Downregulation of RCAS1 and Upregulation of Cytotoxic T Cells Affects Synovial Proliferation and Apoptosis in Rheumatoid Arthritis

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ABSTRACT. ***Objective.*** The main histological change in rheumatoid arthritis (RA) is the villous proliferation of synovial lining cells. This seems to be the result of the proliferation and apoptosis induced by immune balance. We studied the involvement of RCAS1 and the infiltration of cytotoxic T lymphocytes (CTL), and examined the synovium immunohistochemically to determine the involvement of proliferation and apoptosis in synovial lining cells, and their relationship with the activity of RA Treg cells in the germinal center.

Methods. We used double-immunological staining of Ki-67 and caspase-3 to investigate proliferation and apoptosis. We analyzed CTL, regulatory T cells (Treg), and receptor-binding cancer antigen expressed on SiSo cells (RCAS1), recently recognized to play a role in immune evasion. Proliferation and apoptosis were more frequently encountered in synovial lining cells in RA than in those in osteoarthritis (OA) that were used as a control.

Results. High expression of RCAS1 was detected more frequently in the synovial lining cells of OA, but CTL infiltration into the synovium was rarely found. In RA, on the other hand, CTL were observed, while RCAS1 expression was lacking. We compared the presence of Foxp3-positive cells with the level of C-reactive protein (CRP) that served as an active inflammatory marker. Foxp3-positive cells in the germinal center and in CRP showed possible correlation in terms of the range of inflammatory states.

Conclusion. In RA, the lack of RCAS1 is thought to induce CTL infiltration through loss of the ability to evade immune attack, thus leading to apoptosis of the synovial lining cells. In addition, Treg cells may play a role in the downregulation of activated T cells. (First Release Aug 1 2008; J Rheumatol 2008;35:1716–22)

Key Indexing Terms:

RCAS1 APOPTOSIS PROLIFERATION RHEUMATOID ARTHRITIS
CASPASE-3 Ki-67 Foxp3 SYNOVIUM

Rheumatoid arthritis (RA) is the most typical and severe type of chronic inflammatory autoimmune disease, which affects the joints and is accompanied by destruction of cartilage and bone. Infiltration of the synovium by inflammatory lymphoid cells and phagocytes produces various proinflammatory cytokines, resulting in synovitis[1-4]. Studies have focused on 2 major pathways that appear to lead to the development of RA[5,6]. The first involves activation of synovial stromal cells, with a progressive proliferative synovitis following the influx of various inflammatory cells. The second implicates intraarticular activated lymphocytes, mainly CD4+ T-cells, in the production of cytokines associated with villous proliferation of synoviocytes.

Disease progression of RA, which is characterized by villous proliferation of synoviocytes, mainly of synovial fibroblasts, results in bone and joint destruction. The spontaneous arrest of proliferation of synovial tissue is of particular interest, strongly suggesting the involvement of apoptosis in this process[7]. Villous proliferation involves both the proliferation and apoptosis of synovial lining cells[8], the latter being affected by various T-cell types, primarily cytotoxic T-lymphocytes (CTL) that infiltrate the synovial lining cell layer.

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CTL recognize antigen-derived peptides presented by MHC class 1 molecules with their T-cell receptors (TCR), whereas natural killer (NK) cells are triggered by cells lacking such molecules. Both CTL and NK cells play important roles in the immunocytotoxic system against virus infection and tumor cells[9]. CTL may use the effector mechanism to kill the synovium of RA, that is, TIA-1-associated cytotoxic granules (perforin and granzyme B)[10,11]. Studies have characterized synovial T-cells of patients with RA using CD4 and CD8 cell-surface markers, and found that the CD4/CD8 ratio varies within the joint, with CD4-positive T-cells generally predominant[12,13]. Expression of CD4, however, does not necessarily imply a "helper" function of these cells, since both CD8- and CD4-positive T-cells have been reported to exert cytolytic functions[14]. Using *in situ* hybridization, Griffiths, *et al* identified in the synovial fluid of RA patients both CD4- and CD8-positive T cells expressing perforin and granzyme, as functional markers of cytolytic cells[15].

