in RA-SNC77 cells (Takeuchi *et al.*, unpublished observation). Other undefined adhesion molecules may therefore play a key role in inducing the enhanced proinflammatory cytokine production by RA-SNC77 cells.

We found that the VLA-4-dependent adhesion pathway was involved in both binding and transmigration of MC/car cells to a cloned stromal cell line, RA-SNC77. VCAM-1, a functional ligand for VLA-4, however, did not appear to contribute to these cellular interactions, suggesting that RA-SNC77 cells express an alternative ligand(s) for VLA-4. Other investigators have also reported the involvement of a VLA-4-dependent/VCAM-1-independent adhesion pathway in the interaction between bone marrow stromal cells and leukocytes [23,24]. Previously identified ligands for VLA-4 include VCAM-1 [25] and the CS-1 isoform of fibronectin [26]. The CS-1 isoform of fibronectin, which has been reported to be expressed in synovial tissues in RA patients [27,28], was detected at mRNA levels in RA-SNC77 cells, and it may function as a ligand for VLA-4, although further study is required to verify this issue.

In the inflamed RA synovial tissue, various inflammatory cytokines other than IL-6 or IL-8 are readily detected [18,29]. Certain proinflammatory cytokines, such as tumor necrosis factor alpha and IL-1, may participate in the dysregulated production of multiple cytokines in the RA synovial tissues. Although production of tumor necrosis factor alpha and IL-1 by RA-SNC cells is limited to low levels even after stimulation with lymphocytes [7], such regulatory cytokines may contribute the cell contact-dependent production of IL-6 and IL-8 observed in this study. Enhanced expression of proteolytic enzymes, such as cathepsins, matrix metalloproteases [30,31], and aggrecanases [32,33], is also seen in the inflamed RA synovial tissue, and these proteolytic enzymes are thought to be involved in the cartilage and joint destruction. Whether there is any functional link between the lymphocyte adhesion-driven cytokine production and the enhanced expression of the proteolytic enzymes in the RA synovium merits future investigation.

Burger *et al.* [11] recently demonstrated that, other than specialized nurse-like stromal cells, conventional fibroblast-like synoviocytes and IL-4-stimulated dermal fibroblast-like cells also can support pseudoemperipolesis of B lymphoid cells. They also found that, irrespective of their origin, the ability of fibroblastic cells to support B-cell pseudoemperipolesis is dependent on their expression of SDF-1 and VCAM-1, both of which are also detected in RA-SNC77 cells. These findings suggest that the specialized nurse-like cells and conventional fibroblast-like cells share some functional similarities to support B-cell pseudoemperipolesis while the nurse-like cells established from synovial tissues of patients with RA are distinct from other stromal cells derived from non-RA patients in both morphology and cellular functions, particularly proinflammatory cytokine production [7]. Further comparative studies are needed to characterize fibroblastic-stromal cells and nurse-like cells at molecular levels.

In summary, the present results indicate that lymphocyte binding *per se* is critical for enhanced proinflammatory cytokine production by RA-SNC77 cells. While transmigration of lymphocytes underneath the RA-SNC cells did not appear to play a significant role in the production of IL-6 and IL-8, this biological process may be involved in the production of other cytokines and/or proteinases. This nurse-like cell activity, which is seen in stromal cells isolated from the synovia of RA patients but not in those from disease-free controls, may alternatively influence the effector functions of infiltrated lymphocytes in RA synovia. Although VLA-4 is involved in both lymphocyte adhesion to and transmigration beneath RA-SNC cells through the interaction with non-VCAM-1 ligand(s), the VLA-4-independent adhesion pathway appears to be important for the cell contact-induced cytokine production by RA-SNC77 cells. Further investigation to identify the adhesion receptors necessary for cell contact-dependent activation of the nurse-like stromal cells may lead to novel therapeutic strategies through regulating the functions of the nurse-like stromal cells in RA patients.

Conclusion

Nurse-like stromal cell lines, which were established from the synovial tissue of patients with RA, abundantly secrete proinflammatory cytokines on coculture with lymphoid cells. We analyzed the molecular events required for the enhanced proinflammatory cytokine production by a RA-SNC line (RA-SNC77), and showed that VLA-4-independent lymphocyte adhesion alone, but not the subsequent Rho-GTPase-dependent transmigration of the lymphocytes, can induce the upregulated cytokine secretion by the nurse cells.

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Bone marrow CD34+ progenitor cells stimulated with stem cell factor and GM-CSF have the capacity to activate IgD− B cells through direct cellular interaction

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Abstract: Recent studies have suggested the involvement of bone marrow in the pathogenesis of rheumatoid arthritis (RA), in which proliferation of monocyte-lineage cells (MLC) as well as local B cell activation in the synovium play an important role. Here, we show that bone marrow-derived MLC have the capacity to activate human peripheral blood IgD− B cells. Bone marrow CD34+ cells from RA patients that had been stimulated with stem cell factor and GM-CSF for 3–4 weeks (>90% CD14+ HLA-DR+ cells, <0.5% CD19+ B cells, and <0.5% CD3+ T cells; MLC) induced the production of IgG much more effectively than that of IgM by highly purified B cells from healthy donors in the presence of IL-2 and IL-10. CD34+ cells from cord blood or from bone marrow of osteoarthritis patients also displayed the capacity to induce IgG production. The induction of IgG production by the bone marrow-derived MLC was markedly decreased when they were separated from B cells by a membrane filter. The bone marrow-derived MLC interacted preferentially with IgD− B cells to induce IgG production. These results indicate that upon stimulation with stem cell factor and GM-CSF, CD34+ progenitor cells differentiate into MLC that activate preferentially IgD− B cells through direct cellular interactions to produce IgG. Therefore, the data suggest that the accelerated recruitment of MLC from the bone marrow to the synovium might play a role in the local B cell activation in RA. *J. Leukoc. Biol.* 71: 987–995; 2002.

Key Words: *human · B lymphocytes · cell-to-cell interactions*

INTRODUCTION

Rheumatoid arthritis (RA) is characterized by chronic inflammation with infiltration of a variety of inflammatory cells, such as those of myeloid origin as well as T and B lymphocytes into the synovium. One of the characteristic features in RA is local B cell activation, leading to the production of large amounts of immunoglobulin (Ig) and autoantibodies [1]. Previous studies have suggested that extralymphoid B cell activation in the rheumatoid synovium may be promoted by fibroblast-like synoviocytes. Thus, B lymphocytes, some of which expressed proliferating cell nuclear antigen, were shown to be in intimate contact with synoviocytes in the subliming layers of the rheumatoid synovium [2], suggesting that such direct interactions might be important in promoting B cell responses in the rheumatoid synovium. Moreover, synoviocytes have been shown to support the terminal differentiation of activated B cells into Ig-secreting plasma cells [3] as well as the survival of B cells [4]. These results suggest a role for synoviocytes in facilitating local B cell responses in RA synovium.

It has been well known that synovial lining cells consist of macrophage-like type A synoviocytes and fibroblast-like type B synoviocytes. Recent studies have suggested that type A synoviocytes are derived from monocyte precursors in the bone marrow [5]. Moreover, it has been shown that the spontaneous generation of CD14+ monocyte-lineage cells (MLC) from bone marrow CD14− precursor cells is accelerated in RA, resulting in the facilitated entry of such CD14+ cells into the synovium [6]. More importantly, previous studies have disclosed that CD14+ cells derived from the bone marrow of RA patients have various influences on B cell activation. Thus, CD14+ cells generated from bone marrow CD14− precursors of RA patients have the capacity to stimulate the production of the-IgM rheumatoid factor selectively [7]. In addition, it has also been shown that CD14+ human leukocyte antigen (HLA)-DR+ cells generated from bone marrow CD34+ progenitor cells of RA patients have the capacity to support survival of B cells, leading to spontaneous transformation of Epstein-Barr virus positive B cell lines [8]. These results also suggest that bone marrow-derived synoviocytes might play a role in facilitating local B cell responses in the RA synovium. However, the precise mechanisms of B cell activation by bone marrow-derived cells have not been delineated completely.

The current studies were therefore undertaken in order to explore in detail the capacity of MLC induced by stimulation with stem cell factor (SCF) and granulocyte macrophage-colony stimulating factor (GM-CSF) from bone marrow CD34+ pro-

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genitor cells to activate and promote B cell responses. The results indicate that the GM-CSF-stimulated bone marrow CD34+ cells induce IgG production much more effectively than IgM production through direct interactions with IgD− B cells in the presence of interleukin (IL)-2 and IL-10. Therefore, the data support the conclusion that bone marrow-derived type A synoviocytes may also play a role in the local and systemic stimulation of memory B cell responses characteristic of RA.

MATERIALS AND METHODS

Patients

Bone marrow samples were obtained from 31 patients with active RA (4 males and 27 females; mean age, 59.7 years; range, 42–73 years) and from 11 patients with osteoarthritis (OA; 2 males and 9 females; mean age, 71.0 years; range, 57–81 years), who gave informed consent, during joint operations through intramedullary reaming by aspiration from a distal femoral canal prepared for implantation of the artificial femoral head or through aspiration from iliac crest. All 31 RA patients fulfilled the American College of Rheumatology (formerly the American Rheumatism Association) 1987 revised criteria for the disease [9]. Cord blood samples were obtained at nondiseased, normal deliveries upon informed consent for research use by parents.

Culture medium and reagents

RPMI-1640 medium (Life Technologies, Grand Island, NY), supplemented with penicillin G (100 units/ml), streptomycin (100 μg/ml), L-glutamine (0.3 mg/ml), and 10% fetal bovine serum (Life Technologies), was used for all cultures. Recombinant human GM-CSF, human IL-10, human SCF, and human BAFF [B cell activating factor belonging to the tumor necrosis factor (TNF) family] were purchased from PeproTech EC (London, UK). Recombinant human IL-2 (TGP-3) was a gift of Takeda Chemical Industries, Ltd. (Osaka, Japan), whose unit activity was determined by the providers (4.2×10^4 U/mg protein). A variety of monoclonal antibodies (mAb) were used, including anti-CD154 (a murine IgG1 mAb, clone 24–31; Ancell, Bayport, MN), anti-CD70 (a murine IgG1 mAb, clone BU69; Ancell), anti-CD106 (a murine IgG1 mAb, clone 1G11; Immunotech, Marseille, France), and a murine IgG1 control mAb MOPC 21 (Cappel Labs, West Chester, PA).

Preparation and culture of bone marrow CD34+ cells

Bone marrow or cord blood mononuclear cells were isolated by centrifugation of heparinized bone marrow aspirates over sodium diatrizoate-Ficoll gradients (Histopaque; Sigma Chemical Co., St. Louis, MO). CD34+ cells were purified from the mononuclear cells through positive selection using magnetic beads (Dynal CD34 progenitor cell-selection system; Dynal, Oslo, Norway). CD34+ cells thus prepared were approximately 95% CD34+ cells and <0.5% CD19+ B cells and were not found to have the altered phenotypic or functional features as described previously [10]. CD34+ cells were incubated in a 24-well microtiter plate with flat-bottomed wells (no. 3524, Costar, Cambridge, MA; $5.0–10.0\times10^4$/well) with the presence of SCF (10 ng/ml) and GM-CSF (1 ng/ml) for 3–4 weeks, at which time most of the cells become CD14+ HLA-DR+ [11]. RA bone marrow nurse-like cell clones (RA87 and RA91) were a generous gift of Dr. Ryuji Suzuki (Shionogi Co., Osaka, Japan).

