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CD44 Is the Physiological Trigger of Fas Up-Regulation on Rheumatoid Synovial Cells

Koichi Fujii,* Yuko Fujii,† Stefan Hubscher,‡ and Yoshiya Tanaka*2

CD44 is a ubiquitous molecule known as a hyaluronan receptor. However, the relevance of CD44 to inflammatory processes, for example, rheumatoid synovitis, remains unclear. In this study, we propose a novel function for CD44 using synovial cells from rheumatoid arthritis (RA) patients and demonstrated that CD44 cross-linking augmented Fas expression and subsequent Fas-mediated apoptosis of the cells: 1) cross-linking of CD44 on RA synovial cells markedly augmented Fas expression and its mRNA transcription; 2) engagement of CD44 up-regulated Fas on the cells within 3 h, much more than IL-1β and TNF-α did; 3) the Fas-mediated early apoptotic change of the cells was amplified by CD44 cross-linking; and 4) hyaluronan, especially when fragmented, also augmented Fas-mediated early apoptosis of the cells. Based on these findings, we postulate a new concept: that interaction of CD44 on RA synovial cells with hyaluronan fragments present in the surrounding extracellular matrix augments Fas expression as well as Fas-mediated apoptosis of synovial cells. This may lead to spontaneous growth arrest through Fas-Fas ligand pathway observed in synovial cells of RA synovitis in vivo. The Journal of Immunology, 2001, 167: 1198–1203.

Rheumatoid arthritis (RA) is a chronic inflammatory disorder involving synovial membranes of multiple joints, characterized by hyperplasia of synovial cells, excessive angiogenesis, and accumulation of mononuclear cells. Synovial cells are markedly activated by cytokines and adhesion molecules as well as a group of genes called protooncogenes, resulting in hyperplasia of the synovial membrane, and the activated synovial cells produce inflammatory cytokines and degradative enzymes that destroy cartilage and bone. RA synovium also shows some features compatible to apoptosis. Accumulating reports indicate that spontaneous growth arrest and remission are observed in RA synovial cells, which express functional Fas (CD95) Ags and show Fas-mediated apoptosis both in vitro and in vivo (1–6).

Fas is a widely expressed 45-kDa cell surface Ag of the TNF/nerve growth factor receptor superfamily (7–9) and is also known to be a target gene of p53, and the p53 mediates down-regulation of bcl-2, which results in cell cycle arrest and subsequent apoptosis. Recent report identified the expression of Fas and presence of apoptotic change of RA synovial cells, suggesting the role of Fas-mediated apoptosis in the inhibition of synovial hyperplasia (1). Thus, these paradoxical features of RA synovium associated with imbalance between cell proliferation and cell death appear to be most relevant to pathological processes of RA synovitis. However, mechanisms of regulating Fas expression and Fas-mediated apoptosis of proliferating rheumatoid synovial fibroblasts are largely unknown.

Overexpression of adhesion molecules, including ICAM-1, VCAM-1, and CD44, is a hallmark of the inflammatory responses such as RA synovitis (10–12). Adhesion molecules play a fundamental role in inflammatory processes by mediating leukocyte-endothelial cell adhesion, leukocyte migration, and T cell-APC interactions. However, recent findings have indicated that certain adhesion molecules not only function as glue, but also regulate several cellular functions by transducing signaling. We have reported that ICAM-1 and CD44 on rheumatoid synovial cells induced transcription of IL-1β and VCAM-1, respectively, by activation of a nuclear factor, AP-1 (13, 14). These results have prompted us to investigate the adhesion molecules involved in Fas up-regulation on rheumatoid synovial cells. Cross-linking multiple adhesion molecules, such as LFA-1, VLA-4, ICAM-1, and CD44, on rheumatoid synovial cells, we report in this work that CD44 was unique in its remarkable up-regulation of Fas expression on synovial cells. Among multiple cell surface receptors for the matrix, CD44, which is a receptor for hyaluronan, a major matrix polysaccharide abundantly existing in synovium, is overexpressed in inflammatory sites in proportion to the intensity of inflammation, implicating CD44 in the pathogenesis of inflammation (10). This report documents a role for CD44 and its ligand hyaluronan in Fas up-regulation on rheumatoid synovial cells. We propose a model for the involvement of an adhesion molecule CD44 in the induction of Fas expression on synovial cells and the subsequent amplification of Fas-mediated apoptosis of the cells in inflammatory processes of RA synovitis.