Few studies have evaluated the role of T-regulatory (Treg) cells in RA. In addition, the relative numbers of CD4+CD25+ T-cells in the peripheral blood of patients with RA remain controversial. Most studies concur, however, that there is an increased number of Treg cells in RA synovium. Some studies have shown that the ability of CD4+CD25+ Treg cells from patients with RA to suppress production of tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) by CD4+ T-cells or monocytes is deficient, even though they can suppress the proliferation of T-effector cells[16].

Tumor-associated antigens and autoimmune disease can be recognized by the immune system. But tumor cells and some normal cells may evade immune attack by expressing FasL or other molecules that induce apoptosis of activated T-cells[17]. In this regard, a study by Nakashima, *et al*[18] describes RCAS1 (receptor-binding cancer antigen expressed on SiSo cells), which appears to act as a ligand for a putative receptor present on various human cells and normal peripheral lymphocytes such as T, B, and NK cells. Since RCAS1 was found to inhibit the *in vitro* growth of receptor-expressing cells, including tumor and normal cells, through apoptotic cell death, it could be involved in the escape of tumor and normal cells from immune attack and induced from immune surveillance[18].

Several reviews have reported an association between apoptosis and autoimmune diseases including RA, and have proposed that Fas-mediated apoptosis of RA synoviocytes is also important for an accurate understanding of the clinical course and pathogenesis of RA. In addition, a novel molecular pathway in the regulation of RA synovial tissue has been discovered[18].

To clarify the immune evasion in RA, we studied the involvement of RCAS1 and the infiltration of CTL. In addition, we examined the synovium immunohistochemically to determine the involvement of proliferation and apoptosis in synovial lining cells, and their relationship with the activity [C-reactive protein (CRP)] of RA Treg cells in the germinal center.

MATERIALS AND METHODS

Human synovium samples were obtained during total joint replacement surgery from 19 patients who met the American College of Rheumatology revised criteria for RA[19]. The control synovium samples were obtained from the knee and hip joints of 10 patients with radiologically diagnosed cases of osteoarthritis (OA) during total joint replacement surgery at Kurume University and Kurume University Medical Center. CRP concentrations were obtained from the patients' preoperative medical charts.

This study was approved by the Kurume University institutional review board, and patients provided informed consent in accord with the Declaration of Helsinki.

Immunohistochemistry. The synovium specimens were fixed in buffered formalin, embedded in paraffin, and then stained with hematoxylin-eosin. The immunohistochemical staining of TIA-1 was performed as the cytotoxic marker of CTL or NK cells (MD, Granada, Spain), of Foxp3 for Treg cells (Abcam, Cambridge, MA, USA), of CD3 for the total T-cell marker (Dakopatts, Copenhagen, Denmark), of CD56 for NK cells (T Cell Diagnostic, Cambridge, MA, USA), and of CD20 for B cells (L26; Dakocytomation, Glostrup, Denmark). For detection of RCAS1, the 22-1-1 hybridoma culture supernatants, diluted to 1:20 in phosphate buffered saline, were utilized as described[20]. Clinical data of all patients, including age, sex, period of onset, presenting symptoms and signs, temperature, and laboratory data, were obtained from medical records at the 2 institutions. Paraffin sections from the specimens were immunostained with 2 types of antibody combinations. The first consisted of monoclonal antibodies against CD3 plus polyclonal antibodies against caspase-3 (CD3+ caspase-3 prediluted double-stain antibodies; Biocare Medical, LLC, Concord, CA, USA). The second mixture consisted of monoclonal antibodies against Ki-67 plus polyclonal antibodies against caspase-3 (Ki-67+ caspase-3 prediluted double-stain antibodies; Biocare Medical). All immunohistochemical double-staining was performed in a single laboratory following the protocol provided by the manufacturer. Briefly, the first reaction products, which were brown, were produced by incubation with diaminobenzidine (DAB), while the subsequent red reaction products were developed with new fuchsin after an alkaline phosphatase reaction. For immunohistochemical analysis, we examined 100 synovial cells in 3 different parts of the synovial lining cell and counted the number of positive cells. To confirm that CD3-positive cells were cytotoxic, we analyzed the relationship between CD3-positive cells and caspase-3-positive cells that were in contact with CD3. Expression of RCAS1 was scored on a 4-point scale as follows: score 0: no staining is observed or is observed in < 10% of synovial lining cells; score 1: staining is detected in > 10% to < 40% of synovial lining cells; score 2: a weak to moderate, complete cell staining in > 40% to < 70% of the synovial lining cells; and score 3: strong, complete cell staining observed in > 70% of synovial lining cells. In addition, we investigated the relationship between RCAS1 and infiltration of CTL.