Preparation of T cells and B cells

Peripheral blood mononuclear cells obtained from venous blood of healthy adult volunteers were depleted of monocytes and natural killer (NK) cells by treating them with 5 mM L-leucine methyl ester HCl (Sigma Chemical Co.) in serum-free RPMI 1640 (Life Technologies) [12]. Highly purified B cells and T cells were obtained from the treated cell population by rosetting with neuraminidase-treated sheep red blood cells, and the CD4+ T cell population was prepared further by a panning technique as described previously [13]. The B cells thus prepared contained <1% CD14+ monocytes, <1% CD2+ CD3+ T cells, <1% CD16+ NK cells, and >90% CD20+ B cells [14]. The CD4+ T cells contained <0.1% esterase-positive monocytes, <0.5% CD20+ B cells, <2% CD8+ cells, and >96% CD4+ T cells [14, 15]. In some experiments, B cells were fractionated further into IgD+ B cells and IgD− B cells using Dynal CELLection™ Pan Mouse IgG kit (Dynal) conjugated with anti-human IgD mAb (murine IgG1; Immunotech). IgD+ B cells thus prepared contained >90% IgD+ B cells. The characteristic features of the IgD+ B cells were comparable with those found in the previous study [16], as determined by stimulation with *Staphylococcus aureus* Cowan I and IL-2. Peripheral blood monocytes were prepared by glass dish adherence, as described previously [17].

Induction of in vitro production of IgM and IgG

Routine cultures were carried out in duplicate in a total volume of 200 μl in wells of a 96-well microtiter plate with round-bottomed wells (no. 3799, Costar). B cells ($5.0–10.0\times10^4$/well) were cultured with or without autologous monocytes (5×10^4/well), autologous CD4+ T cells ($1.0–2.0\times10^5$/well), or bone marrow CD34+ cells stimulated with SCF and GM-CSF ($2.5–5.0\times10^4$/well) in the presence or absence of IL-2 (0.1 U/ml) and IL-10 (10 ng/ml). The cells were incubated routinely for 10 days at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. In some experiments, bone marrow CD34+ cells (1×10^5/well) were stimulated with SCF and GM-CSF in a 24-well microtiter plate for 3–4 weeks, after which culture supernatant was replaced with fresh medium, and fresh B cells (5×10^5/well) were added with culture inserts so that B cells and bone marrow-derived MLC might be allowed to be in contact or be separated from each other.

Measurement of IgM and IgG

Microtiter plates (Dynex, Chantilly, VA) coated with $F(ab')_2$ fragments of goat anti-human IgM or anti-human IgG (Organon Teknika, Durham, NC) were incubated with cell-free culture supernatants or IgM or IgG standards in phosphate-buffered saline (PBS) containing 1% bovine serum albumin (Miles, Elkhart, IN). Bound IgM or IgG was detected with peroxidase-conjugated $F(ab')_2$ fragments of goat anti-human IgM or IgG (Organon Teknika) as described previously [18].

Immunofluorescence staining and analysis

Purified bone marrow CD34+ cells were expanded carefully with SCF (10 ng/ml) and GM-CSF (1 ng/ml) for 3–4 weeks in a 24-well microtiter plate with flat-bottomed wells (no. 3524, Costar; 1×10^5/well). After the incubation, the cells were stained with fluorescein isothiocyanate (FITC)-anti-CD19 mAb (mouse IgG1; Immunotech), FITC-anti-HLA-DR mAb (mouse IgG2b; Immunotech), phycoerythrin (PE)-conjugated anti-CD14 mAb (mouse IgG2a; Immunotech), PE-conjugated anti-CD3 mAb (mouse IgG1; Immunotech), or PE- or FITC-conjugated, isotype-matched control mAb (Dako, Glostrup, Denmark). Briefly, the cells were washed with 2% normal human serum in PBS, pH 7.2, and 0.1% sodium azide (staining buffer), and then the cells were stained with saturating concentrations of a variety of mAb at 4°C for 30 min. The cells were then washed three times with staining buffer and were fixed with 1% paraformaldehyde in PBS for at least 5 min at room temperature. The cells were analyzed using an EPICS XL flow cytometer (Coulter, Hialeah, FL) equipped with an argon-ion laser at 488 nm. A combination of low-angle and 90° light-scatter measurements (forward-scatter vs. side-scatter) was used to generate a bit-map gating to identify bone marrow cells using CYTO-TROL™ control cells (Coulter) and Immuno-Trol™ cells (Coulter) as standards, as described previously [11]. The percentages of cells stained positively for each mAb were determined by integration of cells above a specified fluorescence channel calculated in relation to the staining with isotype-matched control mAb.

RESULTS

Phenotypic analysis of bone marrow CD34+ cells stimulated with SCF and GM-CSF

Figure 1 shows the representative dual-parameter four-quadrant scattergrams of bone marrow CD34+ cells from an RA patient stimulated with SCF and GM-CSF for 3 weeks. At this time, the stimulated bone marrow cells did not express CD34 (unpublished

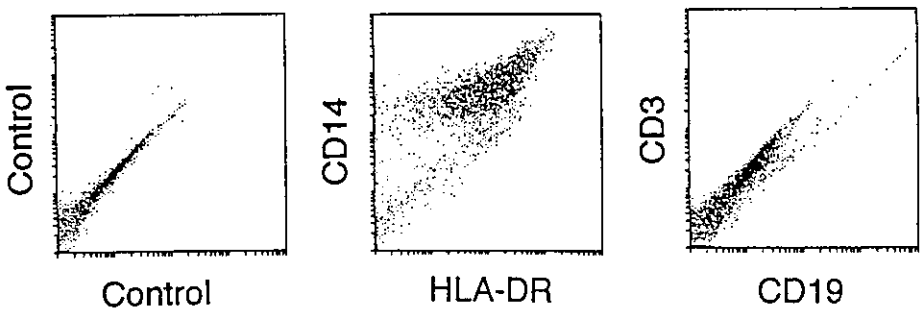

Fig. 1. Two-color flow cytometric analysis of the phenotypes of bone marrow CD34+ cells cultured in the presence of SCF and GM-CSF. CD34+ cells from the bone marrow of a RA patient (1×10^4/well) were cultured in the presence of SCF (10 ng/ml) and GM-CSF (1 ng/ml). After 3 weeks, the cells were harvested and stained with PE-conjugated anti-CD14, FITC-conjugated anti-HLA-DR, PE-conjugated anti-CD3, FITC-conjugated anti-CD19, and PE- or FITC-conjugated, isotype-matched control mAbs. The cells were then analyzed by flow cytometry.

results). More than 90% of the cells expressed CD14 and HLA-DR, whereas there were substantially no CD19+ cells or CD3+ cells (<0.5%) within these populations. Under microscopy, most of the stimulated bone marrow cells had the appearance of monocytes or dendritic cells with small numbers of fibroblast-like cells [11]. Bone marrow CD34+ cells from OA patients or cord blood CD34+ cells showed similar results (unpublished results). These results indicate that bone marrow CD34+ cells differentiate mostly into CD14+ HLA-DR+ MLC without contamination of CD3+ T cells or CD19+ B cells after stimulation with SCF and GM-CSF.

The capacity of bone marrow-derived MLC to induce Ig production by peripheral blood B cells

The next experiments examined the capacity of bone marrow-derived MLC to induce Ig production of peripheral B cells from normal, healthy individuals. Highly purified B cells from normal individuals were cultured with or without bone marrow-derived MLC in the presence or absence of IL-2 and IL-10. As shown in **Figure 2**, RA bone marrow-derived MLC induced the production of IgG modestly but not IgM in the presence of either IL-2 or IL-10. Of note, IL-2 and IL-10 synergistically enhanced IgG production induced by the bone marrow-derived MLC. Similar results were obtained when the bone marrow from the femoral head of an RA patient or that from the iliac crest of the same patient was used (unpublished results). Autologous peripheral blood monocytes also induced the production of IgG in the presence of IL-2 and IL-10 but less effectively than the bone marrow-derived MLC. The RA bone marrow-derived MLC or autologous monocytes alone stimulated by IL-2 and IL-10 could not produce IgG or IgM (unpublished results). These results indicate that RA bone marrow-derived MLC have the capacity to induce the production of IgG much more effectively than that of IgM by peripheral blood B cells. More importantly, the data indicate that the optimal induction of the IgG production by bone marrow-derived MLC requires the presence of IL-2 and IL-10. Furthermore, the data show that peripheral blood monocytes also retain the capacity to stimulate IgG production of B cells.

CD34+ cells from cord blood or from bone marrow of patients with OA have the capacity to induce IgG production after stimulation with SCF and GM-CSF

The next experiments explored whether the capacity of bone marrow-derived MLC to induce the production of IgG might be specific for RA. CD34+ cells were similarly purified from cord

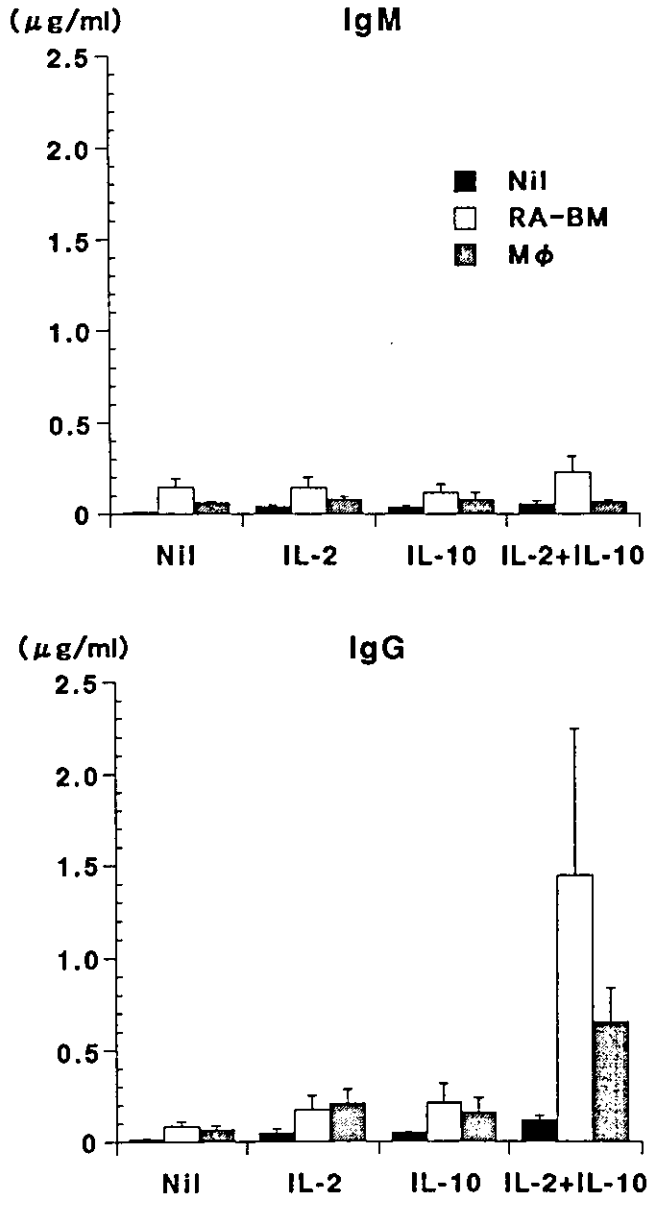

Fig. 2. MLC derived from RA bone marrow (BM) CD34+ cells induce IgG production of peripheral blood B cells in the presence of IL-2 and IL-10. B cells (1×10^5/well) were cultured with or without autologous monocytes (Mϕ) (5×10^4/well) or bone marrow CD34+ cells from an RA patient that had been stimulated with SCF (10 ng/ml) and GM-CSF (1 ng/ml) for 4 weeks (5×10^4/well). IL-2 (0.1 U/ml) and IL-10 (10 ng/ml) were added as indicated. After 10 days of incubation, the supernatants were harvested and were assayed for IgM and IgG contents by enzyme-linked immunosorbent assay (ELISA). The mean and SD values of the results of six independent experiments are shown.

