類風濕關節炎之細胞有秩序性死亡

— 探討滑膜組織內 Fas, Fas L, p53 與 Bcl-2 之表現

APOPTOSIS IN RHEUMATOID ARTHRITIS -EXPRESSION OF Fas, Fas L, P53 AND Bcl-2 IN RHEUMATOID SYNOVIAL TISSUES

計畫編號：NSC88-2314-B-075-096
執行期限：87 年 8 月 1 日至 88 年 7 月 31 日

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中文摘要

最近研究顯示細胞有秩序死亡(Apoptosis)是類風濕關節炎的重要病變機轉。本篇研究,使用單一及雙細胞組織免疫染色法，我們去探討類風濕關節炎(RA)，退化性關節炎(OA)及外傷後關節病變(PT)之 Fas, Fas L, p53 與 Bcl-2 與 Apoptosis 有關蛋白之表現。而 Apoptotic (死亡) 之細胞則以 TUNEL 染色法去偵測。結果顯示在 RA 患者比 OA 與 PT 患者滑膜內皆有明顯之 Fas, Fas L 及 p53 表現，然 Bcl-2 在上述三種病變卻無明顯差異性。RA 患者, Fas 及 p53 在滑膜上皮層下方表現為主，且以類似纖維樣(Fibroblast)滑膜細胞表現居多。 另外, p53 與 Fas 在組織內表現二者具有明顯相關性。RA 患者, 1/3 Fas 陽性與 80% p53 陽性者細胞, TUNEL 亦顯示陽性。上述結果顯示 Apoptosis 在類風濕關節炎與 Fas 及 p53 有明顯相關而與 Bcl 2 無關。

關鍵詞：類風濕關節炎，退化性關節炎，滑膜組織，細胞有秩序死亡，免疫組織化學染色法

ABSTRACT

Recent studies have suggested that apoptosis is one of the pathogenetic mechanisms in rheumatoid arthritis (RA).

In this study, by using single and double immunohistochemical staining assays, we measured Fas, Fas L, p53 and Bcl-2 simultaneously in RA and osteoarthritic (OA) and post-traumatic (PT) synoviual tissues (ST) in order to understand the distribution of those apoptosis-related proteins. The TdT-mediated dUTP-biotin nick end labeling (TUNEL) method was performed to detect apoptotic cells. Our results demonstrated a significant increase of Fas, Fas L, and p53 in RA ST compared with that in OA or PT ST. However, no significant difference of Bcl-2 expression was detected between patient groups. In RA ST, expression of Fas and P53 was detected in

sub-lining layer and the majority of Fas and P53 expressing cells were fibroblast-like synoviocytes. The positive correlation between Fas and P53 was demonstrated in RA ST. In RA ST, one third of Fas-positive and 80% of p53-positive cells were also TUNEL-positive. These results indicate that apoptosis in RA is highly associated with the presence of Fas and p53 but not Bcl-2.

KEY WORDS : rheumatoid arthritis; osteoarthritis; synovial tissue; apoptosis; immunohistochemical stain

INTRODUCTION

Apoptosis, or programmed cell death (PCD), is an important biological function in the human body to maintain homeostasis[1,2]. The PCD is entirely different from necrosis, a pathologic cell death usually caused by tissue hypoxia and the infectious process. Apoptotic cell death is thought to be involved in the negative regulation of immune autoreactive T cells during the differentiation and maturation in the thymus[3]. The Fas antigen (CD95) is a well-known mediator to initiate apoptosis. Binding of Fas and Fas ligand (Fas L) on the cell surface can induce apoptosis[1]. In animal studies, lpr mice express little Fas antigen mRNA immunoreactive cells, which lead to a decreased Fas-mediated apoptosis and immune diseases[3-5]. Bcl-2 is a proto-oncogene that inhibits apoptosis of different cell types[6-8]. P53, a tumor suppressor gene, is a nuclear phosphoprotein, which serves as a critical cell survival and proliferation[9-11]. The overexpression of p53 usually indicates that cells had damage and DNA repair.