Statistical analysis. Clinical and pathological findings for different groups were compared with the Student t test and chi-square test. A p value < 0.05 denoted a statistically significant difference.

RESULTS

Proliferation and apoptosis from synovium with RA. Immunohistochemical analysis was performed to examine the expression and localization of the double-staining of Ki-67 and caspase-3 in synovial tissues derived from patients with either RA or OA. There was strong expression of the double-staining of Ki-67 and caspase-3 by infiltrating CTL in RA synovial lining cells (Figure 1A) compared with that

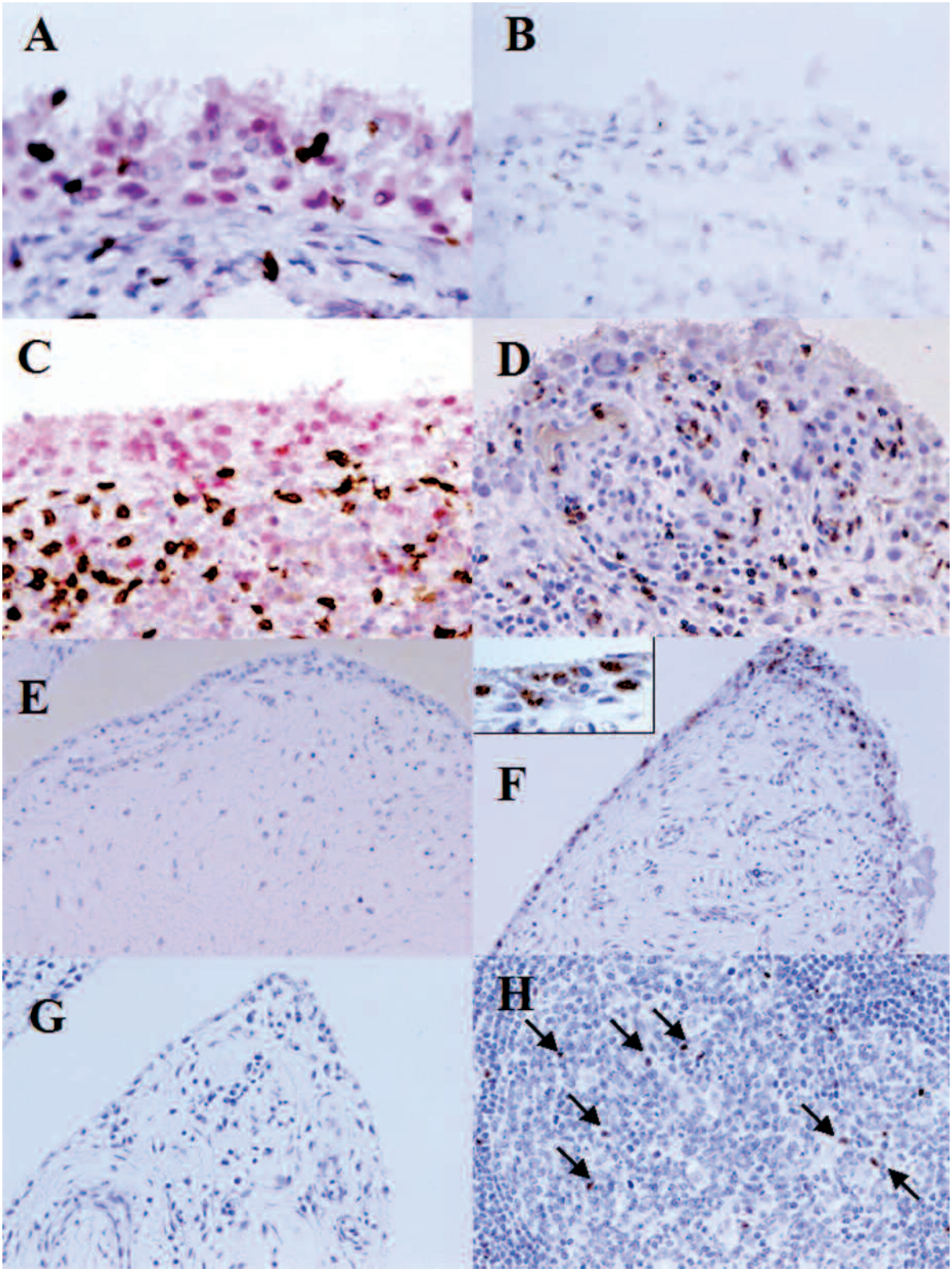

Figure 1. Immunohistochemical staining results. Double-staining of Ki-67 (brown) and caspase-3 (red) show that proliferation and apoptosis were more frequently seen in the synovial lining cells of patients with RA (A) than in those with OA (B). Double-staining of CD3 (brown) and caspase-3 (red) revealed significant apoptosis around CD3 T-cells (C). Widespread infiltration by TIA-1 cells of RA synovium (D); this infiltration was observed far less in OA synovium (E). RCAS1 was strongly expressed in OA synovial lining cells (F) and enlarged image (inset). RCAS1 was not expressed in the RA synovial lining cells (G). Treg cells were detected in the germinal centers of lymph follicles (arrows, H).

in OA cells (Figure 1B). The Ki-67- or caspase-3-positive cells were counted in 100 synovial lining cells, showing increases in both Ki-67 (number: mean 14.5 ± 0.6 SD) and caspase-3 (mean 34.7 ± 11.4 SD). In OA tissues, on the other hand, the immunohistochemical counts of Ki-67 (mean 1 ± 0.5 SD) and caspase-3 (mean 1.6 ± 2.4 SD) were significantly lower compared with RA ($p < 0.000001$; Figure 2A, 2B).