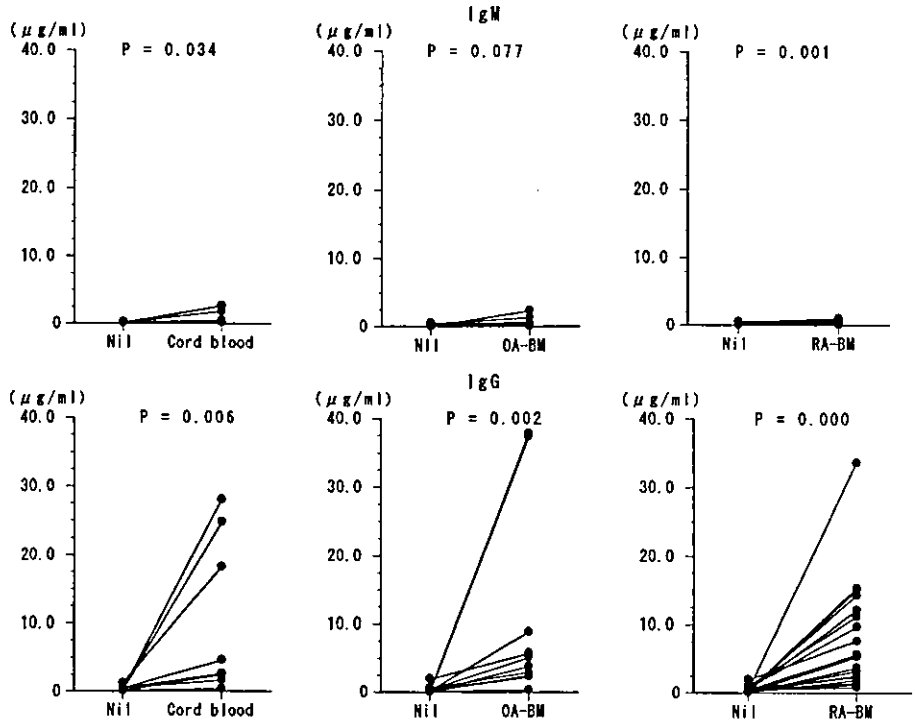

Fig. 3. The comparable capacity of MLC induced from cord blood CD34+ cells or RA or OA bone marrow CD34+ cells by stimulation with SCF and GM-CSF to induce IgG production of peripheral blood B cells. B cells (1×10^5/well) were cultured in the presence of IL-2 (0.1 U/ml) and IL-10 (10 ng/ml) with or without CD34+ cells obtained from bone marrow of 23 RA patients or 11 OA patients or from 8 cord blood samples that had been stimulated with SCF (10 ng/ml) and GM-CSF (1 ng/ml) for 3–4 weeks ($2.5–5.0\times10^4$/well). After 10 days of incubation, the supernatants were harvested and were assayed for IgM and IgG contents by ELISA. The increase in the amounts of IgM and IgG as a result of the addition of MLC induced from CD34+ cells was assessed by subtracting the production of IgM and IgG in cultures without the GM-CSF-stimulated CD34+ cells. The significance of the effects of MLC on the production of IgM and IgG was evaluated by Wilcoxon's signed rank test. The significance of the differences in the enhancement of Ig production among MLC derived from cord blood CD34+ cells and those from RA and OA bone marrow CD34+ cells was evaluated by Student's *t*-test.

blood or from the bone marrow of patients with OA and were stimulated with SCF and GM-CSF for 3–4 weeks. As shown in **Figure 3**, cord blood-derived MLC or OA bone marrow-derived MLC as well as RA bone marrow-derived MLC induced the production of IgG much more effectively than that of IgM. There were no statistically significant differences in the enhancement of Ig production among the three groups. The MLC derived from the RA or OA bone marrow or from the cord blood by themselves produced <10 ng/ml IgG or IgM upon stimulation with IL-2 and IL-10. Therefore, the results indicate that the capacity to induce IgG production by peripheral blood B cells is not specific for the RA bone marrow-derived MLC.

Bone marrow-derived dendritic cells have the capacity to induce IgG production

Previous studies revealed that bone marrow CD34+ cells from RA or OA patients or cord blood CD34+ cells stimulated with SCF and GM-CSF for 4 weeks have the appearance of monocytes, dendritic cells, and fibroblast-like cells [11]. We therefore examined whether bone marrow-derived dendritic cells and nurse-like cells with fibroblast-like morphology might induce the production of IgG from peripheral blood B cells. Bone marrow-derived dendritic cells were induced by stimulation of bone marrow CD34+ cells with SCF, GM-CSF, and IL-4 for 4 weeks as described previously [11]. Nurse-like cells were cloned from RA bone marrow cells as described previously [4]. As can be seen in **Table 1**, bone marrow-derived dendritic cells induced the production of IgG as effectively as bone marrow-derived MLC. By contrast, RA nurse-like cells (RA87 and RA91) did not induce the production of IgM or IgG. Therefore, these results indicate that bone narrow-derived dendritic cells, but not nurse-like cells, also have the capacity to induce IgG production from peripheral blood B cells.

The induction of IgG production by bone marrow-derived MLC requires direct interactions with peripheral blood B cells

The next experiments were carried out to examine whether direct interactions between bone marrow-derived MLC and B cells are required for the induction of the IgG production.

TABLE 1. Comparison of the Effects of Various Bone Marrow-Derived Cells on the Production of IgM and IgG

Expt.	Addition of bone marrow-derived cells	Ig production (μg/ml) IgM	IgG
1.	Nil	0.179	0.781
	bone marrow MLC	0.163	11.170
	bone marrow dendritic cells	0.455	29.825
2.	Nil	0.113	0.092
	bone marrow MLC	0.352	1.708
	bone marrow dendritic cells	0.284	1.025
3.	Nil	0.030	0.024
	bone marrow MLC	0.120	0.896
	RA87	0.040	0.066
	RA91	0.066	0.049
4.	Nil	0.036	0.053
	bone marrow MLC	0.095	1.052
	RA87	0.035	0.101
	RA91	0.097	0.156

B cells (1×10^5/well) were cultured with RA bone marrow CD34+ cells that had been stimulated for 3-4 weeks with SCF (10 ng/ml) and GM-CSF (1 ng/ml) (MLC) or with SCF, GM-CSF, and IL-4 (10 ng/ml) (dendritic cells) (Expts. 1 and 4: 5×10^4/well; Expts. 2 and 3: 2.5×10^4/well) or with cloned, nurse-like cells (RA87 and RA91; 2.5×10^4/well) in the presence of IL-2 (0.1 U/ml) and IL-10 (10 ng/ml). After 10 days of incubation, the supernatants were harvested and assayed for IgM and IgG contents by ELISA.

Highly purified B cells were cultured with the RA bone marrow-derived MLC in the presence of IL-2 and IL-10 such that they were separated by a filter membrane or were allowed to be in contact with each other. As can be seen in **Figure 4**, the production of IgG was decreased markedly when bone marrow-derived monocytes were physically separated from B cells. These results therefore confirm that stimulation of IgG production of peripheral blood B cells requires direct cellular interactions between B cells and bone marrow-derived MLC. It should be noted, however, that there was already increased production of IgG in the presence of IL-2 and IL-10 even when B cells were separated from MLC. It is therefore suggested that there might be an additional soluble factor produced by MLC that enhances IgG production.

The collaboration between bone marrow-derived monocytes and B cells does not involve CD154-CD40 interactions

Some studies have shown that CD154 is expressed on activated T cells and plays a critical role in their capacity to provide contact-dependent help to resting B cells [19, 20]. Of note, mRNA for the human CD154 also has been found to be expressed in mast cells and basophils [21] as well as in NK cells and monocytes [22]. Therefore, it was possible that the interactions between bone marrow-derived MLC and peripheral blood B cells might involve CD154. To address this point, experiments were carried out in which bone marrow-derived MLC and B cells were cocultured with IL-2 and IL-10 in the presence of anti-CD154 mAb or control mAb. As shown in **Table 2**, anti-CD154 did not inhibit the production of IgG induced by bone marrow-derived MLC, although anti-CD154 inhibited Ig production of B cells induced by autologous CD4+ T cells. These results indicate that the interactions between bone marrow-derived MLC and B cells, which lead to IgG production, do not involve the CD154 molecule. Of note, it has been shown that CD27-CD70 interactions are also involved in T cell-dependent B cell activation [23]. However, anti-CD70 did not inhibit the IgG production induced by bone marrow-derived MLC, although it inhibited Ig production stimulated by T cells. It was therefore unlikely that the bone marrow-derived MLC were contaminated with activated T cells. Of note, previous studies showed that bone marrow-derived nurse-like cells can rescue B cells from spontaneous apoptosis and facilitate Ig production in a mechanism that was blocked by anti-CD106 mAb [4]. However, anti-CD106 did not inhibit the IgG production by bone marrow-derived MLC either. In this regard, the interactions between bone marrow-derived MLC and B cells to induce IgG production are different from those between bone marrow-derived nurse-like cells and B cells as described previously [4]. In fact, bone marrow-derived nurse-like cells did not induce IgG production in the presence of IL-2 and IL-10 (Table 1).

