rheumatoid arthritis and osteoarthritis. Scand J Rheumatol 2007t;36:373–7.
18. Matsuda K, Yoshida K, Taya Y, Nakamura K, Nakamura Y, Arakawa H. p53AIP1 regulates the mitochondrial apoptotic pathway. Cancer Res 2002;62:2883–9.
19. Miyashita T, Harigai M, Hanada M, Reed JC. Identification of a p53-dependent negative response element in the bcl-2 gene. Cancer Res 1994;54:3131–5.
20. Atuğ F, Türkeri L, Ozyürek M, Akdaş A. Bcl-2 and p53 overexpression as associated risk factors in transitional cell carcinoma of the bladder. Int Urol Nephrol 1998;30:455–61.
21. Smolewski P, Niewiadomska H, Blonski JZ, Robak T, Krykowski E. Expression of proliferating cell nuclear antigen (PCNA) and p53, bcl-2 or C-erb B-2 proteins on Reed-Sternberg cells: prognostic significance in Hodgkin's disease. Neoplasma 1998;45:140–7.
22. Moul JW. Angiogenesis, p53, bcl-2 and Ki-67 in the progression of prostate cancer after radical prostatectomy. Eur Urol 1999;35:399–407.
23. Bincoletto C, Saad ST, da Silva ES, Queiroz ML. Haematopoietic response and bcl-2 expression in patients with acute myeloid leukaemia. Eur J Haematol 1999;62:38–42.
24. Nakata B, Muguruma K, Hirakawa K, Chung YS, Yamashita Y, Inoue T, et al. Predictive value of Bcl-2 and Bax protein expression for chemotherapeutic effect in gastric cancer. A pilot study. Oncology 1998;55:543–7.