CTL attack synovial lining cells and cause apoptosis. To investigate infiltration of CTL and apoptosis of the RA synovial lining cells, tissue samples were stained with CD3+ caspase-3 prediluted double-stain antibodies. Caspase-3-positive cells were frequently detected surrounding CD3-positive cells in the synovial lining cells (Figure 1C). The CD3-positive cells that had infiltrated the synovial lining cells were strongly positive for TIA-1 (Figure 1D), indicating that they were cytotoxic and activated, and they appeared to attack the synovial lining cells. In these cases, CD56-positive T-cells were rarely observed in the synovial lining cells. This finding demonstrates the relationship between CD3 and apoptosis of synovial cells that are in contact with CD3 (Figure 1C), since apoptotic cells were encountered in the synovial lining cells surrounding CD3, which showed a higher degree of apoptosis than did the cells not in contact with CD3. We detected a strong correlation between CD3 and apoptosis ($p < 0.0001$; Figure 3A). This supports the supposition that CTL may attack synovial lining cells and cause apoptosis. Statistically, a significant correlation between TIA-1 and CD3 was confirmed, and the relationship between CD3 and caspase-3 was also confirmed (Figure 3B).

Expression of RCAS1 in synovial lining cells with RA and OA: CTL infiltration of synovium and lack of RCAS1 expression in RA. For this part of our study, the antibodies TIA-1 and RCAS1 were used. Widespread infiltration by TIA-1-positive lymphocytes was found in synovium of patients with RA (Figure 1D) compared to those with OA (Figure 1E). To investigate infiltration by CTL of the RA and OA synovial lining cells, tissue samples were stained with CD3, CD56, and TIA-1. TIA-1-positive cells were frequently detected in the synovial lining cells in the RA samples, but not in OA samples (Figure 2C). TIA-1-positive cells that

