

Evaluation of Apoptosis Induction in Human Peripheral Blood Mononuclear Cells and Synovial Cells in Patients with Rheumatoid Arthritis

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Rheumatoid arthritis (RA) is a chronic inflammatory destructive disease involving the joint and characterized by T-lymphocyte accumulation within the synovial compartment. It is dominated by the presence of macrophages, plasma cells and synovial fibroblasts which are the main pathogenic factors leading to the destruction of bone and cartilage. The survival of these cells may be promoted by inadequate apoptosis leading to synovial hyperplasia. So, the aim of the present study was to evaluate the apoptosis levels before and after induction of apoptosis using anti-Fas mAb, both in peripheral blood (PB) and synovial fluid (SF) infiltrating mononuclear cells (MCs) of patients with RA. CD₄⁺ T cell subsets and cell survival assays were also done to investigate correlations between these parameters. The study was conducted on 15 patients with RA, 10 individual volunteers as a control group and 10 patients with osteoarthritis (OA) as a control group for SF evaluations (have defective Fas expression on their cells). Results of this work revealed that *in vitro* induction of apoptosis by anti-Fas mAb resulted in increase of: percent (%) reduction of cell viability in PBMCs and SFMCs, % reduction of CD₄⁺ T cell subsets and apoptotic cell % in all studied groups than before induction. The increase in the three parameters is only significant in SF of RA group compared to PB while it is non significant in OA group due to the defective Fas expression on OA cells. Our results also showed a significant positive correlation between CD₄⁺ T cell and viability percentages before induction of apoptosis in SF of RA and between apoptosis levels and CD₄⁺ T cell percentage after induction of apoptosis in the SF of RA group. In conclusion, activated T cells infiltrating SF of RA patients have functional Fas antigen which enable them to undergo *in vitro* apoptosis using anti-Fas mAb. The cytotoxicity of which is more specific to local lesion such as SF of RA patients suggesting that local administration of this anti-Fas mAb may represent a promising new therapy for RA patients.

Rheumatoid arthritis (RA) is a chronic inflammatory disease (Ollier et al., 2001) which is mainly characterized by synovial hyperplasia and pathological immune phenomena including inflammatory cell adhesion and activation (Szekanecz and Koch, 2001), production of mediators such as cytokines and growth factors (Otttenello et al., 2002; Vervoordeldonk and Tak, 2002), angiogenesis (Koch, 1998), bone resorption (Gravallese et al., 1998), and fibrosis and progressive destruction of the affected joints (Szekanecz and Koch, 2001).

T and B lymphocytes, plasma cells, macrophages, mast cells, natural killer (NK) cells and dendritic cells are accumulated in the inflamed synovium (Tak et al., 1997;

Smeets et al., 1998). Viable B cells are present in subintimal clusters associated with vascular cell adhesion molecule-1 (VCAM-1) positive cells, while B cells in the intima are almost invariably undergoing lysis (Otttenello et al., 2002). T cells play a critical role in RA and probably involved in the pathogenesis from initiation to the chronic stage (Dudler and So, 1998).

Inadequate apoptosis (programmed cell death) which may promote the survival of autoreactive T cells and synoviocytes of RA patients (Kawakami and Eguchi, 2002; Eguchi, 2001) may be the mechanism for synovial hyperplasia (Mountz et al., 2001; Leonardo, 1997). Several factors, such as bcl-2 expression (Yang et al., 1997), P53

mutation (Wallace-Brodeur and Lowe, 1999), inhibitory protein signaling and Fas transmission (Hamilton and Piccart, 2000; Perlman et al., 2001) may contribute to the reduced apoptosis. On the other hand, a variety of stimuli act as triggers leading to the onset of the apoptotic program through modulating substances such as cytokines (Wallheim, 2002), genes (Kobayashi et al., 2000) and interacting proteins (Denecker et al., 2001; Hofmann, 1999). From these substances, the Fas molecule and its ligand, Fas L- (Connell, 2001) or anti-Fas mAb, induce apoptosis (Ogawa et al., 2001; Matsuno H et al., 2002).