Bone marrow-derived MLC interact preferentially with peripheral blood IgD− B cells

Bone marrow-derived MLC induced the production of IgG much more effectively than that of IgM. It was therefore possible that the bone marrow-derived MLC might induce class switch. To test this hypothesis, we conducted experiments in which peripheral blood B cells were fractionated into IgD+ B cells and IgD− B cells. The capacity of bone marrow-derived MLC to induce the production of IgM and IgG from each population was then examined. As shown in **Figure 5**, the RA bone marrow-derived MLC induced the production of IgG, but very modestly that of IgM, from unfractionated B cells as well as from IgD− B cells. Although IgD+ B cells produced substantial amounts of IgM in the presence of IL-2 and IL-10, the bone marrow-derived MLC did not affect it significantly. Moreover, the bone marrow-derived MLC induced the production of IgG from IgD+ B cells very modestly. Rather, the

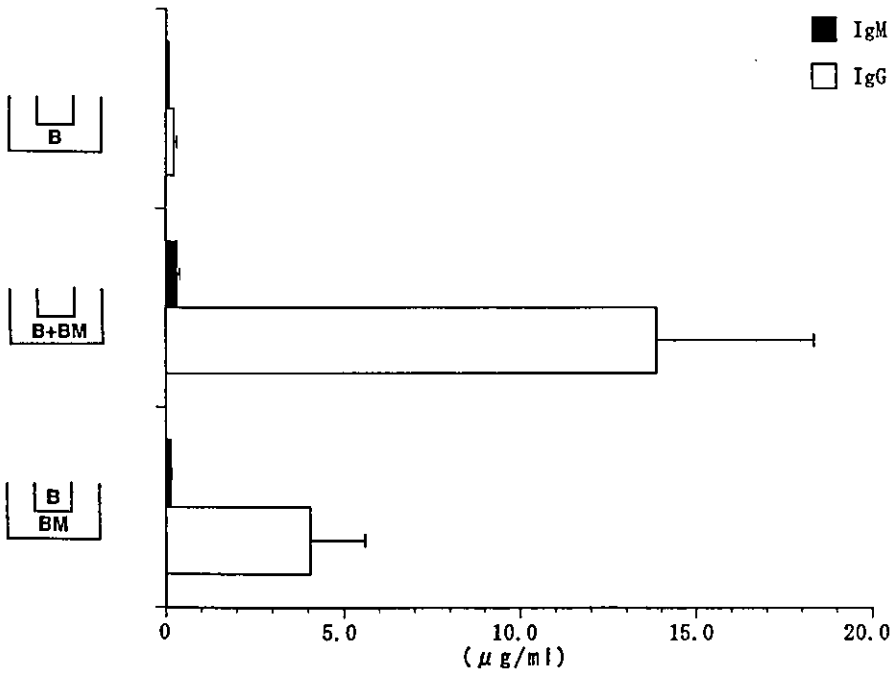

Fig. 4. Optimal induction of IgG production by bone marrow-derived MLC requires physical contact between bone marrow-derived MLC and B cells (B). B cells (5×10^5/well) were cultured in the presence of IL-2 (0.1 U/ml) and IL-10 (10 ng/ml) with or without bone marrow CD34+ cells (1×10^5/well) from RA patients that had been stimulated with SCF (10 ng/ml) and GM-CSF (1 ng/ml) for 3–4 weeks in wells of 24-well microtiter plates equipped with an inner chamber with membrane filter so that B cells (B) and the bone marrow-derived MLC (BM) might be allowed to be in contact or be separated from each other. The cultures were carried out in a total volume of 1 ml. After 10 days of incubation, the supernatants were harvested and were assayed for IgM and IgG contents by ELISA. The mean and SD values of five independent experiments are shown.

TABLE 2. Differential Effects of Anti-CD154 mAb on the Induction of the Production of IgG by Bone Marrow-Derived MLC and by CD4+ T cells

Expt.	mAb	IgM production (μg/ml)			IgG production (μg/ml)		
		Nil	RA-BM MLC	CD4+ T cell	Nil	RA-BM MLC	CD4+ T cell
1.	Control IgG1	0.024	0.227	2.047	0.053	4.135	2.683
	Anti-CD154	0.046	0.131	1.010	0.057	4.908	0.813
	Anti-CD106	0.068	0.288	3.147	0.047	6.070	1.931
2.	Control IgG1	0.173	0.167	4.919	0.125	1.420	2.289
	Anti-CD154	0.137	0.299	3.269	0.053	1.720	1.156
	Anti-CD106	0.161	0.656	4.324	0.157	2.005	1.734
3.	Control IgG1	0.016	0.286	1.197	0.065	3.192	1.625
	Anti-CD154	0.050	0.225	0.079	0.030	4.073	0.230
4.	Control IgG1	0.095	0.252	1.002	1.909	17.966	16.506
	Anti-CD154	0.074	0.350	0.763	1.638	21.241	14.129
	Anti-CD70	0.101	0.329	0.600	1.787	19.350	8.510
5.	Control IgG1	0.038	0.094	0.324	0.064	3.439	1.223
	Anti-CD154	0.063	0.088	0.182	0.173	5.235	0.759
	Anti-CD70	0.044	0.073	0.259	0.144	3.130	0.521

B cells (1×10^5/well) were cultured with RA bone marrow (BM) CD34+ cells that had been stimulated with SCF (10 ng/ml) and GM-CSF (1 ng/ml) for 3–4 weeks (Expts. 1 and 2: 5×10^4/well; Expts. 3–5: 2.5×10^4/well) or with autologous CD4+ T cells (Expts. 1 and 2: 2×10^5/well; Expts. 3–5: 1×10^5/well) in the presence of IL-2 (0.1 U/ml) and IL-10 (10 ng/ml). Bone marrow cells in Expt. 5 were fixed with paraformaldehyde before culture. Various mAb were added where indicated (10 μg/ml). After 10 days of incubation, the supernatants were harvested and assayed for IgM and IgG contents by ELISA.

induction of IgG production by the bone marrow-derived MLC turned out to be dependent on the enhancement of IgG production of IgD− B cells. These results obviate the possibility that bone marrow-derived MLC might induce class switch. Moreover, the data demonstrate that bone marrow-derived MLC interact preferentially with IgD− B cells to induce IgG production.

Finally, we compared the capacity of the bone marrow-derived MLC and BAFF to induce the production of IgM and IgG. As shown in **Figure 6A**, soluble BAFF, but not bone marrow-derived MLC, enhanced the production of IgM significantly from IgD+, IgD−, and unfractionated B cells. By contrast, bone marrow-derived MLC enhanced the production of IgG significantly from IgD+, IgD−, and unfractionated B cells, whereas soluble BAFF enhanced IgG production of IgD− and unfractionated B cells. Of note, bone marrow-derived MLC enhanced the production of IgG from IgD+, IgD−, and unfractionated B cells more effectively than soluble BAFF (Fig. 6B). The results suggest that the interactions between B cells and bone marrow-derived MLC might be different from

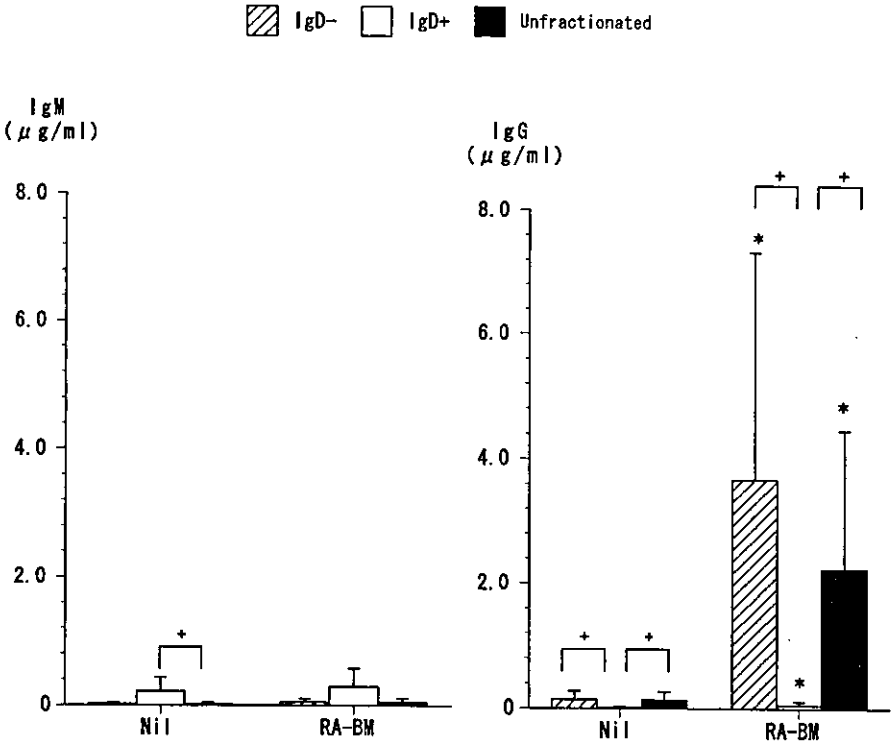

Fig. 5. Bone marrow-derived MLC activate IgD− B cells preferentially to produce IgG. Unfractionated, IgD+, or IgD− B cells (1×10^5/well) were cultured in the presence of IL-2 (0.1 U/ml) and IL-10 (10 ng/ml) with or without bone marrow CD34+ cells from RA patients that had been stimulated with SCF (10 ng/ml) and GM-CSF (1 ng/ml) for 3 weeks. After 10 days of incubation, the supernatants were harvested and were assayed for IgM and IgG contents by ELISA. The mean and SD values of six experiments are shown. The statistical significance was evaluated by Wilcoxon's signed rank test. *, Significant at $P < 0.05$ as compared with cultures without RA bone marrow-derived MLC. +, Significant at $P < 0.05$.

