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Rheumatoid Arthritis Synovial Stromal Cells Inhibit Apoptosis and Up-Regulate Bcl-xL Expression by B Cells in a CD49/CD29-CD106-Dependent Mechanism

Kenji Hayashida,* Yasunori Shimaoka,† Takahiro Ochi,† and Peter E Lipsky²*

Inflammatory sites, such as rheumatoid arthritis (RA) synovial tissue, contain large numbers of activated B cells and plasma cells. However, the mechanisms maintaining B cell viability and promoting their differentiation are not known, but interactions with stromal cells may play a role. To examine this, purified human peripheral B cells were cultured with a stromal cell line (SCL) derived from RA synovial tissue, and the effects on apoptosis and expression of Bcl-2-related proteins were analyzed. As a control, B cells were also cultured with SCL from osteoarthritis synovium or skin fibroblasts. B cells cultured with medium alone underwent spontaneous apoptosis. However, B cells cultured with RA SCL cells exhibited less apoptosis and greater viability. Although SCL from osteoarthritis synovium and skin fibroblasts also rescued B cells from apoptosis, they were less effective than RA SCL. B cell expression of Bcl-xL was markedly increased by RA SCL in a contact-dependent manner, whereas B cell expression of Bcl-2 was unaffected. Protection of B cells from apoptosis and up-regulation of Bcl-xL by RA SCL were both blocked by mAbs to CD106 (VCAM-1), but not CD54 (ICAM-1). Furthermore, cross-linking of CD49d/CD29 (very late Ag-4) on the surface of B cells rescued them from apoptosis and up-regulated Bcl-xL expression. These results indicate that SCL derived from RA synovial tissue play a role in promoting B cell survival by inducing Bcl-xL expression and blocking B cell apoptosis in a CD49d/CD29-CD106-dependent manner. The Journal of Immunology, 2000, 164: 1110–1116.

Materials and Methods

Antibodies and reagents

Biotinylated mouse anti-human CD19 mAb and mouse anti-human CD29 mAb (4B4) were purchased from Coulter (Miami, FL). Mouse anti-human CD20 mAb conjugated with PE, goat anti-mouse IgG conjugated with FITC, mouse anti-human CD14 mAb conjugated with FITC, goat anti-mouse IgG, and goat anti-rabbit IgG conjugated with HRP were obtained from Sigma (St. Louis, MO). Mouse anti-human CD49d mAb (HP2/1) and mouse anti-human CD106 mAb (1G11B1) were purchased from Immunotech (Miami, FL) and Serotec (Oxford, U.K.), respectively. Mouse IgG1 (MOPC) mAb, mouse anti-human IgM heavy chain (DA4-4) conjugated with biotin, mouse anti-human CD11a mAb (TS1/22), and mouse anti-human CD18 mAb (TS1/18) were prepared from hybridoma cell lines purchased from American Type Culture Collection (Manassas, VA). Mouse anti-human CD54 mAb (R6.5) was a gift from Dr. R. Rothlein (Boehringer Ingelheim Pharmaceuticals, Ridgefield, CT). Rabbit anti-human Bcl-2 (C-21) and Bcl-xL (L-19) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Rheumatoid Arthritis Synovial Stromal Cells

The synovial tissue in rheumatoid arthritis (RA) is characterized by infiltration with a variety of inflammatory cells, including T cells, B cells, and plasma cells (1). The lymphocyte accumulation in synovial tissue might be induced by an increased migration from peripheral blood, proliferation of these cells in situ, and/or inhibition of cell death. Increased migration into the synovium has been suggested (2, 3), whereas local proliferation appears to be minimal (4). Programmed cell death is limited in the synovium and rarely involves lymphocytes (5). Therefore, the limited programmed cell death of lymphocytes may contribute to their accumulation in the synovium. A number of mechanisms have been proposed to account for the apparently diminished apoptosis of lymphocytes, including the action of cytokines (6–17), the presence of inhibitors of apoptosis, such as soluble CD95 and Fas ligand (18, 19), and the direct impact of cell-to-cell contact (20). Among the cells that appear to be active in blocking lymphocyte apoptosis is a population of stromal cells with the characteristics of thymus and bone marrow nurse cells (21, 22). Previous studies documented that these stromal cell lines (SCL) blocked B cell apoptosis in vitro (22). The current studies examined the mechanism by which B cells were rescued from apoptosis and demonstrated that SCL derived from RA synovium induced expression of the antiapoptotic protein, Bcl-xL, by a mechanism that involved cell-to-cell contact mediated by CD49d/CD29-CD106 interactions.