So, the aim of this work is to evaluate the *in vitro* apoptosis levels before and after anti-Fas mAb addition, in both PBMC and SFMC populations of patients with RA. In addition, CD₄⁺ T cell subset and cell survival assays were done to determine correlations between these parameters and apoptotic levels before and after induction.

Subjects and Methods

Subjects

The study was conducted on three subject groups (1) 15 consenting patients with chronic RA were chosen according to the American Rheumatism Association (ARA) criteria, (2) 10 normal healthy volunteers matched in age and sex were taken as the control group (C), (3) 10 consenting patients with osteoarthritis (OA) served as another control group since the functional Fas antigen expression, susceptible to anti-Fas mAb, is detected on the synoviocytes of patients with RA but not on the synovial cells of patients with OA.

Patient with other diseases which might affect the immunological status or joint inflammation were excluded.

All RA and OA patients were subjected to : thorough history and clinical examination, laboratory investigation (CBC, ESR, RF) and X-ray examination.

Methods

I. Separation of mononuclear cells

Peripheral blood mononuclear cells (PBMCs) and synovial fluid MNCs (SFMCs) were isolated by density

gradient centrifugation over Ficol-Hypaque (Perper et al., 1977). SFMCs were isolated only from RA and OA patients while PBMCs were separated from all subjects under study. The procedure was carried out at room temperature and under aseptic conditions.

I. Induction of apoptosis (Hoa et al., 1996)

Freshly isolated PBMCs or SFMCs were suspended at 1×10^6 cells/ml of RPMI-1640 tissue culture medium supplemented with glutamine (Gibco, Scotland), 10% heat inactivated fetal calf serum, 100 IU/ml penicillin and 100 µg/ml streptomycin. Cells were then either treated with 1 µg/ml of anti-Fas m Ab (advanced Immunochemical, Inc., USA) or with culture medium. Both cell populations were incubated for 24 hours at 5% CO₂ humid incubator at 37°C. Then the cells were centrifuged at 1800 rpm for 10 minutes and the culture supernatants were harvested and stored at -70°C until apoptosis assay was carried out.

The isolated cells of both the anti-Fas IgM mAb treated and untreated were used for the assessment of cell viability and CD₄⁺ T cell subset as well as evaluation of apoptosis.

1-Viability test (Paul, 1968)

The dye exclusion method was used. Briefly, one drop of cell suspension was added to one drop of 0.2% Trypan blue solution, left for 2 minutes at room temperature and examined microscopically. Non viable cells stain blue. A total of two-hundred cells were counted and the % of viable cells were calculated:

$$\% \text{ of viable cells} = 100 - \frac{\text{Number of dead cells} \times 100}{\text{Total number of cells}}$$

2-CD₄⁺ T cell subset assessment (Thomas et al., 1981)

One hundred µl of the cell suspension (1×10^6 cell/ml) were added to 10 µl of the fluorescense-labeled anti-CD₄⁺. After incubation for 30 minutes at room temperature, the cells were washed 3 times with PBS. The pellet was resuspended and examined under the fluorescent microscope. A total of two-hundred cells were counted,

$$\% \text{ CD}_4^+ \text{ T cells} = \frac{\text{number of +ve stained cells} \times 100}{\text{total number of cells}}$$

3-Evaluation of apoptosis

Apoptosis levels were evaluated in 20 µl of cell culture supernatant (corresponding to a cell equivalent of 5×10^4 cell/ml) before and after anti-Fas mAb treatment by *in vitro* determination of cytoplasmic-histone-associated-

DNA fragments (mono and oligonucleosome using cell death detection ELISA plus (Roche Diagnostics) according to the manufacturer's procedures.