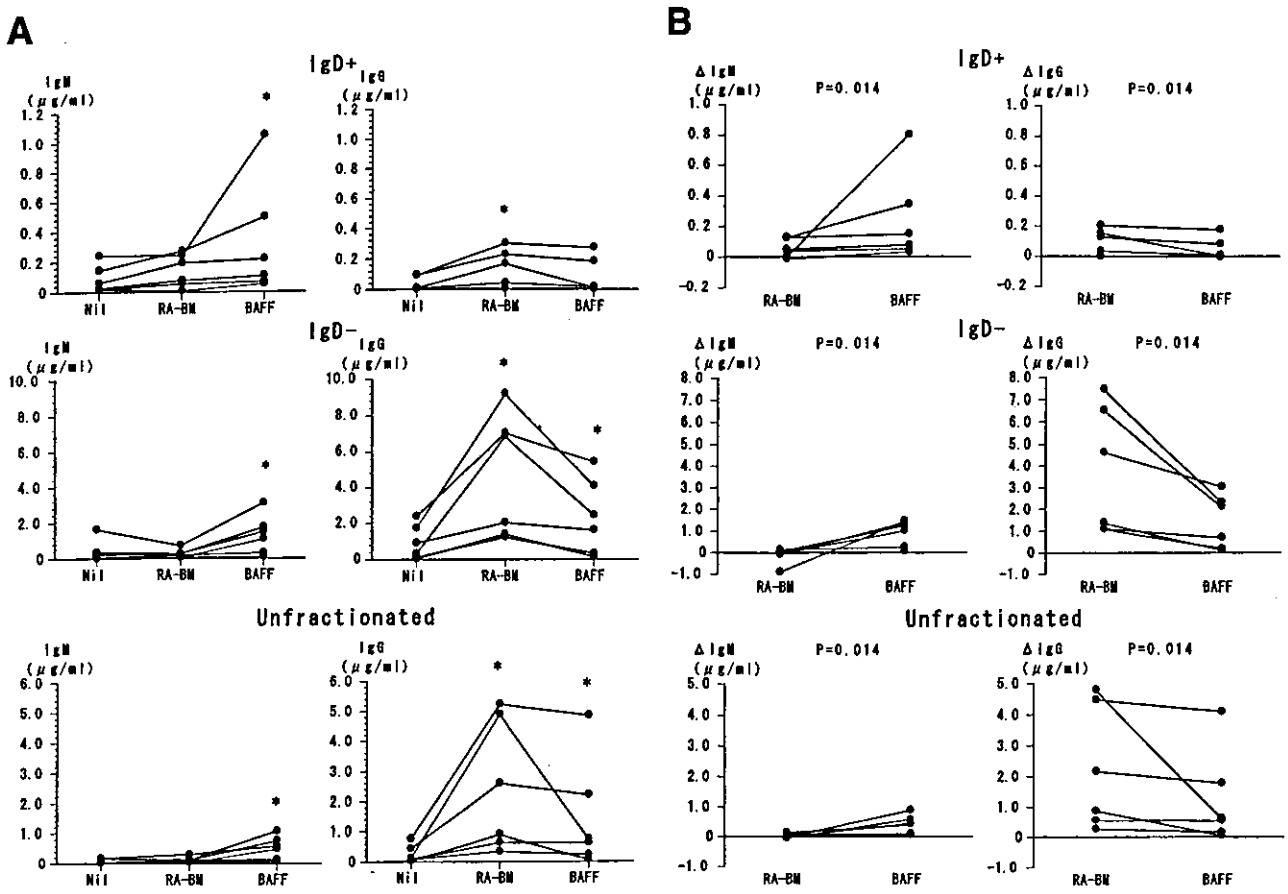

Fig. 6. Differential effects of bone marrow-derived MLC and BAFF on the production of IgM and IgG. (A) Unfractionated, IgD+, or IgD− B cells (1×10^5/well) were cultured with RA bone marrow CD34+ cells that had been stimulated with SCF (10 ng/ml) and GM-CSF (1 ng/ml) for 3 weeks (2.5×10^4/well) or with soluble BAFF (10 ng/ml) in the presence of IL-2 (0.1 U/ml) and IL-10 (10 ng/ml). After 10 days of incubation, the supernatants were harvested and assayed for IgM and IgG contents by ELISA. The statistical significance was evaluated by Wilcoxon's signed rank test. *, Significant at $P < 0.05$ as compared with cultures without BAFF or RA bone marrow-derived MLC. (B) Comparison of the enhancement of the production of IgM and IgG from IgD+, IgD−, and unfractionated B cells by BAFF or RA bone marrow-derived MLC. ΔIgM/ΔIgG were calculated by subtracting the production of IgM/IgG in cultures without BAFF or RA bone marrow-derived MLC from that in cultures with BAFF or RA bone marrow-derived MLC. The statistical significance was evaluated by Wilcoxon's signed rank test.

those involving BAFF in that bone marrow MLC and BAFF display differential capacities to induce the production of IgM and IgG.

DISCUSSION

The results in the current studies demonstrate clearly that after stimulation with SCF and GM-CSF for 3–4 weeks, bone marrow CD34+ cells give rise to MLC, which display the capacity to activate resting peripheral blood B cells to produce IgG. Of note, the IgG production was decreased markedly when the bone marrow-derived MLC were physically separated from B cells by a filter membrane. Moreover, the capacity of the bone marrow-derived MLC to stimulate IgG production was preserved after fixation with paraformaldehyde. Therefore, the results indicate that activation of B cells by bone marrow-derived MLC requires direct contact-dependent interactions. In this regard, the activation of B cells by bone marrow-derived MLC is comparable to that by activated T cells [13]. Conversely, it should be pointed out that bone marrow CD34+ cells stimulated with SCF, GM-CSF, and IL-4, which have the characteristics of dendritic cells morphologically and phenotypically [11], also displayed the comparable capacity to induce the production of IgG. Because several studies showed that dendritic cells are generated from bone marrow-derived monocytes [24–26], the data indicate that the capacity to induce IgG production is preserved after differentiation of MLC into dendritic cells.

Analysis on flow cytometry revealed that more than 90% of bone marrow CD34+ cells stimulated with SCF and GM-CSF were CD14+ HLA-DR+. There were less than 0.5% of CD3+ cells or CD19+ cells within these populations. Moreover, no CD3+ cells were detected in 5- to 10-day cultures of the bone marrow-derived MLC and B cells in the presence of IL-2 and IL-10 (unpublished results). Therefore, it is most likely that CD14+ HLA-DR+ MLC, but not contaminating T cells, can activate resting B cells. In fact, the activation of B cells induced by bone marrow-derived MLC is different from that induced by activated T cells in several aspects. For example, it has been disclosed that the activation of B cells by activated T cells induces the production of IgM and IgG [27], as has also been shown in the present study. By contrast, the activation of B cells by bone marrow-derived MLC induced the production

of IgG, but very modestly that of IgM. Furthermore, B cell activation induced by activated T cells was inhibited by anti-CD154 mAb as well as anti-CD70 mAb as is consistent with previous studies [23, 28], whereas B cell activation induced by bone marrow-derived MLC was not blocked by anti-CD154 mAb or anti-CD70 mAb.

Of note, previous studies disclosed that nurse-like cells from bone marrow and synovium of RA patients promote survival and enhance the function of human B cells [4]. Although most of bone marrow CD34+ cells stimulated with SCF and GM-CSF were phenotypically CD14+ HLA-DR+, it was possible that this population was contaminated with nurse-like cells. It should be noted that the function of bone marrow-derived nurse-like cells to support B cell responses was blocked by anti-CD106 mAb [4]. However, anti-CD106 mAb did not inhibit the production of IgG bone marrow-derived MLC. Moreover, bone marrow-derived nurse-like cell clones could not elicit the production of IgG from peripheral blood B cells in the presence of IL-2 and IL-10. It is therefore unlikely that the capacity of the bone marrow-derived MLC to induce IgG production might be mediated by contaminating nurse-like cells. Taken together, the data suggest that the activation of B cells induced by bone marrow-derived MLC might involve unique cellular interactions that have not been identified yet.

Recent studies have disclosed the presence of a novel ligand of the TNF family designated BAFF [29] or BLyS (B-lymphocyte stimulator) [30] on T cells [29], dendritic cells [29], and monocytes [30]. BAFF and BLyS have been found to induce proliferation of peripheral blood B cells stimulated with anti-IgM or *S. aureus* Cowan I, whereas they were unable to activate resting B cells without stimulation of anti-IgM or *S. aureus* Cowan I [29, 30]. Therefore, it is suggested that BAFF and BLyS might be only a costimulator of B cells, although they play an important role in monocyte-driven B cell activation [29, 30]. Moreover, treatment of BALB/cAnNCR mice by BLyS resulted in the elevation of IgM and IgA, but not IgG in the serum [30]. Of note, in the present study, bone marrow-derived MLC induced IgG production more effectively than BAFF, although they were less potent in inducing IgM production than BAFF. Consistently, the results disclosed that soluble BAFF alone induced the production of IgM by IgD+ B cells as well as that by IgD− B cells in the presence of IL-2 and IL-10. Thus, the results demonstrate for the first time that beyond its role as a costimulator of B cells shown in the previous studies [29, 30], BAFF by itself can induce Ig production from resting B cells in the presence of IL-2 and IL-10. However, the data suggest that the interactions between B cells and bone marrow-derived MLC shown in the current studies might be different from those that involve BAFF or BLyS.

Previous studies have shown that dendritic cells provide naive B cells with signals that are essential for class switch [31]. In the present study, the bone marrow-derived MLC were found to contain a small proportion of cells of dendritic cell morphology (unpublished results), which are considered to correspond to $CD14^{dull}$ $HLA\text{-}DR^{bright}$ cells on flow cytometry [32]. Thus, it was possible that such dendritic cells within the bone marrow-derived MLC might induce class switch in naive B cells. In fact, bone marrow-derived cells, which had been generated by stimulation of bone marrow CD34+ cells with SCF, GM-CSF, and IL-4, also induced the production of IgG. However, the bone marrow-derived MLC only modestly stimulated IgD+ B cells to produce IgG in the presence of IL-2 and IL-10. Moreover, IgG production could not be elicited from IgD+ B cells or cord blood B cells even when they had been cocultured with the bone marrow-derived MLC and thereafter restimulated with immobilized anti-CD3-activated CD4+ T cells (unpublished results). Therefore, the results indicate that the bone marrow-derived MLC in the current studies do not provide signals essential for class switch. Rather, the data support the conclusion that the bone marrow-derived MLC stimulate IgD− B cells preferentially through direct cellular interactions. Thus, it is suggested that IgD− B cells might express such molecules that are involved in the interactions with bone marrow-derived MLC more abundantly than IgD+ B cells.

Of note, the optimal IgG production induced by bone marrow-derived MLC required the presence of IL-2 and IL-10. Previous studies showed that IL-2 and IL-10 synergistically enhance Ig production of B cells stimulated with anti-CD40 or *S. aureus* Cowan I [33, 34]. Moreover, it has been shown that the synergistic effects of IL-2 and IL-10 involve a mechanism that is different from the up-regulation of the expression of IL-2 receptors [34]. In fact, IL-2 and IL-10 still displayed additive effects on Ig production in the presence of high doses of cyclosporine that blocked the IL-10-mediated up-regulation of IL-2 receptors, indicating that IL-2 and IL-10 provide signals that are mutually independent [34]. Thus, it is possible that the production of IgG induced by bone marrow-derived MLC might require two mutually independently signals delivered by IL-2 and IL-10. Further studies to delineate the nature of signals in B cells delivered by IL-2 and IL-10 would be important for a complete understanding of the mechanism of synergy between IL-2 and IL-10 as well as the mechanism of activation of memory B cells by bone marrow-derived MLC.

It has been shown that the spontaneous generation of CD14+ MLC from bone marrow CD14− precursor cells is accelerated in RA [6], possibly resulting in the facilitated entry of such CD14+ cells into the synovium [6]. Although the features of bone marrow-derived MLC shown in the current studies were not specific for RA, it is likely that the promoted entry of such cells into the synovium might result in the sustained activation of B cells. It is therefore likely that the capacity of bone marrow-derived MLC to activate B cells might play a role in the local B cell activation and the collection of plasma cells in the synovium, which are characteristic features in RA [35–37].

