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Establishment of nurse-like stromal cells from bone marrow of patients with rheumatoid arthritis: indication of characteristic bone marrow microenvironment in patients with rheumatoid arthritis

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Abstract

Objective. To investigate the microenvironment of bone marrow (BM) of patients with rheumatoid arthritis (RA).

Methods. Nurse cell-like BM stromal cell lines were established from BM mononuclear cells of patients with RA. We examined the various characteristics of these cell lines, including morphology, pseudoemperipolesis activity, cell surface markers, cytokine production and hyaluronan (HA) production.

Results. These RA BM nurse cell-like lines (RA-BMNC) were of mesenchymal origin and positive for CD44, CD54 and HLA-DR. They were defined as nurse cells because of pseudoemperipolesis activity that allowed lymphocytes to migrate underneath. RA-BMNC lines produced HA and multiple cytokines including interleukin (IL)-6, IL-7, IL-8 and granulocyte-macrophage colony-stimulating factor (GM-CSF). HA production by BM stromal cells was correlated with pseudoemperipolesis activity. RA-BMNC produced significantly higher levels of IL-6, IL-8 and GM-CSF by co-culture with lymphocytes. The cells also produced IL-1β, G-CSF and tumour necrosis factor only when co-cultured with lymphocytes. The RA-BMNC maintained the growth of CD14+ myeloid cells unique to severe RA.

Conclusion. The present results both indicate that RA-BMNC are nurse cells and suggest that they may play an important role in the pathogenesis of RA.

KEY WORDS: Rheumatoid arthritis, Nurse-like cells, Bone marrow.

Rheumatoid arthritis (RA) is a systemic autoimmune disease characterized by destructive polyarthritis, chronic proliferation of synovial cells, and abnormal immune reactions including activation of autoreactive T cells and polyclonal B cells [1–3]. Although the pathogenesis of RA remains unknown, there is evidence that an abnormality of bone marrow (BM) cells contributes to the pathogenesis of RA in humans and in experimental arthritis models [4–16]. Bone marrow stromal cells play important roles in lymphopoiesis through the production of a number of factors essential for early lymphocyte growth and development [17–21]. In a previous study, we observed an increase in mononuclear cells, especially lymphocytes and myeloid cells, in the haematopoietic BM of patients with severe RA [22]. We also previously reported the presence of an abnormal myeloid cell population in epiphyseal BM adjacent to joints affected by severe RA [4]. Those abnormal myeloid cells could be distinguished from normal myeloid cells by the expression of the difucosyl type 2 chain structure (dimetric Lex, a specific marker of human undifferentiated cells [23]) or CD14 antigen. These cells were observed to develop into polymorphonuclear cells (i.e. developed functional cells of myeloid lineage) [24], and thus may play an important role in connective tissue destruction in severe RA. However, it was difficult to maintain these myeloid cells *in vitro*, although we

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induced a little proliferation and maintained these cells for a short period using the BM serum of patients with severe RA [25]. This finding indicates to us that there must be a special microenvironment which supports these cells. Moreover, BM stromal cells derived from multiple myeloma and RA patients supported murine pre-B-cell growth [26, 27]. These findings led us to investigate the characteristics and functions of the BM microenvironment in patients with RA.

Nurse cells were first recognized in a cell suspension from the thymus in 1980 [28, 29]. Thymic nurse cells form complexes with thymocytes, and are thus believed to play an important role in thymocyte differentiation. After initially adhering to thymic nurse cells, thymocytes crawl underneath them. This phenomenon has been referred to as pseudoemperipolesis. Pseudoemperipolesis has also been observed in the interaction between murine lymphocyte progenitors and murine BM stromal cells, and between human pre-B ALL cells and murine BM stromal cells [30–32]. We recently reported the establishment of nurse cell-like clones from human skin [33] and synovial tissue of patients with RA [34, 35]. The observed clones also showed pseudoemperipolesis. A previous study demonstrated that in an experimental arthritis model, BM stromal cells migrated from the BM into the affected joint cavity and thus appeared to contribute to synovial proliferation. Thus, abnormal stromal cells in the BM might directly influence intra-articular lesions during the course of arthritis [36].