Apoptosis is characterized by activation of an endogenous endonuclease and internucleosomal DNA fragmentation and degradation. The enrichment of mono and oligonucleosomes in the cytoplasm of the apoptotic cells is due to the fact that DNA degradation occurs several hours before plasma membrane break down. Thus, the assay is based on a quantitative sandwich-enzyme-immunoassay-principle using mouse monoclonal antibodies directed against DNA and histones, respectively. This allows the specific determination of mono-and oligonucleosomes in the cytoplasmic fraction of cell lysates or supernatant.

Working procedures

Cell culture supernatant samples was placed into a streptavidin-coated microtiter plate (MTP). Subsequently, a mixture of anti-histone-biotin and anti-DNA-POD were added and incubated for 2 hours. During the incubation period, the anti-histone antibody bound to the histone-component of the nucleosomes and simultaneously fixed the immunocomplex to the streptavidin coated MTP via its biotinylation. Additionally, the anti-DNA-POD (monoclonal antibody from mouse, peroxidase conjugated) reacted with the DNA-component of the nucleosomes. After removal of unbound antibodies by a washing step, the amount of nucleosomes was quantified by the POD retained in the immunocomplex. POD was determined photometrically with ABTS (2, 2'-Azino-di [3-ethylbenzthiozolin-sulfonate]) as substrate.

Interpretation

The specific enrichment of mono-and oligo-nucleosomes released into the cytoplasm was calculated using the following formula:

$$\text{Enrichment factor} = \frac{\text{mU of the sample after apoptosis induction}}{\text{mU of the corresponding sample before induction}}$$

$$\text{mU} = \text{absorbance} [10^{-3}]$$

Statistical analysis

The obtained results were analyzed using SPSS version 9 statistical package. Paired t-test was used to compare values of cell viability, CD₄⁺ T cells and apoptosis before and after anti-Fas induction. A percentage change in values was computed using the following formula,

$$\frac{\text{Value before} - \text{value after}}{\text{Value before}} \times 100$$

Anova test was applied to compare the percentage change in the 3 compared groups. T-test was used in comparison of percentage change in SF between RA and OA. Linear correlation between continuous variables within each group was tested by Pearson's correlation coefficient.

Results

The mean values of the % viability of PBMCs before and after anti-Fas mAb addition and the % reduction in RA, OA and normal groups are shown in Table 1. Statistical analysis of this parameter indicates non significant difference between the 3 groups (Table 1).

Table 1. The % viability of PBMCs in rheumatoid arthritis (RA), osteoarthritis (OA) and normal control (NC) groups with and without anti-Fas mAb addition.

	RA		OA		NC	
	Without anti-Fas	With anti-Fas	Without anti-Fas	With anti-Fas	Without anti-Fas	With anti-Fas
Range	94-99	82-96	96-99	90-94	96-99	89-95
Mean	96.47	90.67	97.20	91.90	97.30	92.30
± SE	0.47	1.10	0.29	0.35	0.40	0.67
*p ₁	NS	NS				
**p ₂			NS	NS		
***p ₃					NS	NS

*p₁: Comparison between the mean values of % viability in RA and NC groups.

**p₂: Comparison between the mean values of % viability in RA and OA groups.

***p₃: Comparison between the mean values of % viability in OA and NC groups.

(without vs. without anti-Fas and with vs. with anti-Fas)

A comparison using ANOVA test for the % reduction of PBMCs viability induced by anti-Fas mAb revealed insignificant differences between the 3 groups (Table 2).

Table 2. Comparison of the percentage change in viability (% reduction of viability) of PBMCs induced by anti-Fas mAb in rheumatoid arthritis (RA), osteoarthritis (OA) and normal control (NC) groups.

Groups	Percentage change (reduction %) in viability of PBMCs	
	Range	Mean \pm SE
RA	2.11-17.17	5.99 \pm 1.37
OA	3.09-7.22	5.44 \pm 0.45
NC	3.09-8.25	5.14 \pm 0.49
*P-value	NS	

*Using ANOVA test. NS= Non significant

For the same parameters in SFMC of RA and OA group, the statistical analysis revealed that the only significant difference is detected between the 2 groups after treatment with anti-Fas mAb ($P < 0.001$), table (3).

