The Characteristics of Synovial Fluid from Patients with Rheumatoid Arthritis–I

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Abstract **Objective** To explore the mechanism of the Rheumatoid arthritis mediated by autoreactive T lymphocytes infiltrated in the synovial fluid of patients with rheumatoid arthritis. **Methods** Samples from 22 patients were studied in this report. The phenotype of the lymphocytes infiltrated in the synovial fluid was detected by immune-fluorscence using flow cytometer. The reactive frequency of human Collagen II specific T cells existed in the synovial fluid of rheumatoid arthritis patients was analyzed using a standard split-well method. The cytokine profile was determined by ELISA. **Results** Majority of the T cell subsets infiltrated in the synovial were demonstrated as CD4 and CD8 positive cells, in which the percent of the CD4 positive was 39.6±10.5% and that of CD8 T cells was 36.4± 16.4% respectively. The ratio that CD4/CD8 was much lower than that in the peripheral blood of the RA patients. Majority of them used αβ TCR (69.6±25.7%). There was also a small popular of activated NK with CD16 and CD56 positive (15.5±11.5%). The frequency reactive of the Collagen II specific T cells in synovial fluid was 12.5×10^{-6} while the Collagen II specific T cells in the PBL from same patients showed little lower at the range of 4×10^{-6}. The concentration of IL-12 in the synovium fluid was very high (419.9 ±89.2 pg/ml) and IL-10 was relative lower (187.7±34.5pg/ml). In contrast, the level of both cytokine in the serum from same patients were very low, in which the IL-12 was 65.32±34.2 pg/ml and IL-10 showed 85 ±12.7 pg/ml respectively. **Conclusion** The data indicated that both lymphocyte infiltrated and cytokines involved the lesion of the joint tissues in the RA patients.

Key Words Rheumatoid arthritis; Synovial fluid; Collagen II; Cytokine profile; T Lymphocytes

Rheumatoid arthritis(RA) is a common disease characterized by the chronic lesion of polyarthritides. The etiology and pathogenesis of RA remain unknown. Autoimmunity to cartilage antigens may play a significant role in the pathogenesis of chronic inflammatory polyarthritis. It is commonly accepted that cell mediated immune responses are involved in chronic inflammation since T and B lymphocytes and antigen presenting cells were observed to be enriched in the synovial fluid of RA patients[1]. In vivo studies showed that T cells infiltrating into the synovial fluid expressed IL-2 receptor, IL-10 and IFN-γ[2] and activated CD4 T cells could be detected in the peripheral blood (PBL) of RA patients. The candidate autoantigens may include type II, IX, and XI collagens, human cartilage glycoprotein-39 (HC-gp-39) and proteoglycan aggrecan (PG), among which special attention has been paid for the type II collagen (CII) because there are many reports describing that CII may be target of auto-reactive T cells in the pathogenesis of RA in Caucasian. In this study, we reported the characteristics of CII specific autoreactive T cells which infiltrated into synovium fluid of RA patients by analysis the phenotype and frequency of human Collagen II specificity of these infiltrated T lymphocytes and the level of IL-10 and IL-12 of the synovium fluid. The pathogenic role of the infiltrated T cells and mechanism they involved in the RA were investigated in this study.

MATERIAL AND METHODS

Patients

Twenty Chinese patients with RA, whose ages range from 41 to 69 years old (median 54.0) were studied. Each of them was diagnosed according to the criteria from American College of Rheumatology. All blood and synovial fluid samples from these patients were collected by Shanghai Guanghua Hospital.

Peripheral blood mononuclear cells

The peripheral blood mononuclear cells (PBMC) of patients were obtained by gradient centrifugation on Ficoll-Hypaque (Second chemic factory, Shanghai) at 2500rpm for 20 min, washed twice in RPMI 1640, and counted and subjected to determine the specificity to the CII (for setting up T cell lines, described as below) and phenotype.