Stromal cell lines and fibroblast lines

One RA SCL was established from synovium as previously described (22). In brief, synovial tissues of patients with RA who met American College of Rheumatology criteria (23) were obtained after obtaining informed consent and were dissociated with collagenase and trypsin. Dissociated single

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3 Abbreviations used in this paper: RA, rheumatoid arthritis; SCL, stromal cell line; OA, osteoarthritis; VLA-4, very late Ag-4; FDC, follicular dendritic cells.
FIGURE 1. The SCL derived from RA synovial tissue rescue peripheral B cells from apoptosis. Peripheral B cells \((1 \times 10^6)\) were cultured with or without RA SCL (Sy77; \(4 \times 10^3\)) in 96-well microtiter plates. After various lengths of culture, B cells were harvested with gentle pipetting, and the percentages of viable cells (A) and apoptotic cells (B and C) were determined as described in Materials and Methods. Data are the mean ± SEM of five cultures from one of four independent experiments with similar results. *, \(p < 0.05\); **, \(p < 0.01\) (using Student’s t test).

cells were cultured in DMEM supplemented with 10% FBS and 10% conditioned medium, which was prepared by incubation of PBMC from 10 healthy donors in RPMI 1640 medium with 10% FBS for 48 h. The cultures were then maintained for more than 2 mo, and the SCL were cloned by limiting dilution. Thereafter, clones were maintained and replenished with fresh DMEM with 10 or 20% FBS every 3–4 days. One clone (Sy 77) was used in the present experiments. A second RA SCL (RA6/1) was established from synovial tissue in a similar manner. After three to six passages, this RA SCL contained <2% CD14+ cells. An SCL was also established from osteoarthritis synovium (OA5/26) using the same procedure. Skin fibroblast lines (DT1 and FB-HG) were provided by Dr. Heather Wisbey (University of Texas Southwestern Medical Center, Dallas, TX) and Dr. Herman Girschick (University of Texas Southwestern Medical Center).

B cell separation

The PBMC were isolated from heparinized blood of healthy adult volunteers by density sedimentation using Ficoll/Isopaque. CD19-positive B cells were purified using the CEPRATE streptavidin column (CellPro, Bothell, WA), following staining of the cells with subsaturating concentrations of biotinylated mouse anti-human CD19 mAb. The resultant population of B cells contained >95% CD20-positive B cells.

B cell culture

B cells were cultured in various ways. When cultured alone, B cells \((1 \times 10^6)\) were cultured in 1 ml of RPMI 1640 medium (RPMI 1640 with 10% FBS, 200 U/ml of penicillin G, 10 μg/ml gentamicin, and 0.3 mg/ml l-glutamine) in 24-well culture plates. Alternatively, B cells \((1 \times 10^6)\) were cultured in 200 μl of RPMI 1640 medium in 96-well flat-bottom microtiter plates. In some experiments, B cells were stimulated with anti-IgM Ab. To accomplish this, B cells were incubated with 10 μg/ml of anti-human IgM Ab (DA4.4) conjugated with biotin for 30 min at 4°C and washed twice. Afterward, B cells \((1 \times 10^6)\) in wells of 24-well plates were cultured for 1 h with streptavidin (10 μg/ml) to cross-link surface IgM, harvested, washed, and used for experiments. When SCLs and fibroblasts were incubated with B cells, they were initially cultured in 24-well culture plates by incubating SCL \((4 \times 10^3)\) in 1 ml of DMEM (high glucose DMEM including 10% FBS, 200 U/ml of penicillin G, 10 μg/ml gentamicin, and 0.3 mg/ml l-glutamine) for 1 wk. After this period of time, they became confluent, and B cells \((1 \times 10^6)\) were added in 1 ml of RPMI 1640 culture medium. After various periods of time, B cells were harvested by gentle pipetting. Contamination of harvested B cells with SCLs and fibroblasts could be determined by scatter properties using a flow cytometer and was always <0.5%. Alternatively, SCLs and fibroblasts \((4 \times 10^4)\) were cultured in 96-well flat-bottom microtiter plates for 1 wk, and then B cells \((1 \times 10^6)\) were added, incubated, and harvested as described above.