Table 3. The % viability of SFMCs in rheumatoid arthritis (RA), osteoarthritis (OA) without and with anti-Fas mAb addition.

	RA		OA	
	Without anti-Fas	With anti-Fas	Without anti-Fas	With anti-Fas
Range	93-98	62-79	94-98	86-93
Mean	95	69.13	96	90.20
\pm SE	0.41	0.98	0.39	0.84
* p_1	NS			
** p_2	< 0.001			

* p_1 : Comparison between the mean values of % viability in RA and OA groups without anti-Fas mAb addition.

** p_2 : Comparison between the mean values of % viability in RA and OA groups with anti-Fas mAb addition.

$P < 0.05$ is significant. NS= Non significant

Table (4) indicates the significant decrease ($P < 0.001$) in the mean percentage change of viability in SFMCs of RA as compared to the OA group.

Table 4. Statistical analysis of the percentage change in viability (% reduction) of SFMCs in rheumatoid arthritis (RA) and osteoarthritis (OA) groups.

Group	Percentage change (% reduction) in viability of SFMCs	P-value
	Mean \pm S.E.	
RA	27.21 \pm 1.08	< 0.001
OA	6.02 \pm 1.06	

$P < 0.05$ is significant.

The CD₄⁺ T cells % in PBMCs before and after addition of anti-Fas mAb in the three groups, RA, OA and NC revealed marked decrease after anti-Fas mAb addition in all

groups with significant differences between RA and both NC and OA groups before anti-Fas mAb addition and between RA and NC groups after its addition (Table 5).

Table 5. The CD₄⁺ T cells % of PBMCs in rheumatoid arthritis (RA), osteoarthritis (OA) and normal control (NC) groups with and without anti-Fas mAb addition.

	RA		OA		NC	
	Without anti-Fas	With anti-Fas	Without anti-Fas	With anti-Fas	Without anti-Fas	With anti-Fas
Range	32-47	28-41	29-44	26-35	31-38	28-35
Mean	39.87	34.80	35.80	31.70	34.20	31
± SE	1.12	1.22	1.32	1.15	0.74	0.75
*p ₁	< 0.001	<0.01				
**p ₂			<0.05	NS		
***p ₃					NS	NS

*p₁: Comparison between the mean values of CD₄⁺ cells % in RA and OA groups.

**p₂: Comparison between the mean values of CD₄⁺ cells % in RA and NC groups.

***p₃: Comparison between the mean values of CD₄⁺ cells % in OA and NC groups.

(without vs. without anti-Fas and with vs. with anti-Fas)., P<0.05 is significant. NS= Non significant

On the other hand a non-significant difference is shown between the 3 studied groups concerning the reduction % of CD₄⁺ T cells (Table 6).

Similar results obtained with SFMCs in RA and OA groups expect that a significant difference between them exists with and without anti-Fas mAb addition (Table 7).

Table 6. Comparison of the percentage change in CD₄⁺ T cells (% reduction of CD₄⁺ T cells) of PBMCs induced by anti-Fas mAb in rheumatoid arthritis (RA), osteoarthritis (OA) and normal control (NC) groups.

Groups	Percentage change in CD ₄ ⁺ T cells (% reduction of CD ₄ ⁺ T cells) of PBMCs	
	Range	Mean ± SE
RA	2.44-21.62	12.79±1.54
OA	3.23-18.42	11.27±1.54
NC	6.45-14.29	9.37±0.73
P-value	NS	

NS= Non significant

Table 7. The CD₄⁺T cells % of SFMCs in rheumatoid arthritis (RA), osteoarthritis (OA) without and with anti-Fas mAb addition.

	RA		OA	
	Without anti-Fas	With anti-Fas	Without anti-Fas	With anti-Fas
Range	42-59	23-36	35-40	26-36
Mean	51.60	28.27	38.30	31.80
± SE	1.41	1.19	0.47	1.21
*p ₁	< 0.001			
**p ₂	0.05			

*p₁: Comparison between the mean values of CD₄⁺ T cells % in RA and OA groups without anti-Fas mAb addition.