Rheumatoid arthritis (RA) is a chronic inflammatory joint disease characterized by synovial proliferation and abnormal immune responses, subsequently causing erosion of the affected joint. Marked infiltration of lymphocytes, macrophages, and plasma cells is observed in the synovial tissue of RA[12]. Apoptosis has been observed in rheumatoid synovium using electron microscope and the terminal deoxynucleotidyl transferase-mediated dUTP–biotin nick end labeling (TUNEL) method to detect DNA fragmentation[10,13-15]. Apoptotic cells are mainly fibroblast-like synoviocytes in RA[14,16]. Immunohistochemical studies on rheumatoid synovial membrane have demonstrated that Fas and Fas L express on different cell populations either on fibroblast-like synoviocytes or infiltrated T lymphocytes, or co-express on some mononuclear cells which then develop the apoptosis through the binding of Fas and Fas

L. Firestein et al[14] suggested that the increased proliferation of lymphocytes in the rheumatoid synovium were probably caused by a special synovial microenvironment that favor cell infiltration and accumulation. The defect of Fas gene or the increased Bcl-2 may protect the infiltrated inflammatory cells from apoptosis[7,14]. The expression of p53 was found more frequently in RA than in osteoarthritic (OA) synoviocytes[10]. Both Fas and p53 are involved in the apoptotic pathway, but double staining of both markers in rheumatoid synovium has not been done.

In this study, for further identification of apoptotic-related proteins appearing in rheumatoid or osteoarthritic synovial cells which may or may not undergo apoptosis, we used single and double immunohistochemical staining and the TUNEL method to analyze the *in situ* expression of Fas, Fas L, p53 and Bcl-2 in RA, OA and post-traumatic (PT) synovial tissue (ST). The percentage of each apoptotic protein in cells of different synovial layers was calculated. Moreover, this study will focus on the co-expression of Fas and p53 antigen in RA synovial cells.

MATERIALS AND METHODS

Patients and samples

Synovial tissue was obtained from knee or hip joints of eight patients with rheumatoid arthritis[17] and eight with osteoarthritis[18] and two post-traumatic patients (Table 1). All the synovial pieces were immediately frozen at -70^0 C until biopsy. Mononuclear cells were isolated from synovial fluid (SF) of two RA patients. SF cells were concentrated on the slides after cytospin centrifugation and then treated with 95% alcohol. After drying, slides were stored at -20^0C. Fibroblast-like synoviocytes were isolated from synovium of two RA, two OA and one post-traumatic patients.

Immunohistochemical staining[7,10,14,15]

For immunohistochemical staining, ST samples were cut into 5 μm slices by frozen section. The expression of Fas, Fas L, p53 and Bcl-2 was examined by immunohistochemical staining assay using anti-Fas mAb (Pharmagen), anti-Fas L mAb (Pharmagen), anti-p53 mAb (Oncogen) and anti-Bcl-2 mAb (Dako). Negative control studies were performed for each experiment by isotype control mouse IgG1 mAb. Fas, Fas L, p53 and Bcl-2 was detected using an avidin-biotin-peroxidase complex (ABC) kit (Signet), and diaminobenzidin (DAB) was used as a chromogen. For immunodouble staining, DAB and aminoethyl-carbazole (AEC) (Zymed) were used as chromogens.

Apoptosis detection by TdT-mediated dUTP-biotin nick end labeling

(TUNEL) method

Apoptosis was detected using the TUNEL method *in situ* apoptosis detection kit (Oncor)[15]. Briefly, synovial tissue samples were fixed in 4% buffered formaldehyde and embedded in paraffin. The paraffin sections were treated with xyline for five minutes in slides and then subsequently left in 100%, 95% or 75% EtOH, respectively. The deparaffinized synovial tissue samples were incubated with proteinase K (2mg/ml) at room temperature for 15 minutes. After PBS washing, endogenous peroxidase was blocked by the addition of 3% of H_2O_2. It was then treated with terminal deoxynucleotidyl transferase and biotinylated dUTP. After stopping reaction by TB buffer (30mM sodium chloride, 30 mM sodium citrate), the samples were stained with DAB. Hematoxylin and eosin counterstain was also done
in order to detect cell types that were positive by nick end labeling.