Direct cell-to-cell contact between B cells and SCL was blocked with a 0.4-μm pore size membrane (Falcon, Becton Dickinson, Franklin Lakes, NJ). For these experiments, SCL \((4 \times 10^4)\) were cultured as described above, and B cells \((1 \times 10^6)\) were added to culture contained in the membrane chamber. In the experiments examining the effect of blocking mAb, SCL \((4 \times 10^4)\) were cultured with 5 μg/ml of mAb for 60 min before addition of B cells as described above. In some experiments, B cells were stimulated with anti-CD11a/CD18 and/or CD49d/CD29 mAbs. For these experiments, 5 μg of rabbit anti-mouse IgG mAb were coated in the wells of 24-well culture plates by incubating them in Tris-HCl at pH 9.8 over-night. B cells \((1 \times 10^6)\) were incubated with 2 μg of mAbs for 30 min, washed once, added to the 24-well plates precoated with rabbit anti-mouse IgG, and cultured in RPMI medium as described above.

Analysis of viable cells

The numbers of viable cells were assessed microscopically after staining with trypan blue.

Detection of apoptosis

The hypotonic propidium iodide staining method was used to detect apoptosis of cells by flow cytometry (24). Harvested B cells were resuspended in hypotonic propidium iodide reagent (0.1% sodium citrate containing 50 μg/ml of propidium iodide and 0.1% Triton X-100). Within 60–120 min, the stained cells were analyzed by flow cytometry using the FACScan.
concentration of each sample was adjusted to 2 SD (4%), and stored at 4°C until analysis. Before analysis, the protein
HG, fibroblast cell line from human skin. OA5/26, OA synovial cell line; DT1 and FB
mal cell clone; RA6/1, RA synovial cell line; RA SCL rescued B cell apoptosis a
percentages of viable (A) and apoptotic cells (B) were determined as described in Materials and Methods. Data are the mean ± SEM from the number of replicates indicated (n). Table shows data from three of seven similar experiments.

**Peripheral blood B cells (1 × 10⁶) were cultured with or without RA SCL (Sy77: 1 × 10⁷) in 96-well culture plates. Percent viability was measured after trypan blue staining. Apoptosis was measured by flow cytometry after hypotonic propidium iodide staining. Data indicates mean ± SEM from the number of replicates indicated (n). Table shows data from three of seven similar experiments.

* p < 0.05; ** p < 0.01: statistical difference between B cells with medium alone and B cells with RA SCL using Student’s t test.

**Figure 2.** The RA SCL rescue B cells from apoptosis more effectively than other cell lines. Peripheral B cells (1 × 10⁶) were cultured with various SCLs or fibroblasts (4 × 10⁵) in 96-well microtiter plates. After 4 days of culture, B cells were harvested with gentle pipetting, and the percentages of viable cells (A) and apoptotic cells (B) were determined as described in Materials and Methods. Data are the mean ± SEM of five cultures in concordance with the company’s instructions. One microgram of the extracted RNA was treated with DNase I to eliminate DNA and reverse transcribed by SuperScript II reverse transcriptase at 42°C for 70 min using oligo(dT). The PCR was conducted with Taq polymerase using 1–3 μl of cDNA (1.5 mM MgCl₂; annealing temperature, 56°C; 30–40 cycles). The primer pair for Bcl-2 was CAGAATCCCTGTGAATGAGG (5′) and CTTCAGA CATCGAGGACC (3′), that of Bcl-xL was GTGGAAGAGAACG CATTCGGAGACC (3′) and CTTCCAGA 9