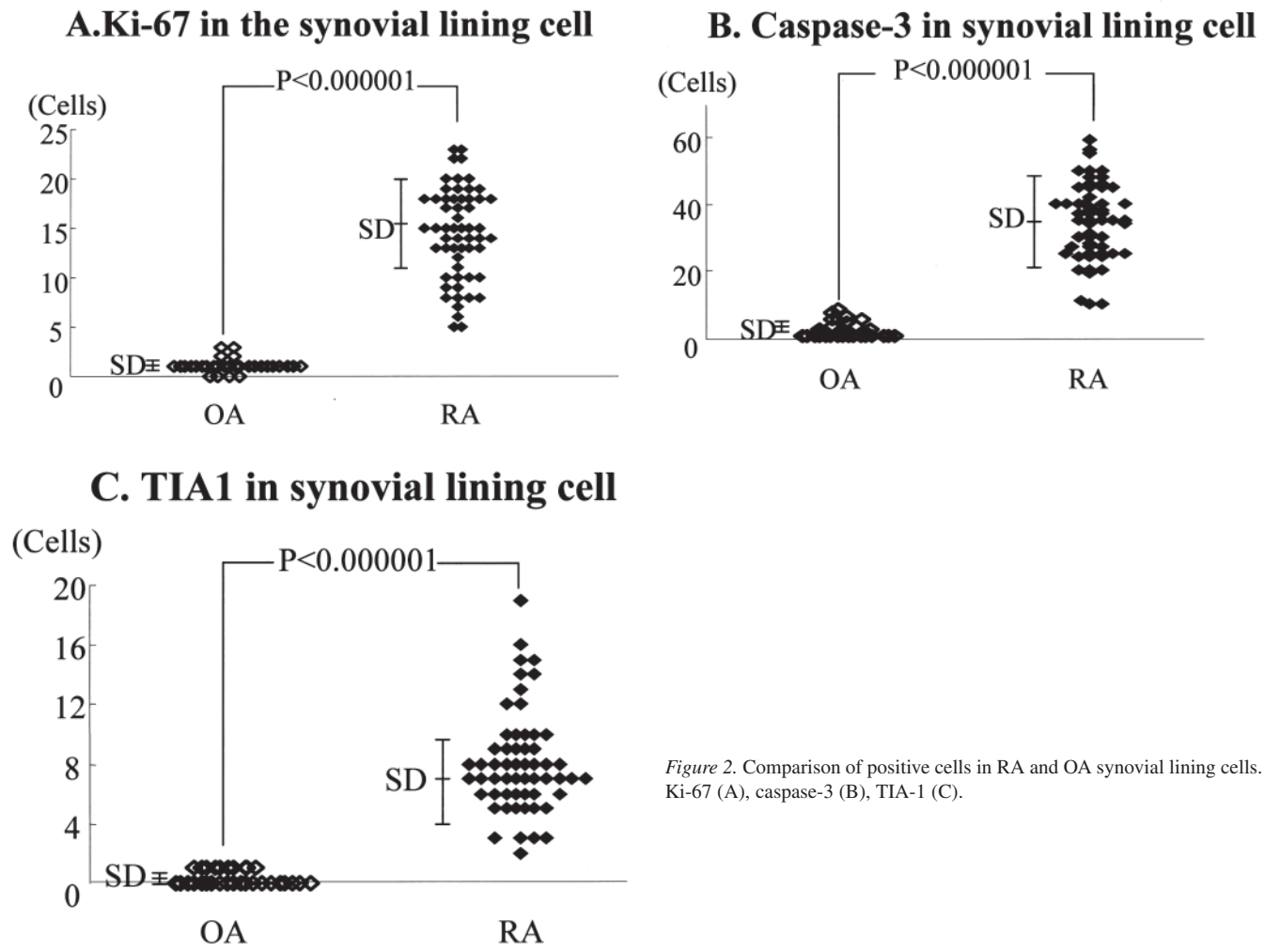

Figure 2. Comparison of positive cells in RA and OA synovial lining cells. Ki-67 (A), caspase-3 (B), TIA-1 (C).