Synovial fluid mononuclear cells

Synovial fluids were spin at 2000 rpm for 10 min. The supernatant were collected for cytokine profile detection. The pellet were re-suspended in RPMI 1640 and loaded on Ficoll-Hypaque. After centrifugation at 2500 rpm for 20 min, the synovial fluid mononuclear cells (SFMC) were obtained and counted and subjected to determine the specificity to the CII (for setting up T cell lines, described as below) and phenotype.

Type II collagen frequency of SFMC

SFMC collected were adjusted to 1×10^6/ml and 100 ul of the cell suspension were added into each well in round-bottomed microtiter plate and co-cultured in RPMI 1640 medium supplemented with 8% fresh heat-inactivated autologous serum in presence of CII (40ug/ml). After 7d in culture, the supernatants were removed completely and 200 ul of fresh medium with human AB serum were added into the cells. After mixed well, the T cells were split into 2 wells. 100ul of fresh APC (PBMC irradiated by 3000 rad) containing 1×10^5 APC were added into the above wells respectively. The cells which included T cells and APC were co-cultured with CII at the final concentration as 40 ug/ml. After 48 h, the cells were labeled with ^{3}H-TdR (Shanghai Institute of Nuclear, 0.2uCi/well) and harvested 16h later with a Skatron Multichannel Cellular Harvester (Norway, PO, Box-3401 LIER). Radioactivity was quantified in a β−scintillation counter. The proliferation was expressed by cpm±SD. Proliferation of CII specific T cells were expressed by stimulation index (SI=cpm with CⅡ / cpm with medium). The CII positive T cells were picked up upon the SI>2, cpm>1500 counts. The frequency of CII positive T cells was assessed by the formula: number of positive well×cell number per well (1×10^5) / total cell number (60×10^5).

The cytokine profile of synovium

The cytokine profile was analyzed quantitatively using Quantikine ELISA Kits (Quantiline™, Minneapolis, MN). In brief, 200 ul of SF and serum coming from same patient were added to the individual wells of flat-bottomed microtiter plates precoated with anti-IL-10 anti-IL-12 antibodies. The plates were aspirated and washed three times with PBS containing 0.1% (v/v) Tween 20 after incubating at room temperature for 2h. 200ul of polyclonal antibody against horse radish peroxidase (HRP) were added to the well and incubated for another 2h. The plates were washed again with wash buffer three times. 200 ul of substrate (DAB) were added to the wells and incubated for 20 min at room temperature and then 50ul of stop solution were added to each well. The concentration of cytokines were determined by reading at 450 nm within 30 min automatically with ELISA reader (Elx-808; Bio-Tek Instruments, Inc., Winooski, VT).

Phenotype and T cell receptor analysis

5×10^5 T cells were labeled by a mixture of monoclonal antibodies against human CD3 (FITC-CD3) and CD4 (PE-CD4) and CD8 (PE-CD8), CD56 (FITC-CD56), αβ(PE-αβ), γδ (PE-γδ) and Ig isotype matched FITC or PE-conjugated antibodies as negative controls. The 2 color fluorescence were determined by a Beckman Cytometer and the data were analyzed by System II software. The results are expressed as percentage of positive cells. All of the antibody used in this report were products of B. D. Pharmingen of U.S.A.

RESULTS

CD8 T cells were found increase in the SF of patients with RA

The SFMC were detected by cytometer. The results revealed that the there were rich of T cell in SF. CD8 T cells were enriched in T cells from SFMC. The percentage of CD4 T cells was 39.6% while CD8 T cells reached to 36.4%. The ratio of CD4 versus CD8 closed to 1 which was much lower than that of in PMBC ($p<0.05$). The T cells infiltrated into SF mainly used αβ-TCR with average reaching to 69.6%. Some activated NK were found in SFMC with percentage of 15.5±11.1% (Table 1).