Microscopic observation

The expression of Fas, Fas L, Bcl-2 and P53 antigen of each positive cell was randomly analyzed and the total number of cells was estimated by comparing the same sections counterstained with Hematoxylin stain. In this study, the synovial membrane was divided into 4 parts; the inner layer was lining layer (LL); the sub-lining layer (SL) which includes the area just below the LL but not to the deeper area; the perivascular (P) area; and the central (C) area which involves more deeper area of SL (Fig. 1). The percentage of positive cells relative to the total cells was calculated for each sample, counting at least 80 and up to 200 cells.

Statistical analysis

One-way ANOVA with Tukey pairwise comparison was used for comparing antigen expression among RA, OA, and PT tissue section. Repeated measurement ANOVA with Tukey pairwise comparison was used for comparing antigen expression among different areas in rheumatic disease tissue section.

RESULTS

Within one year, 18 patients were involved in this study. The demographic data of all patients are shown in Table I. Among them, eight are RA, eight OA, and two PT arthropathy. Thirteen patients were female and five were male. Age ranged from 23 to 74 and tended to be older in OA (66.5 in OA and 41 in RA). Synovial biopsy tissue samples were obtained from 11 knees and

seven hips. The disease duration ranged from two months to 11 years. All the RA patients had positive rheumatoid factor (RF) and increased sedimentation rate (39-122 mm/hr). Non-steroid antiinflammatory drugs (NSAIDs) were used in all patients, and disease-modifying antirheumatic drugs (DMARDs) were administered in all RA patients, except case No. five and seven.

Detection of synovial cells apoptosis by TUNEL method

In RA synovial membranes, on average, 27% of cells were undergoing apoptosis evidenced by brown color staining (Fig. 2). The apoptotic cells were detected mainly in the SL layer. The predominant apoptotic cells were mononuclear cells and few were fibroblasts. For OA and PT, less than 1% of the cells were showing apoptosis, which was significantly lower when compared to RA ($p<0.001$).

Fas, Fas L antigen expression in RA and OA synovial membranes

A significant increase of Fas antigen was found in RA compared with OA and PT samples (17.62% *vs* 3.14% *vs* 2.46%, respectively, $p<0.001$) (Tables II). Cells which expressed Fas antigen-positive were mainly fibroblast-like cells and mainly detected in the SL layer (Fig. 3). For Fas L, similar findings to Fas antigen were presented (Tables III). The co-expression of both Fas and Fas L was examined using double immunohistochemical staining. In this study, a small number of cells (less than 5%) in RA ST can co-express both. A few cells expressing Fas were found in contact with other cells carrying Fas L.

The correlation of Fas antigen and apoptosis

To correlate the presence of Fas antigen and apoptosis, synovial tissue samples were examined after immunohistochemical stain and TUNEL double staining. In RA synovial membranes, approximately 1/3 of cells presented Fas and apoptosis simultaneously (Fig. 4).

Expression of Bcl-2 antigen in RA and OA synovial tissues

Bcl-2, the antiapoptotic antigen, was detected almost equally in RA, OA and PT ST (26.99%, 24.26%, 21.08%, $p>0.05\%$) (Table IV). The majority of those cells with positive Bcl-2 were mainly infiltrated mononuclear cells located in perivascular areas.

Expression of p53 antigen in RA and OA synovial tissues

Like Fas antigen, 20% of LL and SL cells presented p53 antigen. The majority of positive staining cells were located in the SL layer and they were apparently fibroblast-like cells but few of them were infiltrating mononuclear cells. OA and PT samples had less p53 antigen when compared to RA (1.81, 2.75 *vs* 20.09%, $p<0.001$) (Table V).