Materials and Methods

Synovial tissues and culture of synoviocytes

Synovial tissues were obtained from patients with active RA, diagnosed according to the criteria of the American College of Rheumatology, who were treated by joint replacement surgery or synovectomy. Samples were dissected under sterile conditions in PBS, and immediately prepared for culture of fibroblast-like synovial cells. Briefly, the tissue sample was minced into small pieces and digested with collagenase (Sigma Aldrich Japan, Tokyo, Japan) in serum-free DMEM (Life Technologies, Grand Island, NY). After filtering through a nylon mesh, the cells were extensively washed, and suspended in DMEM, supplemented with 10% FCS.
(Bio-Pro, Karlsruhe, Germany) and penicillin-streptomycin (10 U/ml; Sigma Aldrich). Finally, isolated cells were seeded in 25-cm² culture flasks (Falcon, Lincoln Park, NJ) and cultured in a humidified 5% carbon dioxide atmosphere. After overnight culture, nonadherent cells were removed, and further incubation of adherent cells was continued in fresh medium. At confluence, the cells were trypsinized, passaged at a 1:3 split ratio, and recultured. The medium was changed twice each week, and the cells were used after three to seven passages.

Reagents and mAbs

IL-1β, TNF-α (Cosmobio, Tokyo, Japan), annexin V (Immunotech, Marseille, France), and IgM anti-Fas mAb (MBL, Nagoya, Japan) were purchased. Fragmented and native hyaluronan were kindly donated by the Tokyo Research Institute of Seikagaku (Tokyo, Japan). The following mAbs were used as purified IgGs: CD14 (mAb 63D3), anti-glycophorin mAb (mAb 10F7; American Type Culture Collection, Manassas, VA), control mAb (Thy-1.2; Becton Dickinson, San Jose, CA), MHC class II mAb (mAb IVA12; a gift from Dr. J. D. Capra, Dallas, TX), CD11b (mAb NIH11b-1), CD44 (mAb NIH44-1), and CD54 (ICAM-1) (mAb 84H10; gifts from Dr. S. Shaw, Bethesda, MD).

Stimulation of CD44 of synovial cells

Synovial cells were cultured until subconfluence and then incubated with CD44 mAb NIH44-1 (10 μg/ml) for 30 min at 37°C, as already described (8). After washing the cells three times, 1 μg/ml goat anti-mouse IgG-Fc was added as the second Ab for CD44 cross-linking. The cells were cultured until subconfluence and then also incubated with fragmented or native hyaluronan (0.1 μg/ml) for 3 h at 37°C.

Flow microfluorometry

Staining and flow cytometric analysis of synovial cells were conducted by standard procedures, as already described, using a FACScan (Becton Dickinson, Mountain View, CA) (15, 16). Briefly, cells (2 × 10⁵) were incubated with FITC-conjugated specific mAbs at saturating concentrations in the presence of goat anti-mouse irrelevant Ab in FACS medium consisting of HBSS (Nissui, Tokyo, Japan), 0.5% human serum albumin (Greencross, Osaka, Japan), and 0.2% Na₂C₂O₃ (Sigma Aldrich) for 30 min at 4°C. After three washes in FACS medium, the cells were analyzed with FACScan. Amplification of the mAb binding was provided by a three-decade logarithmic amplifier. Quantification of the cell surface Ags on one cell was performed using beads, QIFKIT (Dako Japan, Kyoto, Japan). Briefly, five populations of calibration beads, bearing different, but well-defined numbers of mAb molecules, were analyzed by FACScan. Mean fluorescence intensity of each population of beads was used for construction of the calibration curve. The cell specimen was analyzed by FACScan, and Ag density was calculated by interpolation on calibration curve.