Increasing frequency of CII specific autoreactive T cells in the SF of patients with RA

The SFMC from RA patients were stimulated with CII in vitro and the frequency of the specific auto-reactive T cells were assayed. The PBMC from same patients were treated as same way as control. The results showed that both frequency and number of positive clones were higher in SFMC of patients with RA (Table 2). The frequency of special CII atuo-reactive T cell was 12.5×10^{-6} which was much higher than that of PBMC (only 4×10^{-6}) of same patients with statistics significance ($p<0.05$) also.

The higher level of IL-12 in SF of patients with RA

The cytokine concentration of IL-10 and IL-12 in SF was determined by ELISA. The level of these two cytokine in serum of same patients was detected also as

Table 1 The phenotype of monocytes from PBL and synovial fluid (%)

Phenotype	CD3	CD4	CD8	CD16+56	TCRαβ	TCRγδ
PBMC	49.5±20.2	42.6±12.0	21.0±10.0	15.9±9.6	41.0±11.3	6.9±7.9
SFMC	71.8±21.9	39.6±20.5	36.4±16.4	11.1±15.5	69.6±25.7	3.8±2.2

Table 2 The frequency of the Collagen II reaction T cells in PBL and synovial fluid

sample	case	percentage of positive clones	CII specific frequency
SFMC	22	20	12.5×10^{-6}
PBMC	22	6	4.0×10^{-6}

control. The results indicated that the level of IL-12 in SF was very high which can reach to 419.9±89.2 pg/ml. While the level of IL-10 in SF was very low (187.7± 34.5pg/ml). The concentration of both IL-10 and IL-12 in serum was much lower than that in SF which were 65.32±34.2 pg/ml (IL-12) and 85±12.7 pg/ml (IL-10) respectively (Fig.1).

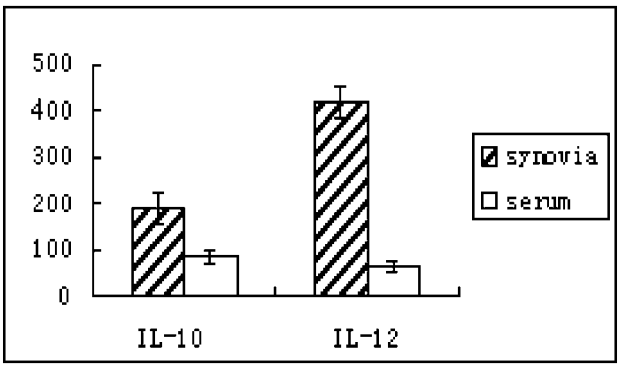

Fig.1 The concentration of IL-12 and IL-10 in the synovial fluid and serum

DISCUSSION

Rheumatoid arthritis is believed as a chronic autoimmune disease with characteristics of inflammatory and lesion of synovium. The type III hypersensitivity reaction in the joints synovia was though to be involved in the pathogenic process of RA. The mechanism which induced the lesion of the synovium included rheumatoid factor (RF) which is anti-auto IgGFc, Ig (IgM, IgG, and IgA) and immune complex which can precipitate on the synovia. These precipitation initiated the chemoattraction of macrophage and lymphocytes into synovia so that the inflammation reaction was raised in the synovia. This inflammation reaction caused hyperplasia, swollen and infiltration with lymphocytes and macrophage which at last induced lesion of synovium. But with development of molecular biology and immunology and setting up RA animal model in past decades, the cartilage specific auto-reactive T cells and producing cytokines were found more important involved in the pathogenesis of RA[2,3].