In summary, the results in the current studies disclosed that bone marrow-derived MLC generated from CD34+ progenitor cells by stimulation with SCF and GM-CSF displayed the capacity to stimulate IgD− B cells preferentially to produce IgG through as-yet undetermined contact-dependent interactions in the presence of IL-2 and IL-10. Further studies to explore the nature of such interactions would be important for our understanding of the pathogenesis of RA as well as the mechanisms of monocyte-driven B cell activation.

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Expression of Extracellular Matrix Metalloproteinase Inducer and Enhancement of the Production of Matrix Metalloproteinases in Rheumatoid Arthritis

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Objective. **To investigate the expression of extracellular matrix metalloproteinase inducer (EMMPRIN) at sites of joint destruction in rheumatoid arthritis (RA) and to correlate it with the production of matrix metalloproteinases (MMPs).**

Methods. **Reverse transcription–polymerase chain reaction was performed to study the existence of EMMPRIN in synovial tissue derived from RA and osteoarthritis (OA) patients. In situ hybridization with a human complementary DNA specific for EMMPRIN and immunohistochemistry were performed to characterize the EMMPRIN-expressing cells at sites of joint destruction, including bone. Northern blot analysis was performed to detect the level of expression of EMMPRIN messenger RNA (mRNA) in synovial tissue. The production of MMP-1 and MMP-3 by synovial tissue from RA patients was examined by enzyme-linked immunosorbent assay.**

Results. **Expression of EMMPRIN mRNA was detected in synovium from 9 of 11 patients with RA and 1 of 5 patients with OA. The presence of mRNA encoding EMMPRIN was recognized in the invasive synovium at sites of joint destruction in RA but not OA. Fibroblast-like synovial cells and granulocytes were demonstrated to express EMMPRIN mRNA. MMP-1 and MMP-3 production by synovial tissue was correlated with levels of expression of EMMPRIN mRNA, as detected by Northern blotting.**

Conclusion. **The expression of EMMPRIN stimulates the production of MMP-1 and MMP-3 in the synovial tissue of affected joints in RA. The results of this study suggest that EMMPRIN may be one of the important factors in progressive joint destruction in RA.**

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by progressive joint destruction. Previous reports have demonstrated that at sites of joint destruction, abnormally expressed matrix metalloproteinases (MMPs) are involved (1–4). Joint degradation involves damage to articular cartilage caused by inflammatory cells, activated fibroblasts in the synovial membrane, and chondrocytes. Several cytokines have been shown to play a role in activating the cells that produce MMPs (5).

MMPs degrade collagens, proteoglycans, and other matrix macromolecules in bone as well as in articular cartilage. Notably, it has been shown that collagenase 1 (MMP-1) and stromelysin 1 (MMP-3) are produced by fibroblasts and macrophage-like cells in the synovium and pannus and have been found to be important in the pathologic destruction of joints in patients with RA (3,4). Suppression of MMPs may be a potential alternative therapeutic target for the treatment of joint destruction in RA. However, the precise pathomechanism of MMP production at the site of joint destruction remains partly unknown.

Extracellular matrix metalloproteinase inducer (EMMPRIN; formerly called tumor cell–derived collagenase stimulatory factor) is a 57-kd transmembrane glycoprotein that is a member of the immunoglobulin superfamily located on the surface of human tumor cells

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and normal keratinocytes (6). EMMPRIN interacts with fibroblasts to stimulate the expressions of several MMPs associated with tissue degradation and remodeling during tumor invasion and wound healing. The expression of EMMPRIN has been shown to be up-regulated in the synovial membrane of RA patients (7). However, the contribution of EMMPRIN to joint destruction in RA is still unknown.

In the present study, we investigated the expression of EMMPRIN and characterized EMMPRIN-expressing cells at sites of joint destruction in RA. We also analyzed the correlation of EMMPRIN expression with the activities of MMP-1 and MMP-3 in synovial tissue.

PATIENTS AND METHODS

Patients and specimens. Joint specimens were obtained from 11 patients with RA who were undergoing joint reconstruction surgery at Osaka University Hospital. All RA patients satisfied the 1987 revised diagnostic criteria of the American College of Rheumatology (formerly, the American Rheumatism Association) (8). Joint specimens were also obtained from 5 patients with osteoarthritis (OA) for use as controls. All study patients gave their informed consent.

Reverse transcription—polymerase chain reaction (RT-PCR). Using a Fast Track messenger RNA (mRNA) isolation kit (Invitrogen, San Diego, CA), RNA was extracted from synovial tissue. The isolated total RNA (3 mg) was reverse transcribed to complementary DNA (cDNA) with a Ready to Go T-Primed First-Strand kit (Amersham Pharmacia Biotech, Buckinghamshire, UK). The completed first-strand cDNA was amplified by PCR with primers specific for EMMPRIN (sense 5′-ACATCAACGAGGGGGAGACG-3′ and antisense 5′-GGCTTCAGACAGGCAGGACA-3′). As a control, β-actin mRNA was used.

Amplification was performed by adding 28.7 ml of sterile diethyl pyrocarbonate–water, 8 ml of 1.25 m*M* of each dNTP mix, 0.25 ml of 10× PCR buffer, and 0.25 ml of AmpliTaq DNA polymerase at 5 units/ml to 3 ml of each completed first-strand reaction. The reaction tubes were heated to 94°C for 5 minutes and incubated in a GeneAmp 1000 PCR system (Perkin Elmer, Foster City, CA) thermal cycler using 28 cycles of 94°C for 30 seconds, 62°C for 30 seconds, and 72°C for 60 seconds. Finally, the samples were incubated at 72°C for 10 minutes. PCR products were electrophoresed on 1.5% agarose gels and visualized by ethidium bromide staining.

Tissue samples. Tissue samples were prepared as previously described (9). Tissue samples with invasive synovium with bone were fixed in 4% paraformaldehyde (Merck, Darmstadt, Germany) in phosphate buffered saline (PBS), pH 7.4 (Sigma, St. Louis, MO), decalcified in 20% EDTA, dehydrated in an ethanol series, and embedded in paraffin. Serial sections (5 μm) were cut with a microtome, and some were stained with hematoxylin and eosin. The remaining sections were prepared for in situ hybridization (ISH) and immunohistochemistry.

In situ hybridization. ISH was carried out as previously described (9). The sections were dewaxed, incubated with 1 mg of proteinase K in TE (0.1*M* Tris, pH 8.0, 50 μ*M* EDTA, pH 8.0) at 37°C for 10 minutes, and fixed with 4% paraformaldehyde in 0.1*M* PBS for 20 minutes at room temperature. The sections were then treated with 0.2*N* HCl to inactivate endogenous alkaline phosphatase, acetylated with 0.25% acetic anhydride in 0.1*M* triethanolamine (pH 8.0), dehydrated again with an ethanol series, and air-dried. Hybridization was performed at 50°C for 16 hours in a moist chamber with 50 μl of hybridization solution (50% deionized formamide, 10% Dextran sulfate, 1× Denhardt's solution), 4× SSC (0.15*M* NaCl, 0.015*M* sodium citrate), and 150 mg of *Escherichia coli* transfer RNA containing ~0.5 mg/ml of RNA probe on each section.

After hybridization, the sections were washed briefly in 5× SSC and in 50% formamide, 2× SSC at 50°C for 30 minutes and then rinsed in 1× TES (10 m*M* Tris HCl, pH 7.6, 1 m*M* EDTA, 0.5*M* NaCl) at 37°C for 15 minutes. The sections were then treated with RNase A (10 mg/ml of 1× TES) at 37°C for 30 minutes, rinsed in 1× TES for 15 minutes at 37°C, and washed twice at 50°C for 20 minutes each with 2× SSC and 0.2× SSC.

Hybridized digoxigenin (DIG)–labeled probes were detected with the aid of a nucleic acid detection kit (Boehringer Mannheim, Mannheim, Germany). DIG–11-UTP–labeled single-stranded complementary RNA was prepared with a DIG RNA labeling kit (Boehringer Mannheim). A human EMMPRIN cDNA was obtained by PCR, and was subcloned into pGEM-T plasmid. The plasmid was linearized by *Sac* II and transcribed by SP6 RNA polymerase to generate a 0.851-kb–long antisense probe. The plasmid was also linearized by *Spe* I and transcribed by T3 RNA polymerase to generate a sense probe.

Immunohistochemical staining. To identify the cells expressing EMMPRIN mRNA on ISH, immunohistochemical staining was performed by the streptavidin–peroxidase technique using Histofine streptavidin–biotin–peroxidase kits (Nichirei, Tokyo, Japan). Monoclonal antibody (mAb) against CD3 (Nichirei) was used to detect T cells, mAb against CD15 (Dako, Santa Barbara, CA) was used to detect granulocytes, mAb against CD20 (Dako) was used to detect B cells, mAb against CD68 (Dako) was used to detect macrophages, and mAb against prolyl-4-hydroxylase (Fuji Chemical, Toyama, Japan) was used to detect fibroblasts.

The serial sections used for ISH were incubated with 10% normal rabbit nonimmune serum for minimizing background staining, and then were incubated with primary antibody for 2 hours at room temperature. Normal mouse serum was used as a control for the primary antibody. After washing in PBS, pH 7.2, the sections were incubated with secondary antibody for 20 minutes at room temperature in a humidified chamber. They were placed in 3% H_2O_2 in methanol to block endogenous peroxidase, then incubated with peroxidase-conjugated streptavidin for 20 minutes at room temperature in a humidified chamber. Finally, sections were washed with PBS, and substrate reagent (3,3′-diaminobenzidine tetrahydrochloride; Dojindo, Tokyo, Japan) was added. Counterstaining was performed with hematoxylin, and the sections were mounted.

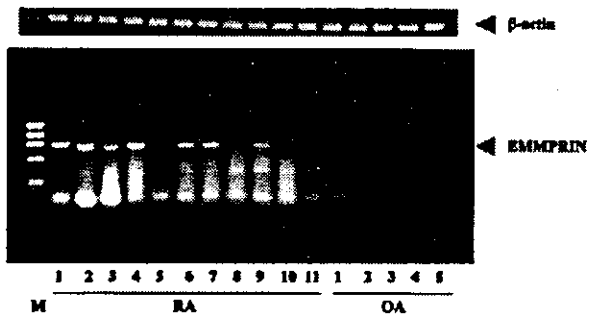

Figure 1. Representative reverse transcription–polymerase chain reaction showing extracellular matrix metalloproteinase inducer bands (492 bp) in synovial tissue samples from 11 patients with rheumatoid arthritis (RA) and 5 patients with osteoarthritis (OA). Lane M contains DNA markers.

Tartrate-resistant acid phosphatase (TRAP) staining. TRAP staining was performed as described previously (10). Briefly, the consecutive sections used for ISH were incubated for 30–90 minutes with medium containing 11.5 mg of disodium tartrate (Wako, Osaka, Japan) and 7 mg of naphthol-AS-TR phosphate and Fast Red TR (Sigma) in 5 ml of 0.2*M* acetate buffer, pH 5.0. Counterstaining was performed with hematoxylin, and the sections were mounted.