In the present study, we establish nurse-like cell clones from BM of patients with RA (RA-BMNC), and show that B and T lymphocytes and myeloid lineage cells migrate under these RA-BMNC. The RA-BMNC were observed to have many characteristics similar to those of nurse cell-like clones established from synovial tissues of patients with RA [34]. RA-BMNC produce multiple inflammatory cytokines and hyaluronan (HA). RA-BMNC also play a role in the differentiation of myeloid lineage cells. Bone marrow stromal cell lines established from healthy donors produce lower levels of cytokines and HA, and did not show strong pseudoemperipolesis activity. The present results indicate that the BM microenvironment of RA patients is different from that of healthy donors, and suggest that a disorder of the BM microenvironment may be involved in the pathogenesis of RA.

Methods

Primary culture of bone marrow cells and establishment of bone marrow stromal cell lines and clones

Heparinized BM aspirate was obtained after informed consent from the anterior iliac crest via a needle puncture from five patients with RA, who met the American College of Rheumatology criteria [37], at the time of joint reconstructive surgery. As a control, heparinized BM aspirate was obtained, with consent, from three age-matched healthy volunteers suffering from trauma, but not showing any evidence of arthritis. All five RA patients were classified as having the more erosive disease subset of RA according to a previous report [38] with an average 12 yr history of RA. Mononuclear cells (MNC) from the BM aspirate were separated by Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) density-gradient (1077 g/ml) centrifugation (30 min, 400 *g*). The cell suspensions were cultured in DMEM supplemented with 10% heat-inactivated fetal calf serum (FCS, Hyqlone) for 2 weeks. After non-adherent cells were removed, long-term culture of adherent cells was maintained in humidified air containing 7% CO_2 at 37°C by changing medium twice a week and passaging the cells at confluence. After 4–5 passages, homogeneous populations of stromal cells were obtained and then defined as cell lines of BM stromal cells. A total of nine clones from RA BM stromal cell lines (RA-BM 1–9) and four clones from healthy donor BM stromal cell lines (HD-BM 1–4) were obtained by the limiting dilution method as previously described [33, 34]. In this study, all cell lines or clones of BM stromal cells were used between 5 and 10 passages.

Cell lines

The human T-cell line Molt-17 was a kind gift from Dr J. Minowada (Fujisaki Cell Centre, Okayama, Japan) and the human B-cell line MC/car was obtained from the American Type Culture Collection (ATCC) cell libraries (Rockville, MD, USA). These cell lines were maintained in RPMI 1640 (Life Technologies, Grand Island, NY, USA) supplemented with 10% FCS. Mouse thymic nurse cell clone IT-79MTNC3 was obtained as previously described [39]. IT-79MTNC3 was maintained with DMEM containing 10% FCS.

Pseudoemperipolesis assay

A total of 30 000 BM stromal cells were incubated in 1 ml of complete medium in 24-well plates overnight. Next, 1×10^6 cells of each lymphoma cell line (Molt-17, MC/car) were added to BM stromal cell culture. After 6 h of incubation, BM stromal cells holding three or more lymphoma cells underneath were considered positive for pseudoemperipolesis. More than 200 BM stromal cells were counted in each experiment [33].

Morphological examination

All procedures for morphological examination with an inverted phase-contrast microscope and a transmission electron microscope were performed as described previously [39].

Hanging drop culture

Hanging drop culture was performed as described previously [39]. After establishment of the cell lines, a mixed population of RA-BMNC and lymphomas was first grown in a 60 mm plastic dish. Cells were detached by trypsin treatment. After the cell number was adjusted, the cells were put into a Terasaki plate. The Terasaki plate was inverted immediately when all wells had received the cells, and incubated overnight in a CO_2 incubator at 37°C, 10% CO_2. The cells were fixed with

glutaraldehyde solution (2.5% in 0.1 M sodium cacodylate buffer) the next morning, and then transferred gently to a 60 mm dish for photography.