With exploring the self-antigen which involved in pathogenesis of RA in past decades, more than one self antigen were thought to be candidate self antigen in this auto-immune disease. The candidate autoantigens may include type II, IX, and XI collagens, human cartilage glycoprotein-39 (HC-gp-39) and proteoglycan aggrecan (PG), among which special attention has been paid for the G1 globular domain and link protein (LP) of PG[5]. Collagen II is mostly studied [4]. The therapy for RA based on tolerance induced by collagen II through oral taking up has been tried both in animal model and clinical patients[6]. To confirm the pathogenic role of collagen II in RA and develop T cell vaccine for RA therapy, we studied characteristics of T cells which infiltrated in synovial fluid in patients with RA in this report. The results indicated that there were rich of T cells infiltrated into SF of RA patients. T subsets in SF was different from that of peripheral blood. The ratio of CD8 was higher than that of in PBMC. The activated NK was found in SF also. This revealed that large number of CD8 and activated NK may triggered stronger autoimmune response by CTL in synovium and enhanced the pathogenic lesion of joint synovium. The higher frequency of specific collagen II T cell isolated from SF demonstrated that there were more auto-reactive T cells in SF. This means that there was collagen II auto-reactive T cell clonal expansion after these T cells infiltrated into synovium. Continually, these auto-reactive T cells recognized the collagen II on synovium and cartilage and started to immune response to these tissue which induced immune pathogenesis and lesion on cartilage and

synovium. According to this mechanism, the frequency may be a useful reference for identifying RA with other arthritis in diagnosis.

It was known that there are two types of TCR, αβ TCR and γδ TCR. In the healthy individuals, 95% of T cells express αβ TCR and only 5 % of the T cells with γδ TCR in the peripheral blood lymphocytes. Usually, most of the γδ T cells distribute under mucosa of intestine and kill the bacteria lived in the cells. They bloom when individuals fall sick or are invaded by pathogenic organisms [7]. It is regarded that γδ T cells play a very important role in the immune regulation. In this report, we showed that most infiltrated T cells in SF of patients with RA were αβ T cells analyzed by monoclonal antibody. Only less 10% of T cells in SF gave γδ T cell phenotype. The role of these αβ and γδ T cells in the pathoarthritis is still under investigation.

According to the cytokine profiles, T cells are divided into two subsets: Th1 and Th2. It is regarded that secreting IFN-γ is a marker of Th1 but IL-4 or IL-5 or IL-10 and IL-13 is an obvious marker of Th2[8,9]. Previous work has showed that in RA patients, the function of Th1 cells was enhanced so that there are higher concentrated Th1 cytokines, such as IFN-γ and TNF et al, in RA patients, especially in synovium and synovial fluid. By contrast, Th2 cells were weak and less Th2 cytokines were concentrated in RA patients. Th1 cytokines induced more Th1 cells migration into synovium and triggered more strong autoimmune response in joint synovium. From the immune reaction in synovium, more chemokines were secreted and induced inflammatory cells chemoattratted to synovium which increased the inflammatory reaction in synovium. These feedback cycle will be continued and heaved lesion of synovium[10].

Recent reportes revealed that inflammatory cells which including macrophage, dendritic cells, B cells and activated T cells also produce IL-12. IL-12 is critical factor to induce proliferation of Th1 and NK. Higher concentrated of IL-12 may induce Th1 function increase so that speeding RA pathogenic and lesion in synovium and cartilage of joints[11]. With contrast by IL-12, IL-10 can suppress IL-12 function described above. We analyzed the profile of IL-12 and IL-10 in 22 RA samples with ELISA and coampared the their different profile with serum of same patients. This results indicated that there was 6 time higher level of IL-12 in SF of RA patients than that in serum. This mean that IL-12 could enhance Th1 cells of synovium and induce more strong inflammatory reaction in joint which increase lesion of joint. As inflammatory down regulation cytokine, IL-10 was much lower concentration than that of IL-12 even though IL-10 was higher in SF than that in serum of same patients. This demonstrated that there was imbalance among Th1 and Th2 cytokine in the SF of RA patients.

The results of this study revealed that there were higher amount of CD8 subset in SFMC compared with that of PMBC. The collagen II specific T cell frequency was higher in SFMC than that of in PBMC. IL-12 in SF was much more than that in serum. This indicated the Th1 was enhanced in SF and involved in the pathogenesis of RA. The mechanism how these collagen II specific Th1 cells induced or triggered RA is under investigation.

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