**p₂: Comparison between the mean values of CD₄⁺ T cells % in RA and OA groups with anti-Fas mAb addition.

P<0.05 is significant.

The % reduction of CD₄⁺ T cells showed a significant difference between the two groups (Table 8). The apoptosis level in PBMCs showed a insignificant increase after induction of apoptosis than before with the same enrichment factor (EF) in the three groups; RA, OA and NC groups (Table 9).

Table 8. Statistical analysis of the percentage change in CD₄⁺ T cells (% reduction of CD₄⁺ T cells) SFMCs in rheumatoid arthritis (RA) and osteoarthritis (OA) groups.

Group	Percentage change (% reduction) in CD ₄ ⁺ T cells % of SFMCs		P-value
	Mean ± S.E.		
RA	44.91 ± 2.27		< 0.001
OA	17.11 ± 2.53		

P<0.05 is significant.

Table 9. Apoptosis level (mU/5 × 10⁴ cells/ml) and enrichment factor (EF) of PBMCs in rheumatoid arthritis (RA), osteoarthritis (OA) and normal control (NC) groups with and without anti-Fas mAb addition.

	RA			OA			NC		
	Without anti-Fas	With anti-Fas	EF	Without anti-Fas	With anti-Fas	EF	Without anti-Fas	With anti-Fas	EF
Range	451-2633	702-2650		573-2689	1080-2362		1263-1882	1323-2079	
Mean	1813.93	1999	1.19	1708.60	1889.30	1.134	1669.90	1799.90	1.08
SE	162.90	134.93	0.09	222.05	223.23	0.03	72.27	71.93	0.02
*p ₁	NS	NS							
**p ₂				NS	NS				
***p ₃							NS	NS	

*p₁: Comparison between the mean values of apoptosis levels in RA and OA groups.

**p₂: Comparison between the mean values of apoptosis levels in RA and NC groups.

***p₃: Comparison between the mean values of apoptosis levels in OA and NC groups.

(without vs. without and with vs. with), NS= Non significant., mU= absorbance X 10³

EF (enrichment factor) = mU of the sample with anti Fas mAb addition/mU of the sample without anti-Fas mAb addition.

The data also revealed in-significant differences in the apoptosis levels with and without anti-Fas mAb as well as in the % of apoptotic cells of PBMCs (Table 10) between the 3 groups.

Table 10. Comparison of the percentage change (% of apoptotic cells) in apoptosis levels (mU/5 × 10⁴ cells/ml) of PBMCs in rheumatoid arthritis (RA), osteoarthritis (OA) and normal control (NC) groups.

Groups	Percentage change (% of apoptotic cells) of PBMCs	
	Range	Mean ± SE
RA	0.18-130.59	19.06±8.61
OA	1.89-29.09	13.39±3.17
NC	2.38-19.34	8.11±2.03
*P-value	NS	

* Chi-square test, NS= Non significant

Apoptosis level in SFMCs showed a marked increase in both RA and OA groups after addition of anti-Fas mAb (Table 11). The statistical comparison indicated insignificant differences between the two groups before apoptosis induction while the difference was

highly significant after the induction using anti-Fas mAb. The EF is significantly increased in RA patients compared to that of OA group. The % of apoptotic cells in RA was significantly higher than that of OA group (Table 12).

Table 11. Apoptosis level (mU/5 x 10⁴ cell/ml) and enrichment factor (EF) of PBMCs in rheumatoid arthritis (RA) and osteoarthritis (OA) groups without and with anti-FAS mAb addition.

	RA		EF	OA		EF
	Without anti-Fas	With anti-Fas		Without anti-Fas	With anti-Fas	
Range	1015-2466	2760-4994		884-2702	998-2728	
Mean	1823	4495.73	2.68	1819.40	1948.70	1.09
SE	113.10	199.39	0.28	223.30	214.74	0.03
*p ₁			NS			
**p ₂			< 0.001			
***p ₃			< 0.001			

*p₁: Comparison between the mean values of apoptosis levels in RA and OA groups without anti-Fas.