Analysis of surface antigen

Antibodies used in this study were fluorescein isothiocyanate (FITC)-conjugated anti-human monoclonal antibodies (mAbs) specific for CD1a (Ortho Diagnostic Systems, Inc., Raritan, NJ, USA), CD1b (Nichirei Co., Ltd, Tokyo, Japan), CD2, CD11a, CD18, CD44, LECAM-1 and HLA-DR (Becton Dickinson), and phycoerythrin (PE)-conjugated anti-human mAbs specific for CD11b, CD11c, CD54 and CD56 (Becton Dickinson). Anti-human mAbs specific for CD21 (Becton Dickinson), CD58 (Cosmo-Bio, Tokyo, Japan), VCAM-1 (Becton Dickinson) and LECAM-2 (Nichirei Co., Ltd) were non-labelled, and FITC-conjugated goat anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA, USA) was used as a second Ab. The stained cells were analysed on FACScan (Becton Dickinson). Scattergates were set to exclude dead cells, cell debris and aggregates. The number of positive cells was obtained from a total population of 10 000 events by comparing a control sample with a test sample.

Measurement of the amount of cytokines and hyaluronan

Bone marrow stromal cells were maintained routinely in DMEM supplemented with 10% FCS until they became confluent. The culture supernatants of BM stromal cells were collected, and the amounts of cytokines in the culture supernatants, interleukin (IL)-1α, IL-1β, IL-2, IL-3, IL-4, IL-6, IL-7, IL-8, granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), tumour necrosis factor alpha (TNF-α) and TNF-β, were measured using an ELISA kit (Quantikine, R & D Systems, Minneapolis, MN, USA), according to the manufacturer's protocol. The concentration of HA in the culture supernatant of BM stromal cells was measured using an HA assay kit (hyaluronic acid 'Chugai', Chugai seiyaku KK, Tokyo, Japan), according to the manufacturer's protocol. The principal feature of this assay is the use of specific hyaluronan binding protein linked with horseradish peroxidase. The detection limit of the assay was 10 ng/ml.

Production and survival of CD14+ myeloid cells by bone marrow nurse cells

A total of 30 000 RA-BMNC were incubated in 1 ml of the complete medium in 24-well plates overnight. Bone marrow mononuclear cells were prepared as described previously [40]. Briefly, to deplete phagocytic cells, BM aspirates were incubated at 37°C for 1 h with the addition of 1/10 volume of silica suspension (JIMRO, Takasaki, Japan). Next, mononuclear cells from the BM aspirates were separated by Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) density-gradient (1.077 g/ml) centrifugation (30 min, 400 *g*). T lymphocytes were depleted by incubation with sheep red blood cells (SRBC) (JIMRO, Takasaki, Japan). The cells were then washed three times with phosphate-buffered saline (PBS) and then incubated with FITC-conjugated CD15 (MX-GA) (Kyowa Medix, Tokyo, Japan) [41] and PE-conjugated CD14 (MY4) on ice for 30 min. The cells were then washed three times with PBS. Using FACStar Plus (Becton Dickinson), CD14– CD15+ cells were collected. To investigate the effect of RA-BMNC on the induction of CD14+ CD15+ myeloid cells, 100 000 apparently normal CD14− CD15+ myeloid cells were obtained from patients with severe RA and then added to RA-BMNC culture. In some experiments, we used a cell culture insert (Falcon® Labware) to avoid any direct cell–cell contact between RA-BMNC and CD14− CD15+ cells in the same well. Following a 5 day incubation, cells were collected and incubated with FITC-conjugated CD15 and PE-conjugated CD14. Then the cells were analysed on a FACScan. Next, to investigate the effect of RA-BMNC on the survival of CD14+ CD15+ myeloid cells, CD14+ CD15+ myeloid cells derived from patients with severe RA were collected using the FACSstar Plus (Becton Dickinson). A total of 100 000 CD14+ CD15+ cells were co-cultured with RA-BMNC. Following a 14 day incubation, the number of living CD14+ CD15+ cells was counted.