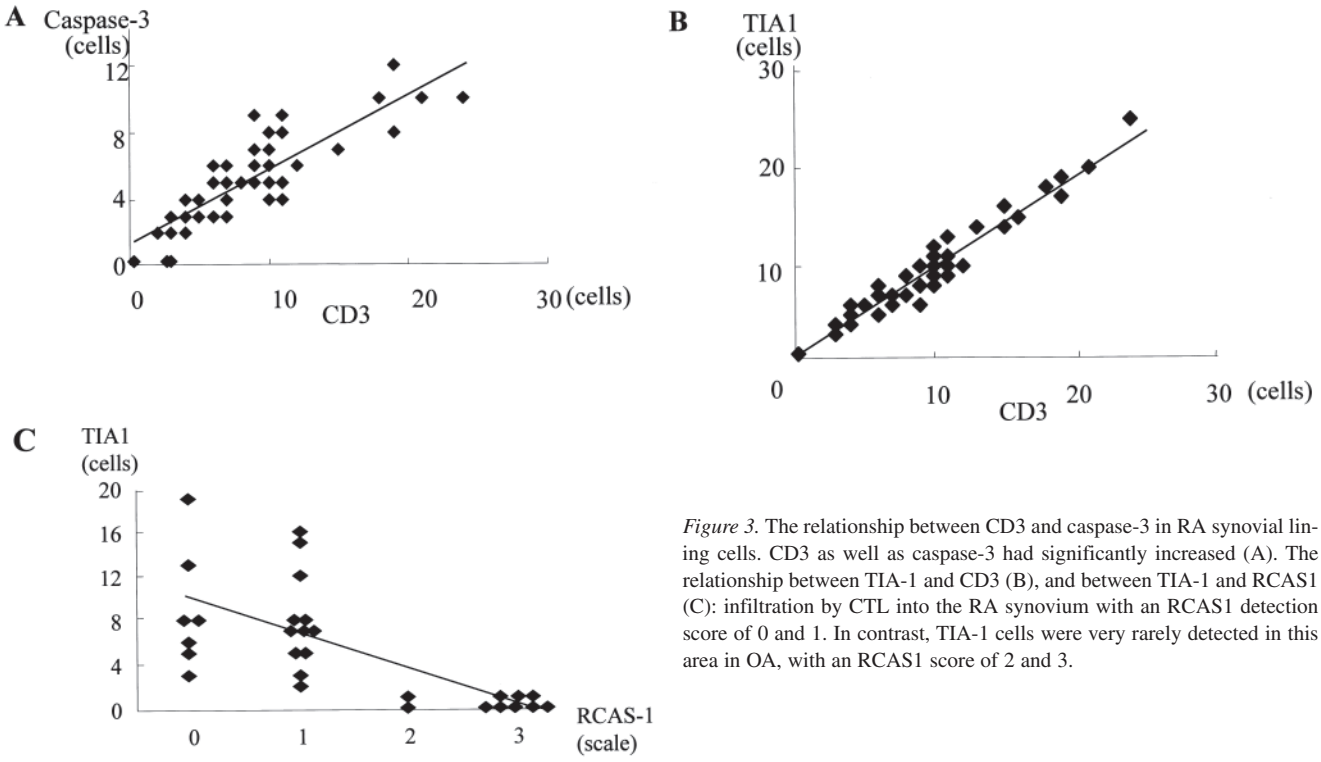

Figure 3. The relationship between CD3 and caspase-3 in RA synovial lining cells. CD3 as well as caspase-3 had significantly increased (A). The relationship between TIA-1 and CD3 (B), and between TIA-1 and RCAS1 (C): infiltration by CTL into the RA synovium with an RCAS1 detection score of 0 and 1. In contrast, TIA-1 cells were very rarely detected in this area in OA, with an RCAS1 score of 2 and 3.

had infiltrated the synovial lining cells were strongly positive for CD3, indicating that they were activated and apparently attacking the synovial lining cells. CD56-positive (NK) T-cells were rarely observed in the RA and OA synovium. TIA-1-positive lymphocytes were counted in 100 synovial lining cells each of RA and OA. CTL were found in the RA synovial lining cells (number: mean 8.1 ± 3.4 SD), but rarely in OA (mean 0.3 ± 0.5 SD; Figure 2C). In addition, strong expression of RCAS1 by synovial lining cells was detected, especially in OA (Figure 1F) but not in RA samples (Figure 1G). Moreover, the synovium of RA patients showed low or no expression of RCAS1 compared with the OA synovial tissue (RA score: 0–1, OA score: 2–3; $p < 0.0001$; Figure 3C). RCAS1 was also expressed in the OA synovial lining cells (Figure 3C), which were rarely infiltrated by CTL. In the RA cases, on the other hand, the expression of RCAS1 was markedly reduced, and CTL frequently infiltrated the synovial lining cells. These findings suggest that a reduction in RCAS1 is closely related to infiltration and activation of CTL in the synovium.