**p₂: Comparison between the mean values of apoptosis levels in RA and OA groups with anti-Fas.

***p₃: Comparison between the mean value of EF in RA and OA groups.

P<0.05 is significant. NS= Non significant., mU= absorbance X 10³

EF (enrichment factor) = mU of the sample with anti Fas mAb addition/mU of the sample.

Table 12. Z value of Mann-Whitney test showing the percentage change in apoptosis level (mU/50 x 10³/ml)(% of apoptotic cells) of SFMCs in rheumatoid arthritis (RA) and osteoarthritis (OA) groups.

Group	Percentage change in apoptosis (% of apoptotic cells) of SFMCs	t-value	P-value
	Mean ± S.E.		
RA	168.39 ± 30.56	308.71	< 0.001
OA	9.08 ± 2.67		

P<0.05 is significant.

The statistical comparison between the obtained data revealed that the increase in % reduction of viability, reduction of % CD₄⁺ T cells and in apoptosis levels of SFMCs compared to PBMCs is significant in RA group while it is non

insignificant in OA group (Table 13). The most important statistical correlations between these parameters are represented in RA group (Table 14).

Table 13. Statistical comparison of % change of % viability, % CD₄⁺ T cells and apoptosis levels in PBMCs and SFMCs of rheumatoid arthritis (RA) and osteoarthritis (OA) groups.

		% changes (mean ±SE)		P-value
		PBMCs	SFMCs	
RA	Viability %	5.99±1.37	27.21±1.08	< 0.01
	CD ₄ ⁺ T cell %	12.79±1.54	44.91±2.27	< 0.01
	Apoptosis level	19.06±8.61	168.39±30.56	< 0.01
OA	Viability %	5.44±0.45	6.02±1.06	NS
	CD ₄ ⁺ T cell %	11.27±1.54	17.11±2.53	NS
	Apoptosis level	13.39±3.17	9.08±2.67	NS

P<0.05 is significant. NS= Non significant

Table 14. The statistical correlations between % cell viability, % CD₄⁺ T cells and Apoptosis level of SFMCs without and with anti-Fas mAb addition in rheumatoid arthritis (RA) group.

	Apoptosis without anti-Fas	Apoptosis with anti-Fas	CD ₄ ⁺ T cells without anti-Fas	CD ₄ ⁺ T cells with anti-Fas	Viability % without anti-Fas
Apoptosis with anti-Fas	r= -0.29 p>0.05				
CD ₄ ⁺ T cells % without anti-Fas	r=0.06 p>0.05	r= -0.04 p>0.05			
CD ₄ ⁺ T cells with anti-Fas	r=0.24 p>0.05	r=0.43 p<0.05	r=0.3 p>0.05		
Viability % without anti-Fas	r= -0.23 p>0.05	r= -0.14 p>0.05	r=0.49 p<0.05	r= -0.14 p>0.05	
Viability % with anti-Fas	r= -0.16 p>0.05	r=0.09 p>0.05	r=0.03 p>0.05	r=0.28 p>0.05	r=0.02 p>0.05

*:P is significant at the level < 0.05.

Discussion

It is well established that mononuclear cells, T-cells in particular, play a critical role and are probably involved in the pathogenesis of RA (Dudler et al., 1998). Significant numbers of T-cells have been detected in the synovial fluid of patients with RA. Failure of apoptosis of T-cells and synoviocytes of RA patients may be the mechanism for synovial hyperplasia (Matiba et al., 1997; Leonardo, 1997). So, we aimed in this study at evaluating the apoptosis process of PBMCs and SFMCs of RA patients through *in vitro* induction of apoptosis in these cells using anti-Fas mAb and investigating the viability of these cells, the % of CD₄⁺ T-cell subset and the apoptosis level as well as the correlations

between these parameters. Subjects of the present study included 10 patients with OA considered as a second control group since they are not known to express functional Fas antigen on their SFMCs (Kobayashi et al., 2000; Perlman et al., 2001).