Statistical analysis

All results were expressed as the mean $\pm$ S.D. Data were analysed using the non-parametric Mann–Whitney *U*-test and *P* values of <0.05 were considered significant. Analysis of statistical correlation was performed using Spearman's test of rank correlation.

Results

Establishment of the RA-BMNC

We established RA-BMNC lines which grew continuously in DMEM culture medium containing 10% FCS, but stopped growing when the monolayer became confluent. The RA-BMNC did not exhibit either a typical fibroblastic (slender and elongated) or macrophage-like (irregular with many membrane rufflings) appearance in a phase-contrast photomicrograph. Under light microscopy, RA-BMNC were large adherent cells with an irregular (rather polygonal) shape, and with a low contrast with multiple long slender cytoplasmic processes (Fig. 1). The cells (average size 213 45 $\times$ 111 28 μm) were much larger than BM stromal cells (average size 115 32 $\times$ 40 20 μm) obtained from the healthy donors.

Under transmission microscopy, RA-BMNC were seen to contain many profiles of Golgi apparatus, some lysosome-like granules, bundles of thin filaments, euchromatic nuclei, and long and branching mitochondria. No tonofilaments, which are characteristic cytoskeletons of epithelial cells, were observed. Very few classical junctional complexes, including zonula occludens or desmosomes, were observed at the site of cell contact. Caveolar structures were also prominent at the surface of the cells (Fig. 2A and B). RA-BMNC were positive

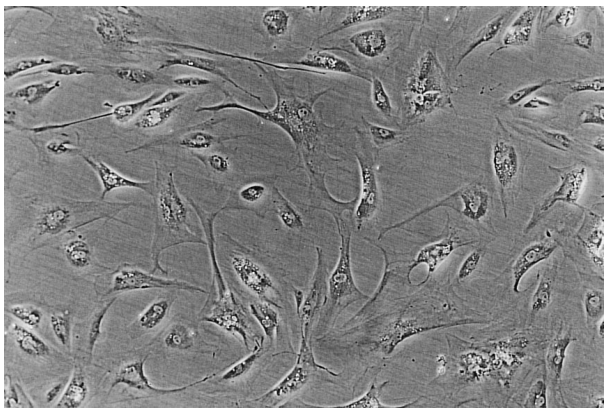

FIG. 1. Photomicrograph of RA-BMNC. RA bone marrow stromal cells are large adherent cells. Original magnification $\times 100$.

for vimentin, which is characteristically expressed in cells of mesenchymal origin. They were, however, negative for cytokeratin, which is unique to cells of epithelial origin (data not shown).

Cell surface markers

The RA-BMNC were positive for CD29 (Int. $\beta 2$), CD44 (Pgp-1; hyaluronic acid receptor), CDw49c (Int. $\alpha 3$), CD51, CD54 (ICAM-1) and HLA-DR. These cells were weakly positive for CDw49a (Int. $\alpha 1$), CDw49b (Int. $\alpha 2$), CDw49d (Int. $\alpha 4$), CDw49e (Int. $\alpha 5$), CD58 (LFA-3) and VCAM-1. These cells were negative for CD1a, CD1b, CD2 (LFA-2), CD11a (LFA-1 α), CD11b (Mac-1α), CD11c and CD18 (LFA-1β) (Table 1). The cell surface markers of two clones of BM stromal cell lines derived from healthy donors were also examined. The expression patterns of the cell surface markers on BM stromal cells derived from healthy donors were similar to those on RA-BMNC.