Measurement of MMP levels produced by synovial tissue. Synovial tissue samples from 4 patients with RA that expressed EMMPRIN mRNA and from 2 patients with RA that did not express EMMPRIN mRNA were studied. Synovial tissue was cut into small pieces (20 mg wet volume), and cultured in 24-well microtiter plates (Becton Dickinson Labware, Franklin Lakes, NJ) with Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (Gibco BRL, Grand Island, NY). After 48 hours, culture medium was collected for the measurement of MMP-1 and MMP-3. The assays for human MMP-1 in the supernatant were performed with an enzyme-linked immunosorbent assay (ELISA) kit (Amersham) according to the manufacturer's protocol. The assays for human MMP-3 in the supernatant were performed with an ELISA using a 1-step sandwich method (Fuji Chemical), as described previously (11).

Northern blot and densitometric analysis. Thirty micrograms of total RNA was subjected to electrophoresis on a 1.5% agarose–formaldehyde denaturing gel and transferred to a nitrocellulose membrane (Amersham). The filter was backed, prehybridized, and hybridized; EMMPRIN oligonucleotide was used as a probe, and the RNA was labeled by 3'-end labeling for Northern blotting. The filter was then washed and exposed to radiographic film. The relative intensities of the bands of interest were analyzed with the use of an NSF-300G scanner (Microtek, Anaheim, CA) and scan analysis software (Biosoft, Palo Alto, CA). Results are expressed as the ratio of band intensities of the sample relative to the band intensities of the GAPDH mRNA as determined by Northern blot analysis.

Statistical analysis. All values are expressed as the mean ± SD. Statistical analyses were performed with the Mann-Whitney U test, and P values less than 0.05 were considered significant. Analysis of statistical correlations was performed using Pearson's correlation coefficient.

RESULTS

Expression of EMMPRIN mRNA in synovium. We first studied the expression of EMMPRIN mRNA in synovial tissue. RT-PCR revealed EMMPRIN mRNA expression in synovial samples from 9 of the 11 RA patients and only 1 of the 5 OA patients (Figure 1).

Localization of EMMPRIN mRNA expression at the site of joint destruction. The results of ISH demonstrated marked expression of EMMPRIN mRNA at the site of joint destruction in RA (Figures 2A and C). This expression was detected in 28.0 ± 7.4% (mean ± SD) of the mononuclear cells of the invasive synovium (Figure 2B) and in 36.3 ± 8.1% of fibroblast-like cells at the site of bone destruction (Figure 2D) in joint specimens from

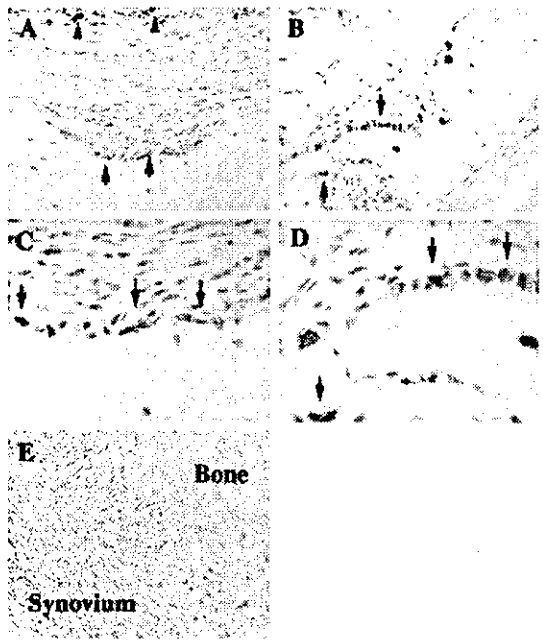

Figure 2. In situ hybridization of extracellular matrix metalloproteinase inducer (EMMPRIN) mRNA in synovium and bone from patients with rheumatoid arthritis (RA) and osteoarthritis (OA). Expression of EMMPRIN mRNA was confirmed in both **A**, the invasive synovium (**arrows**) and **C**, the eroded bone (**arrows**) in sections from a patient with RA and was detected in **B**, mononuclear cells of the invasive synovium (**arrows**) as well as in **D**, fibroblast-like cells at the site of bone erosion (**arrows**). **E**, Little expression of EMMPRIN mRNA was noted in the sample from the patient with OA. (Original magnification × 100 in **A**, **B**, and **E**; × 400 in **C** and **D**.)

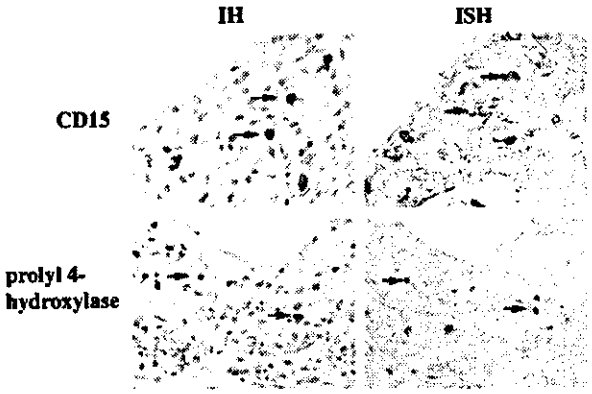

Figure 3. Immunohistochemical (IH) staining for CD15 and prolyl-4-hydroxylase and in situ hybridization (ISH) of synovial tissue sections from a patient with rheumatoid arthritis. Cells that expressed extracellular matrix metalloproteinase inducer mRNA on ISH (arrows) were also positive for CD15 and prolyl-4-hydroxylase (arrows). (Original magnification × 400 for CD15; × 200 for prolyl-4-hydroxylase.)

RA patients. However, little expression of EMMPRIN mRNA was detected in the samples of synovium and bone obtained from the OA patients (Figure 2E).

Characterization of cells expressing EMMPRIN. To characterize EMMPRIN-expressing cells, immunohistochemical staining was performed. The cells expressing EMMPRIN mRNA on ISH were negative for CD3, CD20, and CD68 (data not shown). Of the cells positive for CD15, 28.2 ± 5.8% (mean ± SD) expressed

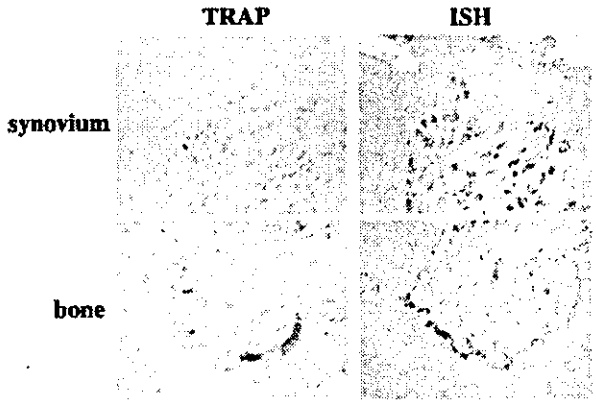

Figure 4. Tartrate-resistant acid phosphatase (TRAP) staining and in situ hybridization (ISH) of serial sections of synovium and bone from a patient with rheumatoid arthritis. Cells in both the synovium and bone that expressed extracellular matrix metalloproteinase inducer mRNA on ISH were negative for TRAP. (Original magnification × 400.)

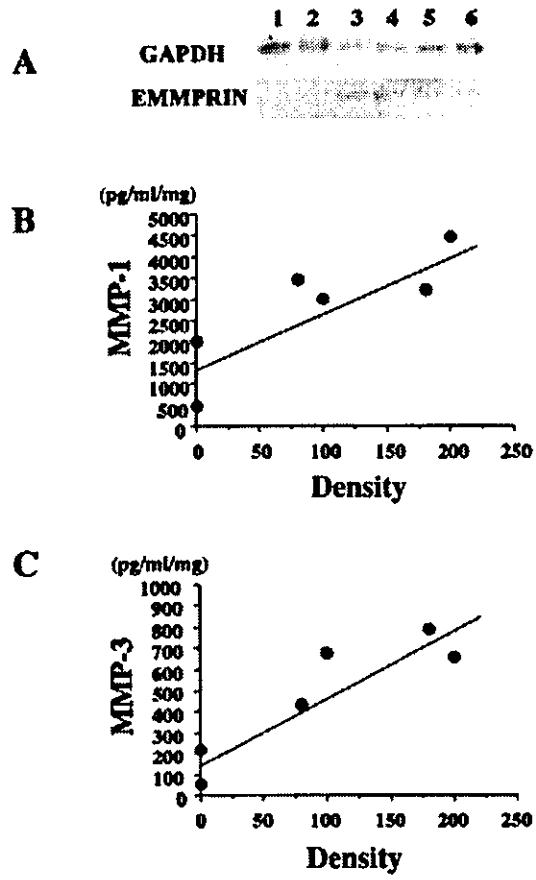

Figure 5. Relationship between the expression of extracellular matrix metalloproteinase inducer (EMMPRIN) mRNA and the production of matrix metalloproteinases (MMPs) 1 and 3 by synovial tissue from 6 patients with rheumatoid arthritis. **A**, Gene expression of EMMPRIN in synovial tissue. **B** and **C**, Relationship between synovial tissue expression of EMMPRIN mRNA and the production of MMP-1 and MMP-3, respectively, by Pearson's correlation coefficient. EMMPRIN mRNA signals were measured by densitometry and were normalized relative to those of GAPDH.

EMMPRIN mRNA on ISH (Figure 3A). Of the cells positive for prolyl-4-hydroxylase, 23.3 ± 9.1% expressed EMMPRIN mRNA on ISH (Figure 3B).

The results of TRAP staining showed that the cells that expressed EMMPRIN mRNA on ISH were negative for TRAP both in the synovium (Figure 4A) and in the bone (Figure 4B). These results demonstrated that the cells expressing EMMPRIN mRNA were synovial fibroblast-like cells and granulocytes, not infiltrating lymphocytes or synovial macrophages, and that neither osteoclasts nor osteoclast-like cells in the synovium and

bone at the site of joint destruction expressed EMMPRIN.

Enhanced production of MMP-1 and MMP-3 by synovial tissue expressing EMMPRIN. To study the relationship between EMMPRIN expression and the production of MMP-1 and MMP-3 in synovial tissue from patients with RA, we performed Northern blot analysis and used an ELISA kit to measure the levels of these metalloproteinases in the culture medium from synovial tissues obtained from 6 patients. In 2 synovial tissue samples, EMMPRIN mRNA was under the detection limit; however, in 4 samples, gene expression of EMMPRIN was confirmed (Figure 5A).

In the culture medium of synovial tissue from an RA patient expressing EMMPRIN mRNA, the average level of MMP-1 was 3,559.0 ± 677.8 pg/ml/mg and the average level of MMP-3 was 588.8 ± 132.5 pg/ml/mg (mean ± SD). In the culture medium of synovial tissue from an RA patient expressing no EMMPRIN mRNA, the average level of MMP-1 was 2,164.2 ± 1319 pg/ml/mg and the average level of MMP-3 was 208.3 ± 125.6 pg/ml/mg. Production of MMP-1 and MMP-3 by synovial tissue correlated with levels of EMMPRIN mRNA expression detected by Northern blotting ($r = 0.850$, $P = 0.0295$ for MMP-1 and $r = 0.903$, $P = 0.0098$ for MMP-3) (Figures 5B and C).