**Table 1. RA SCL rescues B cell apoptosis**

| Expt. 1 (n = 6) | Medium + RA SCL | 92.0 ± 0.8 | 47.0 ± 4.1 | 24.7 ± 3.9 |
| Expt. 2 (n = 5) | Medium + RA SCL | 92.3 ± 1.0 | 82.0 ± 3.3* | 71.0 ± 2.1** |
| Expt. 3 (n = 5) | Medium + RA SCL | 95.7 ± 0.9 | 89.0 ± 2.8 | 82.0 ± 0.6** |
| Apoptosis (%) | Medium + RA SCL | 94.7 ± 0.9 | 84.9 ± 1.4** | 70.5 ± 0.2** |

* p < 0.05; ** p < 0.01: statistical difference between B cells with medium alone and B cells with RA SCL using Student’s t test.
products were transferred onto Zeta-Probe blotting membranes using a vacuum blotting apparatus. $^{32}$P-labeled probes (Bcl-2, GTGACTTCCGATCAGGAAGG; Bcl-xL, GGTATTGGTGAGTCGGATCG; GAPDH, CCTCCAGACCCTGCCTGAGC) specific for PCR products were used to detect PCR products by Southern hybridization. To adjust the amount of cDNA of each sample precisely, G6PD expression was examined first using 32–35 cycles of RT-PCR to amplify 1, 2, and 3 $\mu$L of cDNA. After resolving the PCR products on agarose gels and identifying the relevant bands with ethidium bromide, the optimal amounts of cDNA were determined. Bcl-2 and Bcl-xL expression in this amount of cDNA was examined using 32, 35, 38, and 40 cycles of PCR amplification, and the results in the linear part of the amplification curve are reported in the figures.

**Results**

The SCL from RA synovium can rescue B cells from apoptosis

When cultured alone, B cells spontaneously underwent apoptosis and died (Fig. 1). However, when B cells were cultured with RA SCL, apoptosis was blocked, and B cell viability was preserved. In each experiment (Table I) significant differences in viability and apoptosis between B cells cultured with medium alone and those with RA SCL were noted after 6 days of incubation. However, in some experiments (Expt. 2), but not others (Expt. 1 and 3), differences in viability were not seen on day 3. Similarly, significant differences between apoptosis noted in cultures with and without SCL were not always noted on day 3. To determine whether RA SCL were unique in their ability to promote the viability of B cells, the activities of a variety of other cell lines were tested (Fig. 2). All the cell lines rescued B cells from apoptosis to varying degrees, but the viability-promoting activity of RA SCL was significantly better than those of the other cell lines.

**Bcl-X is up-regulated by B cells cultured with RA SCL**

To determine whether rescue from apoptosis reflected up-regulation of antiapoptotic proteins, expression of Bcl-2 related proteins was investigated before severe apoptosis (Fig. 3A). B cell expression of Bcl-2 protein was unchanged as a result of culture with or without SCL. In contrast, Bcl-xL protein was not detected in fresh B cells, but was markedly up-regulated within 1 day of culture with SCL, but not when B cells were cultured alone. Increased B cell viability and decreased apoptosis were again observed upon coculture with SCL on day 3, but the changes were less marked because of the short length of the incubation. RA-SCL also rescued anti-IgM-stimulated B cells from apoptosis (Fig. 3B). Moreover,
RA-SCL up-regulated Bcl-x<sub>L</sub>, but not Bcl-2, expression by anti-IgM-stimulated B cells. Finally, the impact of coculture with other cell lines on B cell expression of Bcl-x<sub>L</sub> was examined (Fig. 4). Bcl-x<sub>L</sub> expression was more markedly up-regulated by coculture with RA-SCL. These results show that RA SCL can induce Bcl-x<sub>L</sub> protein expression in resting and stimulated B cells more effectively than in other cell lines tested and, in addition, can rescue B cells from apoptosis more effectively.