Pseudoemperipolesis activity

We co-cultured BM stromal cells with human T-cell lymphoma cell line Molt-17 or B-cell line MC/car, and evaluated nurse cell activity. RA-BMNC were evaluated as positive for nurse cell activity when pseudoemperipolesis was observed. Pseudoemperipolesis, migration of Molt-17 and MC/car under RA-BMNC, was determined by observation under a light microscope. Molt-17 and MC/car formed characteristic complexes with RA-BMNC (Fig. 3A). RA-BMNC were defined as nurse cells because the mixed culture showed obvious pseudoemperipolesis. In the hanging drop culture system, RA-BMNC and Molt-17 or MC/car formed a typical complex (Fig. 3B). Bone marrow stromal cell lines derived from normal donors, however, did not form the typical complexes. The percentages of pseudoemperipolesis-positive cells in BM stromal cell lines are shown in Table 2. The average percentages of pseudoemperipolesis-positive cells in BM stromal cells derived from healthy donors were $4.0 \pm 1.4\%$ with MC/car cells and $1.5 \pm 0.6\%$ with Molt-17 cells, while those in RA-BMNC were $39.4 \pm 4.5\%$ with MC/car cells and $23.8 \pm 2.5\%$ with Molt-17 cells. The percentages of pseudoemperipolesis-positive cells in RA-BMNC were significantly higher compared to BM stromal cell lines derived from healthy donors ($P < 0.01$).

Cytokine production by RA-BMNC

The production of cytokines by RA-BMNC and BM stromal cell lines derived from healthy donors was

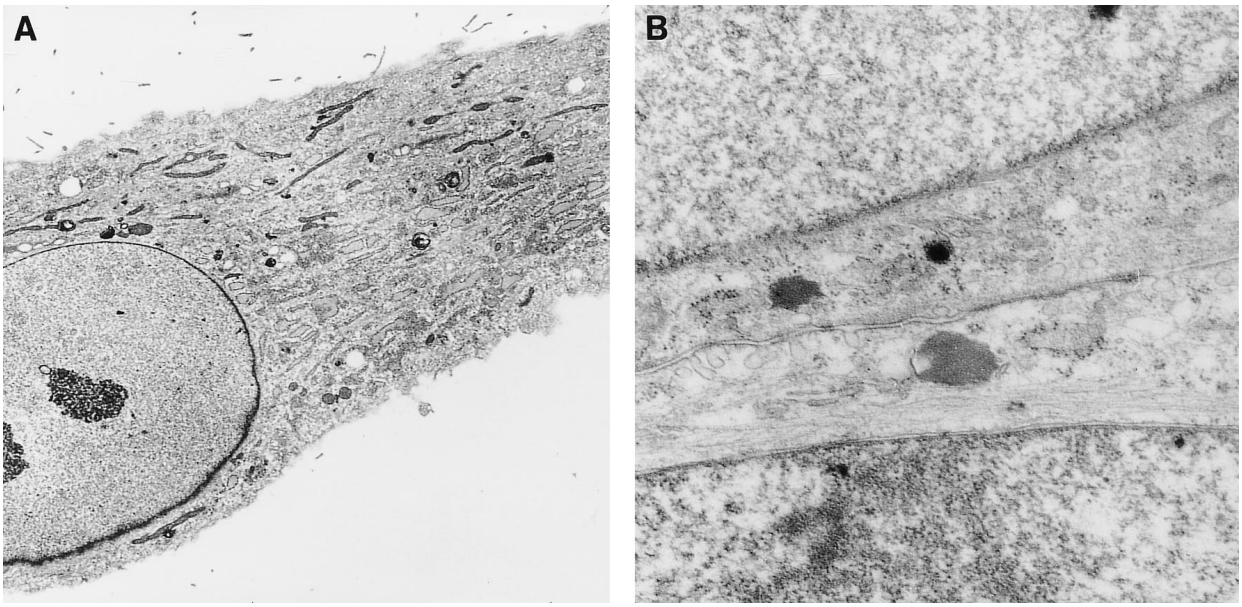

FIG. 2. Transmission electron micrograph of RA-BMNC. (A) RA bone marrow stromal cells had many profiles of Golgi apparatus, some lysosome-like granules and bundles of thin filaments. Tonofilaments, which are characteristic cytoskeletons of epithelial cells, were not observed. Original magnification $\times 4500$. (B) Nuclei were euchromatic. Long and branching mitochondria were conspicuous. Original magnification $\times 36\,000$.