DISCUSSION

In this study, we clearly showed the presence of mRNA encoding EMMPRIN in synovium at the site of joint destruction in tissue from RA patients. However, little EMMPRIN expression was found in synovium from OA patients. These results are consistent with the previous report by Konttinen et al (7). By RT-PCR, only 1 of the 5 synovial samples from OA patients revealed a very weak band indicating the presence of EMMPRIN mRNA. We studied the clinical background and laboratory data of the patient from whom this synovial sample was taken and found no striking difference between this patient and the other 4 OA patients examined. Previous studies have demonstrated that most normal adult tissues, including epidermis, retinal pigment epithelium, and breast lobules and ductules, express very low levels of EMMPRIN, which suggests that EMMPRIN may play a physiologic role in tissue remodeling by inducing stromal MMPs (6,12–14). The EMMPRIN mRNA found in the synovium of the patient with OA may reflect tissue remodeling, localized inflammation, or wound healing occurring in that patient.

To characterize the EMMPRIN-expressing cells, we performed immunohistochemical staining of serial sections of tissues. EMMPRIN expression was found in the synovial fibroblast-like cells and granulocytes, but not in the infiltrating lymphocytes or macrophage-like cells. In tumors, EMMPRIN has been detected on the surface of tumor cells, but not on adjacent fibroblasts (15–17). In RA, however, fibroblast-like synovial cells are the primary cells that express EMMPRIN. In addition to a paracrine stimulation by granulocytes expressing EMMPRIN, there may be an autocrine stimulation of fibroblasts to produce MMPs in the synovium. A recent study has demonstrated that EMMPRIN expression was present at the surface of both tumor epithelial cells and peritumor stromal cells from breast and lung tissues (18). Granulocytes from the peripheral blood of patients with RA express higher levels of EMMPRIN compared with healthy donors (19). Granulocytes that have migrated to affected joints are peripheral granulocytes, and our histologic results confirmed their increased expression of EMMPRIN.

Although the precise function of EMMPRIN remains unknown, recent experimental data have demonstrated that EMMPRIN stimulates the production of interstitial collagenase, stromelysin 1, and gelatinase A in fibroblasts (20,21). These results suggest a connection between EMMPRIN and progressive joint destruction, because synovial cells from patients with RA have been demonstrated to be the principal source of several MMPs. The results of the present study demonstrated that synovial tissue that expressed EMMPRIN mRNA produced significantly more MMP-1 and MMP-3 than did synovial tissue that did not express EMMPRIN mRNA, and the findings strongly suggested that MMP-1 and MMP-3 production was stimulated by EMMPRIN in affected RA joints.

In this study, we showed a correlation between the expression of EMMPRIN and the production of MMP-1 and MMP-3 on Northern blot analysis. It would be difficult, however, to clearly demonstrate regulation of the expression and activity of MMPs by EMMPRIN. Previous studies have shown that EMMPRIN stimulates fibroblasts to produce 3–10 times the usual level of MMP-1 and MMP-3 in tumor cells and Chinese hamster ovary cells (20–22). The present study showed a 1.5–3 times increase in the production of MMP-1 and MMP-3. Of course, EMMPRIN expression alone does not appear to regulate MMP expression; the results of this study suggest that EMMPRIN may be involved in the regulation of MMP expression in the RA synovium. In addition, EMMPRIN may play a role in the degradation

of bone and cartilage associated with synovium invasion by stimulating the synthesis of several MMPs by synovial fibroblast-like cells.

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綜　説

骨吸収におけるTNF関連サイトカインの役割

——慢性関節リウマチにおける骨吸収機構の解明を目指して——

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I. はじめに

破骨細胞は高度に石灰化した骨組織を破壊・吸収する唯一の細胞で，その起源は生体に広く分布するマクロファージ系の造血細胞であると考えられている。近年，FosやSrcなどのがん関連遺伝子を欠失させたマウスが破骨細胞の分化あるいは骨吸収機能に異常を来し，大理石骨病を呈することが報告され，破骨細胞による骨吸収のメカニズムが今まで骨に関心がなかった多くの人によっても注目されている。

破骨細胞研究の有利な点は，多核巨細胞としての破骨細胞の形成を視覚的に判別できるとともに，その骨吸収機能を細胞培養系で簡単に再現できるところにある。われわれは，これまでにマウスを用いた破骨細胞の分化・融合・活性化（機能発現）を解析する各種の細胞培養系を確立し，その解析を行ってきた。その結果，破骨細胞の分化と機能は，骨芽細胞あるいは骨髄間質細胞の細胞膜上に発現する破骨細胞分化因子（osteoclast differentiation factor：ODF）によって厳格に調節されていることを提唱してきた[1-3]。ODFの同定は20世紀中には無理であると思われていたが，1997年に思いもよらない方向からその解明が進み，1998年破骨細胞分化因子（ODF/RANKL）の同定とその遺伝子クローニングという劇的な結末を迎えた[4-8]。

われわれの研究グループが破骨細胞の形成に関する研究を本格的に開始した今から15年前には，現在のような破骨細胞研究の分子レベルでの急速な解明はまったく想像できなかった。われわれは慢性関節リウマチ（RA）の病態における骨破壊にも注目して研究を行ってきたが[9,10]，receptor activator of NF-κB ligand（RANKL）の発見はRA患者の関節破壊における破骨細胞の役割にも一つの示唆を与えた。本稿では，RAの骨吸収機構について，われわれの成績を中心に最近の研究成果を概説したい。

II. RANKLの発見

1997年，世界の3つの研究グループ（雪印乳業，Amgen，インディアナ大学）によって破骨細胞形成を抑制する新規因子が発見され，そのcDNAがクローニングされた。この新規物質はTNF受容体に共通した構造を有していたが，膜貫通領域を持たない分泌性の蛋白質であった[8]。この新規受容体はosteoclastogenesis inhibitory factor（OCIF），TNFレセプター1（TR1）などとも呼ばれていたが，その後骨を防御する因子として命名されたosteoprotegerin（OPG）という名称を使用しようという提案が米国骨代謝学会で採択された[11]。

続いて1998年，OPGが結合するリガンドとしてTNFファミリーに属する膜結合型蛋白質のcDNAがクローニングされた。この分子こそ，われわれが10年以上追い求めてきた破骨細胞分化因子（ODF）そのものであった[4,8]。ODFは316個のアミノ酸から成る

Possible Roles of TNF Family Cytokines in Bone Destruction of Rheumatoid Arthritis
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〈Keywords〉 GM-CSF：IFN-γ：IL-17：IL-18：receptor activator of NF-κB ligand（RANKL）

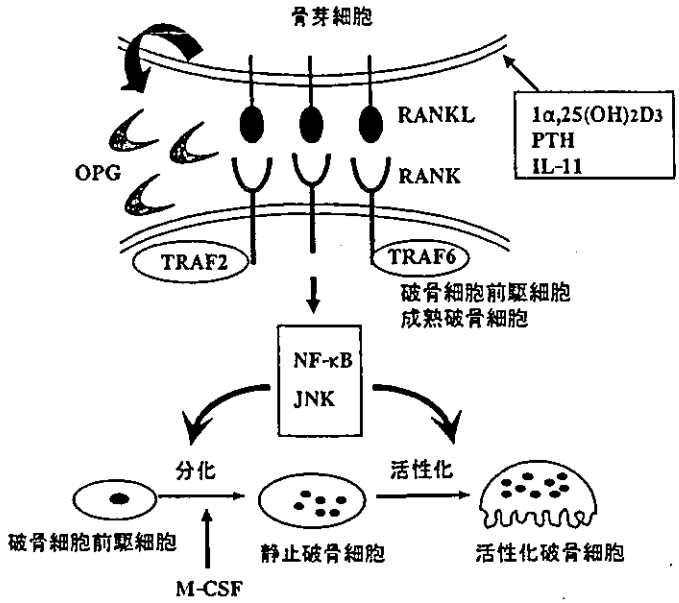

図 1　破骨細胞形成の分子機構
活性型ビタミン D(1 α,25(OH)$_2$D$_3$)，副甲状腺ホルモン(PTH)，IL-11 等の骨吸収促進因子の刺激により骨芽細胞の細胞膜上に発現誘導される RANKL を，破骨細胞前駆細胞または破骨細胞の RANK が認識することによって，TRAF を介したシグナル伝達により破骨細胞の分化と活性化が行われる。TRAF を介したシグナル伝達には NF-κB と JNK が関与する。OPG は RANKL のおとり受容体として RANK 以降のシグナル伝達を遮断する。

膜貫通領域を有する TNF ファミリーに属する蛋白質であった。骨芽細胞における ODF 遺伝子の発現は，破骨細胞の分化を促進する因子である活性型ビタミン D，副甲状腺ホルモン，IL-11 などの刺激によって著しく増強された。また，ODF の細胞内領域と膜貫通領域を欠如した可溶性 ODF を遺伝子工学的に作製し，破骨細胞分化誘導活性をマウスあるいはヒトの造血細胞だけの培養系を用いて調べたところ，骨芽細胞の非存在下でも可溶性 ODF とマクロファージコロニー刺激因子 (M-CSF) の添加によって破骨細胞が多数形成された。これらの破骨細胞形成促進活性は OPG の添加によって完全に阻害された[4,7,12,13]。

ODF の真の受容体は，すでに報告されていた RANK (receptor activator of NF-κB) と呼ばれる TNF 受容体ファミリーに属する膜結合型蛋白質であることが種々の実験により明らかになった (図 1)。現在，ODF は RANK リガンド (RANKL) という名称で統一されつつある。

一方，OPG は RANK と構造が類似していることからおとり受容体(decoy receptor)として働き，RANK よりもはるかに高い親和性を持って RANKL に結合することにより，RANKL の活性を強く抑制することが明らかとなった[8] (図 1)。

III. IL-17 による破骨細胞形成促進作用

IL-17 は活性化 T 細胞が産生するサイトカインであり，RA 患者由来の線維芽細胞に作用し IL-6，IL-8，G-CSF などの産生を促進することや，線維芽細胞が支持する CD 34 陽性の造血細胞の増殖を促進することが知られている[14~16]。そこで，破骨細胞形成に対する IL-17 の作用を検討したところ，骨芽細胞と骨髄細胞の共存培養系における破骨細胞の分化を IL-17 は強力に促進することが明らかとなった[17]。IL-17 のこの促進活性はプロスタグランジン E_2 合成酵素であるシクロオキシゲナーゼ (COX-2) の選択的阻害剤 (NS-398) または OPG の添加により完全に阻害された[17]。また，IL-17 は骨芽細胞における RANKL mRNA の発現を促進した。そこで，RA 患者の関節液中の IL-17 濃度を測定したところ，変形性関節症 (OA) に比較して RA 患者の方が有意に高値を示した。