Northern blot analysis

For Northern blot analysis, total cellular RNA was isolated from cultured RA synovial cells by a single-step isolation procedure. The RNA (10 μg) was electrophoresed through a 1% agarose gel and blotted onto nylon filters (Amersham, Arlington Heights, IL). Fas cDNA (Biognostik, Gottin- gen, Germany) was labeled with [32P]dCTP (Dupont NEN, Boston, MA), and Northern blot analysis was subsequently performed.

Statistical analysis

Significant differences among groups within each experiment were determined by ANOVA, followed by post hoc Scheffé’s F test.

Results

CD44 and Fas were expressed on RA synovial cells

We initially characterized cultured synovial cells derived from the synovium of RA patients. The cells were spindle shaped and grew in a cobblestone pattern. Flow cytometric analysis of these cells in a confluent culture indicated that they lacked macrophage markers such as MHC class II Ags, CD14, and CD11b, suggesting they are type B fibroblast-like cells (Fig. 1). These fibroblast-like cells express Fas in monomodal pattern as well as CD44. CD44 was positive on all the synovial cells examined by FACScan, and 17 of 20 CD44 positive cells also coexpressed Fas.

Cross-linking of CD44 on RA synovial cells up-regulated Fas expression

To characterize the function of CD44, we assayed the cell surface molecule expression by CD44 cross-linking using a specific mAb and second cross-linker Ab. Flow cytometry showed that Fas expression was markedly augmented by the CD44 cross-linking on synovial cells. As shown in Fig. 2 and Table I, Fas was moderately expressed on nonstimulated synovial cells. However, CD44 cross-linking significantly up-regulated Fas expression, whereas cross-linking of ICAM-1 using its specific mAb had no effect. The results were consistent in five patients with RA. Time-course experiments showed that Fas expression on RA synovial cells reached maximum levels within 3 h of CD44 cross-linking, but the expression returned to almost basal levels after 24 h of incubation (Fig. 3). The results indicate that the CD44 cross-linking induced a marked, but transient amplification of Fas expression on RA synovial cells.

Cross-linking of CD44 triggered Fas mRNA transcription

CD44 cross-linking also induced transcription of Fas mRNA in RA synovial cells. After CD44 cross-linking, RNA was extracted from RA synovial cells, and specific mRNA was detected by Northern blot analysis using primers specific for human Fas. RA synovial cells barely expressed Fas mRNA without stimulation.

FIGURE 1. Phenotypic analysis of synovial cells. Cells were stained with CD11b mAb (NIH11b-1), CD14 mAb (63D3), MHC class II mAb (IVA1), CD44 mAb (NIH44-1), Fas mAb, and ICAM-1 mAb (84H10). Flow cytometric analyses were performed using FACScan. Light line, Thy-1.2 mAb as a negative control; heavy line, labeled cells. The histogram is a representative result of five similar experiments.

FIGURE 2. Fas up-regulation by CD44 cross-linking on RA synovial cells. Synovia were obtained from five patients with RA, and synovial cells were isolated. Cells were cross-linked with control medium ([]), anti-CD44 mAb, NIH44-1 (■), and anti-ICAM-1 mAb, 84H10 (□), at a concentration of 10 μg/ml for 3 h. Fas expression was analyzed by FACScan. Each value represents the number of molecules expressed per cell, calculated using standard QIFKIT beads, as described in Materials and Methods. Data are mean ± SD. *, p < 0.05 compared with controls.
CD44 cross-linking markedly augmented Fas mRNA transcription, which was maximal within 2 h of stimulation, but subsequently gradually diminished (Fig. 4).

**CD44 was the most potent stimulator of Fas expression among stimuli**

IL-1β is known to induce Fas expression on pancreatic β cells (9). In the next series of experiments, we compared the magnitude of Fas up-regulation induced by CD44 and other stimuli, including several cytokines on synovial cells. When CD44 was cross-linked by specific mAb and second cross-linker Ab, the expression of Fas on RA synovial cells was markedly augmented, whereas cytokines such as IL-1β and TNF-α, which are known to be abundantly produced in RA synovium and to be involved in the pathogenesis of RA synovitis, had no effect on Fas expression at 3 h of stimulation of RA synovial cells (Fig. 5A). When ICAM-1, MHC-class I, or VCAM-1 Ag were cross-linked by their specific mAb and second cross-linker Ab, the expression of Fas on RA synovial cells was unchanged, whereas CD44 cross-linking markedly augmented Fas expression (Fig. 5B). All these studies were reproducible among three different RA patients. These results suggest that CD44 appears to play a pivotal role in Fas up-regulation on the cell surface during inflammatory process like rheumatoid synovitis.