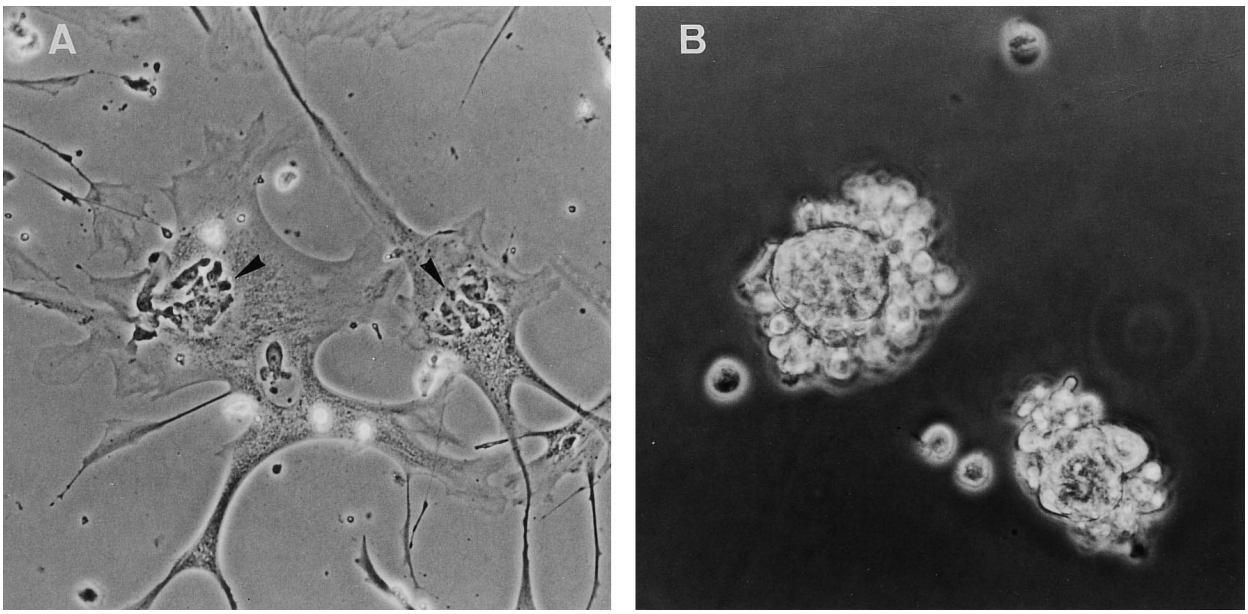

FIG. 3. Interaction between RA-BMNC and human lymphocytes. (A) 1×10^4 cells/well of RA bone marrow stromal cells were cultured overnight, and 5×10^5 cells of a human B-lymphoma cell line were added. The cells were photographed under a phase-contrast microscope after 6 h incubation. Arrowheads show migration of B-lymphoma cells under RA-BMNC. Original magnification ×200. (B) Phase-contrast micrograph of RA bone marrow stromal cells and human lymphocytes in hanging drop culture. Human lymphoma MC/car cells were embraced by large RA bone marrow stromal cells. Original magnification ×400.

examined. A total of 10 000 BM stromal cell lines were incubated in 1 ml of the complete medium in 24-well plates for 48 h. The culture supernatants were collected and the amounts of cytokines were measured using ELISA kits. All nine RA-BMNC clones produced detectable levels of IL-6 and IL-8 (Table 3). The levels of IL-6 (930 ± 274 pg/ml) and IL-8 (2845 ± 1438 pg/ml) in the culture supernatants of

TABLE 1. Surface phenotype of BM stromal cell lines derived from RA patients and healthy donors[a]