Our results revealed that the % cell viability is decreased after induction of apoptosis in the three studied groups. the decrease is statistically significant only in the SFMCs of RA group compared to that of SFMCs of OA group. This finding is in agreement with Hoa et al (1996) and Hasunuma et al. (1996) who attributed the decrease in cell viability to the induction of apoptosis by treatment with anti-Fas mAb. On the other hand, the insignificant reduction in % viability may be due to spontaneous

apoptosis occurs normally as a result of incubation for 24 hours.

The mean CD₄⁺ T cells % in PBMCs of RA patients was significantly higher than that of both control groups before treatment with anti-Fas mAb indicating the important role of CD₄⁺ T-cells in the pathogenesis of RA and the autoactivation of T-cells (Schirmer et al., 1998). This role is confirmed by the positive correlation existed between CD₄⁺ T-cell % before and after apoptosis induction in PBMCs of RA.

Correlation studies in the present work revealed that % CD₄⁺ T-cell and the % viability in the SF of RA patients (before and after addition of anti-Fas mAb) has increased, indicating that the CD₄⁺ T-cells are the main cells present in SF of RA patients which have the susceptibility to apoptosis after addition of anti-Fas mAb. This goes in agreement with the finding of Hasunuma et al. (1996) who reported that CD₄⁺ T-cells are the most prominent cells in rheumatoid synovium and are the main population undergoing apoptosis by anti-Fas mAb.

The significant increase in the % reduction of CD₄⁺ T-cells in SF of RA than PBMCs after induction of apoptosis could be due to the infiltration of those activated Th cells into the affected joints. This decrease in CD₄⁺ T-cells % in the PBMCs of RA, OA and C groups (insignificant) and in SFMCs of RA (significant) and OA (insignificant) groups after apoptosis induction could be explained by the presence of minimal Fas expression on the CD₄⁺ T-cells of PB and SF of OA and C group, in addition to PB of RA patients. These results also suggest the ineffectiveness of the anti-Fas mAb for induction of apoptosis in these groups, and the presence of functional Fas antigen expression susceptible to anti-Fas mAb on the active T cells infiltrated into the SF of RA patients inducing significant cytotoxicity in this group. This suggestion is supported by many studies. Hoa et al. (1996) stated that some T-cells in the SF of RA

express functional Fas-antigen and play a crucial role in the pathogenesis of RA. Griffith (1995) demonstrated that Fas antigen is a major target molecule for CD₄⁺ T-cell cytotoxicity. Hasunuma et al. (1996) reported that more than 50% of synovial infiltrating cells died within 24 hours of incubation with anti-Fas IgM mAb, while almost all cells remained alive in the case of control IgM mAb. Nishioka et al. (1998) had identified two distinct populations of synovial T cells; one population is susceptible to anti-Fas mAb and another being resistant to it in the RA group.

The significant increase in the % CD₄⁺ T-cells of RA than of OA group before treatment with anti-Fas mAb may be due to the increase in the inhibitory protein level signaling Fas transmission such as Fas-associated phosphatase-1 (FAP-1) or survival protein bcl-2 in RA. This explanation is supported by Schirmer et al. (1998) who demonstrated that patients with RA have a subset of CD₄⁺ T lymphocytes characterized by a defect in CD28 expression, and a dysregulation of bcl-2 and subsequent resistance to apoptosis. This may favour the clonal outgrowth of autoreactive T-cells. Autoreactive T-cells in RA synovium express both Fas and Fas-L on their surfaces but these cells are not completely eliminated by Fas/Fas L interaction, resulting in chronic inflammation. This failure in immunologic surveillance might be due to a dysfunction of Fas-L positive T cells or an inhibitory effect on Fas-Fas L interaction resulted from the presence of soluble Fas antigen or an inhibitory Fas signal transmission such as FAP or bcl-2 (Connell, 2001). Accordingly, we can suggest that CD₄⁺ T-cells may express functional Fas antigen and are susceptible to apoptosis when treated with anti-Fas mAbs in SF of RA patients supporting the obtained results of cell viability and apoptosis studies in the present work.