Fas and p53 co-expression in RA synovial tissues and correlation with apoptosis

To understand whether the cells with p53 antigen underwent apoptosis, the TUNEL method was used. It is apparent that 80% of p53-positive cells presented were undergoing apoptosis. Regarding the co-expression between Fas, Fas L, Bcl2 and p53, the double immunohistochemical stain showed that a strong correlation between Fas and p53 was demonstrated ($r=0.926$, $p<0.001$) (Table VI).

Expression of Fas, Fas L, p53 and Bcl-2 antigen in RA synovial fluid mononuclear cells

Except for p53, the majority of SF mononuclear cells presented Fas, Fas L and Bcl-2 antigen.

DISCUSSION

RA is pathologically characterized by synovial lining cell hyperplasia and inflammatory cell infiltration. The proliferation or hyperplasia of synovial lining cells is not endless, and the inhibition or proliferation can be regulated by the apoptotic mechanism. The defect of apoptosis can lead to abnormal cell proliferation and infiltration. Synovial tissues from RA patients in this study showed 30% cells (mainly synovial fibroblast-like cells and less mononuclear cells) undergoing apoptosis, which was confirmed by the TUNEL method. The findings were entirely different from either OA or PT. Our results have no significant difference as reported by Matsumoto[19] and Ashara et al[20].

The immunohistochemical stain demonstrated the increased Fas and Fas L expression in RA ST compared with OA or PT. However, Fas was mainly presented on synovial fibroblast-like cells and Fas L on infiltrated mononuclear cells. In our RA patients, the location of Fas and Fas L was apparent in the sub-lining layer which is closed to the findings reported by Hasunuma et al[21] but different from Matsumoto[19] and Ashara et al[20] who found that the lining layer was the main location to present Fas and Fas L. Upon further examination of RA ST with double staining, some Fas L positive fibroblast-like cells in close adjacent to Fas L on some mononuclear cells were observed. The apoptosis of T cells or other cells in RA ST is achieved after binding of Fas and Fas L with T-fibroblast or T-T cell pairs. This corresponds to the findings that 30% of our RA LL and SL cells had apoptosis. Nozawa[22] also confirmed that Fas L on synovial T cells in RA SF could bind Fas on fibroblast-like cells to develop apoptosis.

The study of Fas antigen and apoptosis in RA patients in this study could not convince us that Fas-Fas L interaction is the only way to develop apoptosis, because only 1/3 of Fas-positive cells presented apoptosis and besides, some cells had apoptosis but were Fas antigen-negative. Other routes through p53 or TNF α also develop apoptosis[1,13]. Another possibility is the

increased Bcl-2 antigen in RA synovium which may inhibit apoptosis[14,19]. However, two histologic findings in this study could not convince us of that. One was that the percentage of Bcl-2 appearing in synovial cells did not show a significant difference between RA and OA, OA and PT, RA and PT. Another disapproved point was the location of Bcl-2, which was mainly expressed in lymphocytes and vascular endothelial cells, a similar result to the one reported by Isomaki et al[7], but in contrast to the Fas antigen, which was mainly expressed in fibroblast-like cells. Therefore, the anti-apoptotic protein, Bcl-2, may not play an important role in promoting synovial fibroblast proliferation in RA. Nozawa et al[22] found the increased soluble Fas in patients with systemic lupus erythematosus may decrease the apoptosis through the interference of Fas and Fas L binding. But there was no clear evidence that soluble Fas which was produced locally in RA synovium inhibited apoptoisis. Another possibility was raised by the Salmon et al[23] that an integrin-ligand interaction incorporating the Arg-Gly-Asp motif involved in fibroblast-mediated synovial T cell survival. In this study, the presence of anti-Fas antibody did not accelerate apoptosis in cultured RA synovial fibroblast-like cells (unpublished result) and the cause is unknown yet.

The relationship between RA and tumor-associated antigen, including c-myc, c-fos and p53, has been studied by many investigators[10,24,25]. Among those, p53 is the main focus of study in recent years because its presence in RA or OA synovium usually indicates tissue damage, DNA repair and synovial cell survival[10]. The finding of higher p53 expression in RA than in OA or PT in the present study does not differ from previous reports[10,14]. In the local inflammatory joint, the increased nitric oxide products may contribute to the synovial cell DNA damage and then the overexpression of p53 marker [26].