	% Positive						
Antibodies	RA-BM 1	RA-BM 2	RA-BM 3	RA-BM 4	HD-BM 1	HD-BM 2	IT-79MTNC3[b]
Control	0.3	0.4	0.3	0.5	0.4	0.6	0.9
CD1a	0.4	0.4	0.3	0.6	0.6	0.5	ND
CD1b	0.6	0.3	0.5	0.3	0.4	0.6	ND
CD2 (LFA-2)	0.4	0.5	0.3	0.3	0.5	0.6	1.1
CD11a (LFA-1a)	0.9	0.9	0.7	0.4	0.4	0.6	0.7
CD11b (Mac-1a)	0.5	0.6	0.6	0.6	0.3	0.5	0.8
CD11c	0.7	0.8	0.7	0.5	0.9	0.7	ND
CD18 (LFA-1b)	0.3	0.3	0.5	0.6	0.6	0.4	0.9
CD29 (Int. β1)	91.5	64.6	90.3	97.2	90.7	23.5	ND
CDw49a (Int. α1)	18.6	12.7	17.6	10.5	6.5	6.7	ND
CDw49b (Int. α2)	26.3	11.4	25.2	60.4	36.8	3.6	ND
CDw49c (Int. α3)	92.0	ND	91.2	95.6	59.4	29.5	ND
CDw49d (Int. α4)	17.5	6.7	57.6	24.5	32.5	2.0	ND
CDw49e (Int. α5)	36.2	78.6	27.6	25.9	78.6	5.6	ND
CDw49f (Int. α6)	0.5	6.7	7.2	7.1	6.3	2.4	ND
CD44	95.5	78.6	95.6	94.2	86.2	89.4	99.5
CD51	78.2	ND	94.1	95.3	77.1	23.7	ND
CD54 (ICAM-1)	54.0	39.9	38.7	22.1	42.7	31.5	31.9
CD56 (NCAM)	0.9	0.5	0.3	0.2	0.3	0.6	ND
LECAM-1	0.8	0.3	0.6	0.3	0.4	0.4	1.6
VCAM-1	21.5	20.4	28.4	4.2	8.6	7.8	20.9
CD58 (LFA-3)	71.6	15.5	75.6	51.8	40.7	11.8	ND
MHC class I	98.0	98.3	99.0	98.6	91.5	93.0	ND
MHC class II	0.8	0.5	0.6	0.8	0.7	0.9	1.1

[a]RA-BMs, HD-BMs or IT-79MTNC3 were detached with 0.05% EDTA in PBS at 4°C for 1 h. They were stained with anti-human or anti-mouse mAbs, and analysed on FACScan.
[b]IT-79MTNC3, murine thymic nurse cell clone.

TABLE 2. Pseudoemperipolesis activity of BM stromal cell lines derived from RA patients and healthy donors[a]

Cell lines	Pseudoemperipolesis (%)	
	MC/car	Molt-17
IT-79MTNC3[b]	50	25
HD-BM 1	3	1
HD-BM 2	6	2
HD-BM 3	3	1
HD-BM 4	4	2
RA-BM 1	35	25
RA-BM 2	40	21
RA-BM 3	34	29
RA-BM 4	40	24
RA-BM 5	45	24
RA-BM 6	41	23
RA-BM 7	36	21
RA-BM 8	39	26
RA-BM 9	35	25

[a]3×10^4 cells/well of RA-BMs, HD-BMs or IT-79MTNC3 were inoculated and cultured overnight, and 1×10^6 cells of each lymphoma cell line were added to the culture. After 6 h, >200 stromal cells were counted. Stromal cells that had more than three lymphoma cells were counted as positive cells with pseudoemperipolesis.
[b]IT-79MTNC3, murine thymic nurse cell clone.