Expression of Treg in the germinal center may be associated with activator (CRP) of RA. Foxp3-positive cells were detected in the germinal center (GC) of synovial ectopic lymph follicles. They were lymphocytes, and between 0 and 10 Foxp3-positive cells were counted per field in the GC (Figure 1H). We also analyzed the relationship between CRP and expression of Treg cells in GC. In the CRP-negative group (0.0–1.0), as shown in Figure 4, the number of Treg cells in the GC had increased (number: mean 9.8 ± 5.4

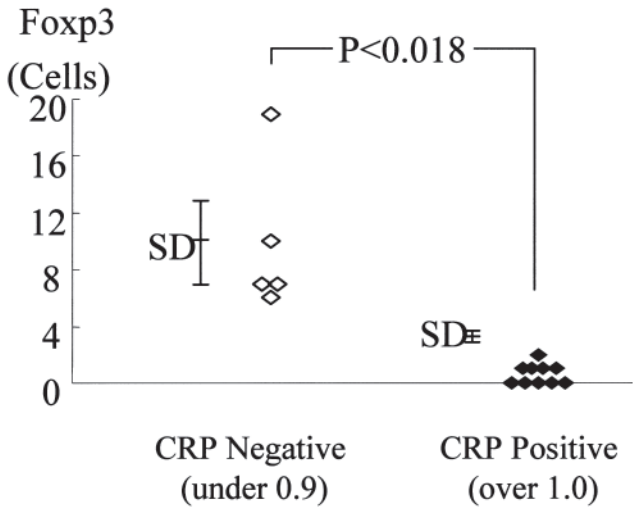

Figure 4. Comparison of CRP-positive and CRP-negative groups in terms of Foxp3 expression in the germinal center of lymph follicles. Treg cells were detected with much higher frequency in the CRP-negative group than in the CRP-positive group.

SD). In the CRP-positive group (1.1–8.0), on the other hand, expression of Treg cells was rarely detected in the GC (mean 0.6 ± 0.7 SD). There was thus an interesting and significant difference in the number of Treg cells between the CRP-positive and negative groups. Thus, we observed that the expression of Treg cells may be downregulated in the inflammatory mechanism.

DISCUSSION

In our study, proliferation and apoptosis were more frequently encountered in the synovial lining cells of patients with RA than in those with OA, while high expression of RCAS1 was detected significantly more frequently in the synovial lining cells of patients with OA. CTL were found to have infiltrated the synovium of the patients with RA, who lacked expression of RCAS1. Moreover, analysis of Foxp3 in the germinal center and CRP levels showed a possible relationship in terms of the extent of the inflammatory state. These findings indicate that RCAS1 should be considered to play an important role in the synovium by evading immune surveillance, while Treg cells may be involved in the downregulation of activated T cells.

Rheumatoid synovium is characterized by the proliferation and infiltration of a variety of inflammatory cells, resulting in bone and joint destruction. However, synovial proliferation is not infinite, and spontaneous arrest of the growth of hypertrophied synovial tissue has occasionally been observed[21,22]. This paradoxical finding reflects 2 clinical processes. The first is spontaneous remission, while the second is intractable proliferation of the synovium, which leads to pannus formation and ultimately to destruction of the bone and cartilage. The spontaneous arrest of proliferation of synovial tissue is of particular interest and strongly suggests the involvement of apoptosis[7]. We were able to demonstrate that there is a relationship between CD3 and apoptosis of synovial cells that are in contact with CD3 (Figure 1E). We detected a strong correlation, indicating that CTL may attack synovial lining cells and cause apoptosis.