In RA, p53 point mutation has been found by Firestein *et al*[27]. The synvoial cell proliferation was probably a result of p53 gene mutation. We have extracted DNA from cultured synovial fibroblasts of three RA and used PCR-SSCP and DNA sequencing to measure p53 Exon 4 and Exon 5. The mutation of p53 in our RA patients has not been demonstrated (unpublished result). This differs from the findings presented by Firestein[27], perhaps due to the restricted numbers of our RA patients.

No matter what the location and frequency of antigen presentation in RA synovium, p53 and Fas had very similar findings during the immunohistochemical examination. In this study, positive correlation between Fas and p53 was found, and this has not been reported before. For apoptosis, the significance of co-expression of Fas and p53 in RA synovial cells should be identified by future experiments.

Synovial fluid mononuclear cells from our RA patients expressed Fas, Fas L and Bcl-2 antigen. Over 90% of cells were $CD45RO^{+}Fas^{+}$ [23]. The presence of large amounts of cytokines, particularly IL1 and

TNF α in RA synovial fluid, enhance the Fas antigen expression in RA synovial fluid cells more than in synovial tissue cells.

In summary, the defect of apoptosis in RA synovial cells, including synovial lining cells, fibroblast-like cells, macrophages, and infiltrating lymphocytes, may develop the sustained inflammation in RA. In this study, apoptosis in RA is highly associated with the presence of Fas and p53 bot not Bcl-2.

ACKNOWLEDGEMENTS

Supported by grants from the National Science Council (NSC 88-2314-B-075-096).

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Table I —The demographic data of 18 patients

Patient	Gender	Age (yr)	Involved joint	Disease duration (yr)	RF IU/ml	ESR mm/hr	Current Therapy
RA 1	M	23	Knee	3	200	122	NSAID; Steroid; Cyclosporin
RA 2	F	42	Knee	7	116	85	NSAID; Steroid; M; H
RA 3	F	37	Hip	10	95.8	39	NSAID; Steroid; M; H
RA 4	M	23	Hip	3	200	122	NSAID; Steroid; Cyclosporin
RA 5	F	48	Knee	4	372	92	NSAID; Steroid
RA 6	M	68	Knee	7	218	100	NSAID; Steroid; H
RA 7	F	48	Knee	2 months	120	95	NSAID
RA 8	F	59	Knee	3	130	55	NSAID; Steroid; Cyclosporin
OA 1	F	70	Knee	1	ND	ND	NSAID
OA 2	F	65	Hip	1	ND	ND	NSAID
OA 3	F	70	Knee	5	ND	ND	NSAID
OA 4	F	73	Knee	2	ND	ND	NSAID
OA 5	M	69	Hip	5	ND	ND	NSAID
OA 6	F	59	Hip	5	ND	ND	NSAID
OA 7	F	52	Hip	11	ND	ND	NSAID
OA 8	F	74	Knee	1	ND	ND	NSAID
PA 1	F	31	Knee	6 months	ND	ND	NSAID
PT 2	M	53	Hip	2 months	ND	ND	NSAID

RA : Rheumatoid arthritis
OA : Osteoarthritis
PT : post-traumatic
NSAID : Non-steroid antiinflammatory drugs
RF : Rheumatoid factor
ESR : Erythrocyte sedimentation rate
ND = Not determined
M : Methotrexate
H : Hydroxychloroquine

Table II—Fas expression (%) in different synovial layers : comparison among RA, OA and PT patients.

Layer	RA (n = 8)	OA (n = 8)	PT (n = 2)	F value	Pairwise comparison
LL	4.38 ± 2.07	2.16 ± 1.75	0.01 ± 0.00	5.63*	RA>PT
SL	41.91 ± 10.74	5.30 ± 3.11	3.95 ± 3.74	51.03**	RA>OA; RA>PT
Perivascular	1.79 ± 1.82	2.70 ± 1.23	1.20 ± 0.85	85.51**	RA>OA; RA>PT
Central	12.38 ± 8.29	2.38 ± 0.94	4.70 ± 5.09	6.02*	RA>OA
Total	17.62 ± 4.53	3.14 ± 0.93	2.46 ± 1.99	46.35**	RA>OA; RA>PT

LL : Linging layer SL : Sublining layer

* : $p < 0.05$; ** : $p < 0.001$

Table III —Fas L expression (%) in different synovial layers : comparison among RA, OA and PT patients.