**CD44 augmented Fas-mediated apoptosis of RA synovial cells**

Apoptosis of RA synovial cells is known to be induced by anti-Fas mAb. We next investigated the effect of CD44-induced Fas up-regulation on Fas-mediated apoptosis of RA synovial cells. Stimulation of RA synovial cells with anti-Fas mAb induced early apoptotic change of RA synovial cells, which was detected by double staining with propidium iodide (PI) and annexin V and stained for PIlow annexin Vlow. Cross-linking of CD44, followed by Fas mAb stimulation, markedly increased the number of PIlow annexin Vhigh, early apoptotic synovial cells (Fig. 6 and Table II). These results suggest that Fas up-regulation by CD44 may contribute to the augmentation of Fas-mediated apoptosis of RA synovial cells.

**Fas-induced early apoptosis was augmented by fragmented hyaluronan**

Hyaluronan is a major ligand for cell surface CD44. We finally assessed the biological activity of hyaluronan on Fas-induced early apoptosis of RA synovial cells. As shown in Fig. 7 and Table II, soluble full-length hyaluronan had almost no effect on apoptotic change of RA synovial cells, and the vast majority of the cells remained PIlow annexin Vlow. However, the 6.9-kDa fragmented hyaluronan apparently increased the Fas-mediated early apoptotic change of RA synovial cells, PIlow annexin Vhigh cells. Fragmented hyaluronan, but not full-length hyaluronan, also enhanced Fas expression (data not shown). This suggests that hyaluronan, especially when fragmented, is a possible ligand augmenting Fas-mediated apoptosis of RA synovial cells.

**Discussion**

Fas was identified as an apoptosis-mediating molecule, and its expression is crucial in controlling tissue homeostasis. Rheumatoid synovium shows an extreme character comprised of two diverse phenomena of RA synovial cells, intractable proliferation, and growth arrest of the cells, and the mechanisms altering its balance may lead to the pathogenesis of RA. Recent reports indicate that anti-Fas mAb accelerates apoptotic change of the RA synovial cells (17–20). On the basis of the results presented in the current study, we propose a new concept, that stimulation of the adhesion molecule CD44 plays a pivotal role in the regulation of Fas expression.
in RA synovial cells. We deduce this from the following novel findings: 1) CD44 cross-linking on RA synovial fibroblast-like cells up-regulated Fas expression and Fas mRNA transcription, more than did stimulation with inflammatory cytokines, including IL-1β and TNF-α; 2) Fas-mediated apoptosis of RA synovial cells was markedly augmented by the CD44 cross-linking on synovial cells; and 3) fragmented hyaluronan effectively augmented Fas-mediated apoptosis compared with native hyaluronan. Based on these findings, we postulate a new concept, that interaction of CD44 on RA synovial cells with fragments of extracellular hyaluronan present in the surrounding extracellular matrix augments Fas expression and Fas-mediated apoptosis of synovial cells.

Recent findings indicate that certain adhesion molecules not only function as a glue, but also regulate several cellular functions by transducing signals. Several reports demonstrate that cell-extracellular matrix adhesion also regulates Fas expression. For example, interaction of type IV collagen and epithelial cells augments Fas expression and enhances Fas-mediated apoptosis (21).