Direct contact is necessary for RA SCL-mediated rescue of B cell from apoptosis and induction of Bcl-x<sub>L</sub> in B cells

As shown in Fig. 5, both up-regulation of Bcl-x<sub>L</sub> and rescue of B cells from apoptosis were inhibited when direct contact between RA SCL and B cells was blocked by interposition of a 0.4-μm pore size membrane. Importantly, B cells in the upper chamber could not be rescued from apoptosis by soluble factors even when they were generated by RA SCL cultured with B cells in the lower chamber. It should be noted that the effect of the membrane separation appeared to be somewhat greater for up-regulation of Bcl-x<sub>L</sub> than for rescue from apoptosis, in that there was minimal up-regulation of Bcl-x<sub>L</sub> when the cells were separated, but there was some rescue from apoptosis. These results indicate that direct cell-to-cell contact between B cells and SCL cells is importantly involved in both up-regulation of Bcl-x<sub>L</sub> by B cells and rescue from apoptosis, with the former more dependent than the latter.

Interactions mediated by CD106 (VCAM-1) and CD49d/CD29 (VLA-4) play a significant role in the rescue of B cells from apoptosis and induction of Bcl-x<sub>L</sub>

The RA SCL expresses both CD54 and CD106 (22). Blocking CD54-mediated interactions with an mAb that blocks binding of both CD11a/CD18 and CD11b/CD18, had no effect on the ability of SCL to up-regulate B cell Bcl-x<sub>L</sub> and rescue B cells from apoptosis (Fig. 6). By contrast, an mAb to CD106 inhibited both SCL-induced up-regulation of Bcl-x<sub>L</sub> and rescue of B cells from apoptosis, but not completely.

Cross-linking CD49/CD29 rescues B cells from apoptosis and induces Bcl-x mRNA and protein

Cross-linking CD49/CD29 with mAb up-regulated Bcl-x<sub>L</sub> mRNA and protein in the absence of RA SCL and also rescued B cells from apoptosis. Cross-linking CD11a/CD18 modestly rescued B cells from apoptosis, but had no impact on expression of Bcl-x<sub>L</sub> (Fig. 7). Because of the smaller amount of Bcl-x<sub>L</sub> induced by cross-linking CD49d/CD29, it was only detected with a more prolonged exposure. In this circumstance, some Bcl-x<sub>L</sub> expression could also be detected in B cells cultured with medium alone (Expt. 1). It should be noted that mRNA and protein expressions were regulated in a qualitatively similar manner, although the quantitative effects were markedly different, with a much greater impact on mRNA levels noted. This suggests that there may be translational or post-translational regulation of Bcl-x<sub>L</sub> expression in these cells that is controlled differently from the regulation of mRNA levels.

Discussion

The results of this study indicate that SCL derived from RA synovial tissue can induce Bcl-x<sub>L</sub> and rescue peripheral blood B cells from apoptosis, and this ability of RA SCL is greater than that of OA SCL and skin fibroblasts. The data also indicate that a direct physical interaction between RA SCL and B cells mediated by the
correspondence pair VCAM-1 and VLA-4 provides signals that induce Bcl-xL and plays a role in the rescue of B cells from programmed cell death. A similar phenomenon in the rheumatoid synovium may contribute to the local accumulation and activation of B cells at this inflammatory site.

It has become apparent that maintenance of the viability of resting B cells is a dynamic process, involving the active countering of proapoptotic mechanisms. One of the major mechanisms to maintain the viability of lymphocytes involves the activity of the Bcell family of molecules that inhibit programmed cell death following mitochondrial disruption (25). Previous results had indicated that a population of SCL isolated from bone marrow or rheumatoid synovium could prevent apoptosis of resting B cells and also stimulate their differentiation into Ab-forming cells (22). These SCL constitutively expressed VCAM-1 (22). The current studies demonstrate that engagement of B cell VLA-4 by VCAM-1 expressed on SCL up-regulates Bcl-xL, an antiapoptotic Bcl-2 family member, and protects resting B cells from programmed cell death. These results are the first demonstration that engagement of VLA-4 on B cells can induce Bcl-xL and also the first mechanistic explanation of the means by which interaction with SCL can protect B cells from apoptosis.

In RA synovial tissue, minimal apoptosis of lymphocytes has been noted despite intensive infiltration (5). Previous reports have suggested that soluble factors produced by RA synovial fibroblasts might promote the viability of synovial T cells (26). However, the previous studies clearly showed that the viability of resting B cells could be maintained not by fibroblast cell lines, but, rather, by a specific population of synovial SCL (22). Moreover, the maintenance of B cell viability could not be explained by soluble factors produced by SCL. In the current studies, soluble factors produced by RA SCL could maintain B cell viability modestly, although the major contribution of RA SCL to the rescue of B cells from apoptosis involved direct cell-to-cell contact mediated by VCAM-1.