Layer	RA (n = 8)	OA (n = 8)	PT (n = 2)	F value	Pairwise comparison
LL	4.38 ± 2.38	1.46 ± 0.57	2.75 ± 1.77	5.63 *	RA > OA
SL	30.25 ± 3.80	3.88 ± 1.80	4.50 ± 2.12	178.26 **	RA > OA ; RA > PT
Perivascular	11.25 ± 3.92	1.88 ± 1.80	0.00 ± 0.00	24.63 **	RA > OA ; RA > PT
Central	11.12 ± 1.25	1.50 ± 1.31	3.00 ± 1.41	117.61 **	RA > OA ; RA > PT
Total	14.25 ± 1.75	2.18 ± 1.08	2.56 ± 1.33	154.31**	RA > OA ; RA > PT

LL : Lining layer SL : Sub-lining layer

* : $p < 0.05$; ** : $p < 0.01$

Table IV —Bcl-2 expression (%) in different synovial layers : comparison among RA, OA and PT patients.

Layer	RA (n = 8)	OA (n = 8)	PT (n = 2)	F value
LL	4.41 ± 1.14	2.46 ± 2.31	1.35 ± 0.49	3.68
SL	44.03 ± 7.31	38.54 ± 5.09	32.80 ± 12.45	2.63
Perivascular	42.25 ± 9.81	43.38 ± 12.33	40.00 ± 14.14	0.07
Central	17.25 ± 5.11	12.65 ± 4.12	11.00 ± 4.24	2.63
Total	26.99 ± 4.64	24.26 ± 3.74	21.08 ± 7.58	1.55

LL : Lining layer SL : Sub-lining layer

Table V—p53 expression (%) in different synovial layers : comparison among RA, OA and PT patients.

Layer	RA (n = 8)	OA (n = 8)	PT (n = 2)	F value	Pairwise comparison
LL	16.63 ± 3.62	1.63 ± 2.38	1.00 ± 1.41	57.05 *	RA > OA; RA>PT
SL	40.12 ± 9.42	2.50 ± 2.20	4.00 ± 1.40	0.74 *	RA > OA; RA >PT
Perivascular	11.00 ± 2.62	2.00 ± 2.33	3.00 ± 2.83	27.59 *	RA > OA; RA > PT
Central	12.63.± 3.74	1.13 ± 1.25	3.00 ± 1.41	37.63 *	RA > OA; RA > PT
Total	20.09 ± 3.26	1.81 ± 1.75	2.75 ± 0.35	111.54 *	RA > OA; RA > PT

LL : Lining layer SL : Sub-lining layer

* : $p < 0.001$

Table VI — The correlation between different antigens expression in eight rheumatoid arthritis synovial tissues .

	Fas positive cells (%)	Fas L positive cells (%)	p53 positive cells (%)
Bcl-2 positive cells (%)	0.629	0.469	0.694
Fas positive cells (%)	1	0.690	0.926*
Fas L positive cells (%)	0.690	1	0.588

* : $p < 0.001$

Figure Legend

Fig. 1. Synovial membrane was divided into 4 parts; lining layer (LL), sub-lining (SL), perivascular (P) and central (C) area.

Fig. 2. Detection of synovial cells apoptosis by TUNEL method. Apoptotic cells were stained with brown color.

Fig. 3. Cells, which expressed Fas antigen, were mainly fibroblast-like synoviocytes.

Fig. 4. Double staining of synovial tissues to detect cells positive for Fas antigen and apoptosis. Cells stained with blue were TUNEL + Fas-positive (arrow) and red were Fas antigen-positive (arrow head).