From a survey of cross-linking of multiple adhesion molecules, we found that one such molecule, CD44, was unique in its remarkable up-regulation of Fas expression and enhancement of Fas-mediated apoptosis of them. The principal known ligand for CD44 is hyaluronan, which is a high m.w. linear repeating disaccharide-β-D-glucuronyl-β-D-n-acetylglucosamine and is the major extracellular glycosaminoglycan found in almost all types of extracellular matrix, including RA synovium (22–24). The nonaggregated form of hyaluronan is continuously secreted into the joint space by elements of the synovium. The presence of short chain molecules of hyaluronan in the synovium is due to degradation after synthesis (25, 26). Our present results show that fragmented hyaluronan was more effective in Fas-mediated apoptosis of RA synovial cells than native hyaluronan. These results suggest that hyaluronan, especially the degraded form, is far from an inert space filler, but has important biological activities such as regulation of Fas-mediated apoptosis. IL-1β and TNF-α are also known to induce Fas expression. For instance, exposure of β islet cells to IL-1β induces Fas expression on the cells through the production of nitrogen oxide (9, 20). IL-1β and TNF-α are abundantly produced in RA synovium and play a central role in the process of rheumatoid synovitis. However, engagement of CD44 on RA synovial cells up-regulated Fas expression, which reached maximum levels within 3 h, whereas IL-1β and TNF-α did not in the same kinetics. Furthermore, other reports indicate that IL-1β and TNF-α rather inhibit Fas-mediated apoptosis of RA synovial cells (27, 28). Thus, CD44 stimulation might function in a different signaling manner from inflammatory cytokines in synovial cells.

We have previously reported that CD44 cross-linking on RA synovial cells enhances their adhesion to T cells by up-regulation of VCAM-1 (13). Our previous report and current data imply that CD44-mediated signaling augments adhesion of T cells with synovial cells through VLA-4/VCAM-1 pathway, resulting in more efficient interaction through surface molecules such as Fas/Fas ligand (FasL) pathway. T cells, including autoreactive T cell clones,
are known to play a central role in an initiation of RA synovitis. However, in chronic and proliferating RA synovium, the significance of T cell functions for the persistent inflammation is unclear. Several reports suggest that T cells in RA synovium might not be as active, less amounts of both cell surface activation Ags and cytokine production (29, 30). In SCID mouse model of RA, depletion of T cells rather amplifies synovial cell proliferation, the growth of pannus, and subsequent degradation of bones (31). Furthermore, a minority of patients have to date benefited from the therapy using anti-CD4 mAb, which failed to control growth of synovium in vivo (32, 33). These reports suggest that T cells might rather restrain hyperplasia of the synovial membrane in chronic RA synovitis. Several reports demonstrated that FasL gene transfer to RA synovium induces remarkable apoptosis to synovial cells through Fas/FasL pathway (34–36). These reports and our current data suggest that ligation of CD44, highly expressed on synovial cells, by surrounding hyaluronan enhances adhesion and interaction of synovial cells with synovial FasL-positive cells, resulting in amplified apoptosis of synovial cells through Fas/FasL pathway. In vivo, synovial cells are surrounded by and encounter extracellular matrix such as hyaluronan mainly through their receptors, including CD44, indicating that the engagement of CD44 by matrix protein always occurs in RA synovium. However, several possibilities are raised, for example, soluble Fas, soluble VCAM-1/ICAM-1, and soluble CD44 might interfere with the interaction of RA synovial cells and T cells, which may inhibit Fas/FasL-mediated apoptosis of RA synovial cells (38–40). Hyperplasia of RA synovial cells may result from the incomplete apoptosis of synovial cells, despite abundant Fas expression.

Taken together, these results indicate that CD44 is deeply concerned in Fas expression and Fas-mediated apoptosis of RA synovial cells in the following novel characteristics: 1) the involvement of cell surface functional molecules per se (in this study, CD44), in the induction or amplification of other functional molecules (in this study, Fas) on the same cell; 2) interaction of CD44 and degraded products of hyaluronan play an important role in biological activities, such as induction of apoptosis; 3) CD44-mediated signaling might be different from cytokine-mediated one in regulating Fas expression and Fas-mediated apoptosis; 4) CD44 further augments Fas/FasL-mediated apoptosis of synovial cells by augmenting the adhesion of synovial cells with T cells through up-regulation of VCAM-1 on synovial cells. Several clinical studies are underway to test various strategies to regulate activation of the synovial cells in RA patients, and the rational design of future therapeutic strategies for RA synovitis may thereby include the exploitation of CD44 and Fas death pathway to directly reduce growth of synovial cells in vivo.

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