Numerous reports of the presence of Treg cells in animal models and in humans point to a wide diversity of populations and mechanisms involved in RA. Thymically derived CD4+CD25+ Treg cells are essential for maintenance of self-tolerance and the prevention of autoimmune disease. This "natural" subset expresses the transcription factor Foxp3, which has emerged as an important functional marker of Treg cell activity. This importance is underscored by the fact that ectopic Foxp3 expression is sufficient to empower naive T cells with a regulatory function[23]. Manipulation of the peripheral pool of Treg cells has been a specific focus for the treatment of autoimmune diseases and transplantation. Data showing that Treg cells from patients with RA are functionally defective, and that after infliximab therapy this defect is reversed, have emphasized the potential benefits of biological therapy[16]. An intriguing finding is the increased number of peripheral blood CD4+CD25hi Treg cells seen only in RA patients responding to infliximab (anti-TNF-α therapy).

We focused on Treg cells in the germinal center in synovial ectopic lymph follicles. We were particularly interested in the group of patients with negative CRP, as it has been suggested that the immune system is probably most effective at controlling inflammation, and we indeed found a high expression of Treg cells in the germinal center in this group, while the positive CRP group showed low levels of Treg cell expression. An interesting possibility suggested by these findings is that Treg cells may downregulate the immune response by attenuating activated T cells. These results support the hypothesis of an association between Treg cells and the extent of the inflammatory state in RA.

Tumor cells may also evade immune attack by expressing FasL or other molecules that induce apoptosis in activated T and NK cells. Nakashima, *et al*[18] described RCAS1 as a candidate molecule for the immune evasion system of human cancer cells. In normal organs, RCAS1 has been shown to be expressed in the liver[24], thyroid gland[25], prostate[26], stomach[27,28], and lung[29].

It has also been suggested that expression of RCAS1 and FasL in the uterine glands and cytotrophoblasts may play a role in the downregulation of the maternal immune response, for maintenance of pregnancy in the early stage[30]. Reasoning that the RCAS1 signaling system might play a defensive role against immune attack, we hypothesized that RCAS1 might perform a similar etiologic role in autoimmune diseases. We therefore examined RCAS1 expression in the synovial lining cells of patients with RA and OA. We found that RCAS1 was expressed in the synovial lining cells of subjects with OA, but was strikingly reduced in cases with RA, and that this reduction was accompanied by marked infiltration and activation of CTL, and seems to be closely associated with CTL activation and destruction of synovial lining cells. There is also a possibility that the downregulation of RCAS1 and activation of CTL might be a consequence of the inflammatory process of RA rather than its cause. It could not be clarified in our study whether RCAS1 or FasL plays a more important role in maintenance of the synovial lining cells. However, RCAS1 may evade immune attack and induce apoptosis of activated T cells, so that RCAS1 would function as one of the "failsafe" mechanisms of inhibition of synovial immune attacks. This would account for the synovial lining cells with OA expressing RCAS1, which prevents CTL from infiltrating the synovium. On the other hand, RCAS1 expression was defective in RA, thus inducing CTL to infiltrate the synovium.

In conclusion, our findings demonstrated that, compared to patients with OA, those with RA show heightened apoptosis and proliferation in the synovial lining cells. The mechanism of apoptosis and proliferation in the synovial lining cells was identified as increase in CTL (TIA-1+ CD3+), which showed widespread infiltration of the synovium and appeared to attack the synovium. Most of the synovial lining cells in contact with the CTL showed caspase-3-positive apoptosis. We considered that the activated T cells (TIA-1+ CD3+) attack and damage the synovial lining cells, causing apoptosis and leading to the growth of new synovial cells to replace the damaged cells. We therefore hypothesize that the mechanism of villous proliferation is

caused by apoptosis and proliferation of synovial lining cells. An important finding in this connection is that the synovial lining cells of patients with RA in our study were found to express low levels of RCAS1, while those of patients with OA expressed high levels. This result supports the notion that normal synovial lining cells of patients with OA expressed RCAS1 to evade immune attack and induce apoptosis of activated T cells. RCAS1 therefore represents an important target for future therapeutic protocols for RA.

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