Concerning apoptosis, the defect in apoptosis in the SFMCs of RA patients

compared to OA group before *in vitro* induction of apoptosis which may lead to hyperplasia of synoviocytes can be explained by Kawakami & Eguchi (2002) who denoted that humoral factors including cytokines and growth factors present in RA patients modulate the expression of apoptosis regulating molecules in the cells, which inhibit the apoptotic process of synovial cells and osteoclasts. The decrease in Fas mediated apoptosis may be due to increased endogenous inhibitor such as the proinflammatory cytokines IL-1 β , TNF α and IL-6 (EL Hallous, 1998; Hasunuma, 1996). Mutations of P53 suppressor gene, deficient functional Fas L expression, over expression of anti-apoptotic molecules such as sentrin, and activation of nuclear factor K β , all may cause inadequate apoptosis (Tak et al., 1999; Collins et al., 1992). Over expression of inhibitor bcl-2 is suggested by Tak et al. (1999), Zhang et al. (1997) and Firestein et al. (1995) to be the main inhibitor of apoptosis of SFMCs in RA patients. Synovial impairment of Fas/Fas L due to low level of Fas L expression may lead to SFMCs survival in RA patients and soluble Fas was found to be capable of binding Fas L and inhibiting apoptosis in those patients (Hasunuma et al., 1996).

The significant increase in the % of apoptotic cells in SF of RA compared to OA after *in vitro* induction of apoptosis by anti-Fas mAb in our work could be explained according to the finding of Hoa et al. (1996) who showed that RA synovial stromal cells, but not PBMCs, were highly susceptible to anti-Fas mAb and underwent apoptosis, while in OA patients these cells did not undergo apoptosis. Similarly, Nakajima et al. (1995) go in agreement with our data as they determined the presence of Fas antigen on synovial RA cells. The DNA ladder formation which is characteristic to apoptosis is detected only in samples from patients with RA not with OA.

The positive correlation between apoptosis levels and % CD $_4^+$ T-cell after addition of anti-Fas mAb in SF of patient with RA indicated that CD $_4^+$ T-cells are the main cells in the SF of RA patients. These cells expressed functional Fas antigen and have the susceptibility to anti-Fas mAb. On the other hand, the negative correlation between apoptosis levels before addition of anti-Fas mAb and % viability both before and after apoptosis induction (as apoptosis level increases, the viability % of SFMCs decreases), confirm our previously mentioned data about % cell viability and % CD $_4^+$ T-cell in RA patient. Using local anti-Fas mAb in the treatment of RA has been suggested by many workers: Ogawa et al. (2001) revealed that administration of anti-Fas mAb for CDIF1 mice may be a useful therapeutic strategy for RA. Sedrak et al. (1999) reported that factors governing apoptosis in RA synovium as the local release of cytokines, the liberation of free oxygen radicales through hypoxia and reperfusion are not manifested in the peripheral blood, but are restricted to the joints. Nishioka et al. (1998) demonstrated that various Fas expressing cells such as hepatocytes and stem cells may undergo apoptosis by systemic administration of anti-Fas mAb. Motsuno et al. (2002) denoted that the administration of the humanized anti-Fas mAb may provide a new treatment for RA by inducing Fas-mediated apoptosis in inflammatory cells.

In conclusion, activated T cells infiltrating the SF of RA patients have functional Fas antigen and can be induced to undergo apoptosis *in vitro* using anti-Fas monoclonal antibody, the cytotoxicity of which is more specific to local lesions such as SF of RA patients. So, local administration of anti-Fas mAb may serve as an effective tool for the treatment of RA. However, the clinical safety and usefulness of this approach has to be more proven.

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