The mechanism, by which B cells are rescued from apoptosis, has been extensively investigated in secondary lymphoid tissue (20, 27–29). In germinal centers, B cell expression of Bcl-xL is increased (20, 30). Various combinations of surface molecules and cytokines have been reported to induce Bcl-xL in B cells or B cell lines. In peripheral B cells, CD40 ligand (CD154) and IL-13 can induce Bcl-xL (31). CD40 ligand and IgM cross-linking can also induce Bcl-xL on tonsil B cells and/or WEHI-231 cells (27, 32), whereas the combination of the polyclonal activator, SAC, and IL-10 can also induce Bcl-xL on tonsil B cells (29). Finally, anti-IgM stimulation induced Bcl-xL protein and rescued murine A20 cells from apoptosis (33). Besides interactions with Ag and activated T cells, direct contact with follicular dendritic cells (FDC) could contribute to B cell survival. In this regard, FDC and SCL have certain similarities, including the constitutive expression of the adhesion molecules, ICAM-1 and VCAM-1, and their involvement in spontaneous interactions with B cells (22). Because SCL do not express CD40 ligand (22), the possibility that adhesion molecules play a role in maintaining the viability of resting B cells was considered. The results of the mAb blocking experiments indicated that direct cell contact with SCL through VCAM-1-VLA-4 interactions is important in rescuing resting B cells from apoptosis.

In view of the central importance of VCAM-1-VLA-4 interactions between B cells and FDC (34–36), a similar process could be involved in up-regulating Bcl-xL and protecting against apoptosis in the germinal center. We have previously noted the functional and phenotypic similarities between SCL and FDC. However, there are differences between these cell types, including the observation that SCLs do not express CD21 and CD35 on their surface.

It should be noted that the finding that VCAM-1-mediated interactions could not block B cell apoptosis completely suggested that other mechanisms could be involved. SCL are known to produce IL-6, IL-8, GM-CSF, G-CSF, and hyaluronic acid and might also express surface molecules such as CD157 that could contribute to B cell survival (22, 37). Despite the possible contributions of these other molecules, it is clear that VCAM-1-VLA-4 interactions play a central role in the SCL-dependent rescue of resting B cells from apoptosis.

Bcl-xL expression is largely regulated at the level of gene transcription (25). In this regard, it has been reported that leukemia inhibitory factor induced Bcl-xL via the gp130 and STAT1 signaling pathway in cardiac myocytes (38). In addition, an erythropoietin-stimulated Jak2 kinase-initiated signal pathway has been reported to inhibit radiation-induced apoptosis and induce Bcl-xL in the DA3 murine myeloid cell line (39). However, there were no previous reports of a relationship between Bcl-xL expression and VLA-4 stimulation. VLA-4 stimulation has been reported to result in phosphorylation of focal adhesion kinase, which can then activate phosphatidylinositol 3-kinase and the generation of PIP3 (40–42). Subsequently, Akt (activated form serine/threonine kinase) can be phosphorylated and activated, and provide a cell survival signal (43). Besides this putative pathway of activation, the promotor region of human and mouse Bcl-xL contains several transcription binding sites, including sites for Ets-1, AP-4, NF-E2, Lyf-1, AP-1, Oct-1, GATA-1, and Evi-1, which are candidates to be involved in up-regulation of transcription (44). Current studies are focusing on the mechanism of Bcl-xL up-regulation following VLA-4 engagement.

Synovial stromal cells in RA have many potential functions. These cells induce B cell activation and Ig production and also secrete many cytokines and chemokines related to inflammation (22, 37, 45–48). Additionally, they can protect naive B cells from apoptosis by the VLA-4-VCAM-1-dependent mechanism, as described here. These finding suggest that synovial stromal cells may play a central role in propagating the inflammatory response characteristic of RA, especially the accumulation of B cells and their activation to produce Ig locally within the synovium.

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