RA-BMNC clones were significantly higher than those in the culture supernatants of BM stromal cell clones derived from healthy donors (IL-6, 181 ± 60 pg/ml; IL-8, 1037 ± 155 pg/ml) ($P < 0.01$ for both IL-6 and IL-8). Four of the six clones produced GM-CSF and IL-7 (GM-CSF, 8.0 ± 3.2 pg/ml; IL-7, 9.5 ± 7.0 pg/ml). In contrast, none of them produced detectable levels of IL-1α, IL-1β, IL-2, IL-3, IL-4, G-CSF, TNF-α or TNF-β. Bone marrow stromal cell clones derived from healthy donors also produced IL-6 and IL-8, but the levels of these cytokines were much less than those produced by RA-BMNC clones.

Enhanced production of inflammatory cytokines by RA-BMNC lines after incubation with B-lymphoma cell line

We examined the effect of co-culture with lymphoma cell lines on cytokine production by RA-BMNC. A total of 30 000 RA-BMNC-1 and -2 were incubated in 1 ml of the complete medium in 24-well plates overnight. A total of 1×10^6 cells of B-lymphoma cell line MC/car or T-lymphoma cell line Molt-17 were added to RA-BMNC culture. Following a 5 day incubation, the culture supernatants were collected and the amounts of cytokines measured using ELISA kits. The average levels of IL-6, IL-8 and GM-CSF in the culture supernatants of RA-BMNC alone were 31 030, 1845 and 185 pg/ml, respectively. The average levels of IL-6, IL-8 and GM-CSF in the supernatants of co-culture of RA-BMNC and MC/car were 77 228, 26 230 and 683 pg/ml, respectively. The average levels of IL-6, IL-8 and GM-CSF in the supernatants of co-culture of RA-BMNC and Molt-17 were 66 590, 12 558 and 540 pg/ml, respectively. Notably, the levels of IL-6, IL-8 and GM-CSF in the supernatants of co-culture of RA-BMNC and MC/car or Molt-17 were significantly higher when compared with the culture of RA-BMNC alone ($P < 0.01$ for IL-6, IL-8 and GM-CSF). Moreover, IL-1β, G-CSF and TNF-α were detected in the supernatants of co-cultures of RA-BMNC clones and MC/car or Molt-17 (IL-1β, 430 pg/ml, 355 pg/ml; G-CSF, 635 pg/ml, 507.5 pg/ml; TNF-α, 567.5 pg/ml, 415 pg/ml) (Table 4). The above results indicate that RA-BMNC produced higher levels of cytokines by co-culture with lymphoma cell lines.

Hyaluronan production by RA-BMNC

Overproduction of HA has been observed in patients with RA, although the role of HA in the pathogenesis of RA is not understood. In the present study, we

TABLE 3. production of cytokines from BM stromal cell lines derived from RA patients and healthy donors[a]

Cell lines	Cytokines in culture supernatant (pg/ml)											
	Il-1α	IL-1β	IL-2	IL-3	IL-4	IL-6	IL-7	IL-8	G-CSF	GM-CSF	TNF-α	TNF-β
Medium	–	–	–	–	–	–	–	–	–	–	–	–
HD-BM 1	–	–	–	–	–	205	–	1250	–	–	–	–
HD-BM 2	–	–	–	–	–	250	–	1050	–	–	–	–
HD-BM 3	–	–	–	–	–	160	–	950	–	–	–	–
HD-BM 4	–	–	–	–	–	110	–	900	–	–	–	–
RA-BM 1	–	–	–	–	–	740	9	5600	–	20	–	–
RA-BM 2	–	–	–	–	–	720	–	2100	–	7	–	–
RA-BM 3	–	–	–	–	–	1400	12	4000	–	6	–	–
RA-BM 4	–	–	–	–	–	630	5	1800	–	–	–	–
RA-BM 5	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
RA-BM 6	–	–	–	–	–	870	6	1100	–	–	–	–
RA-BM 7	–	–	–	–	–	840	–	2900	–	5	5	–
RA-BM 8	ND	–	ND	ND	ND	1280	ND	3250	ND	ND	–	ND
RA-BM 9	ND	–	ND	ND	ND	960	ND	2010	ND	ND	–	ND

[a]1×10^4 cells/well of RA-BMs or HD-BMs were inoculated and cultured for 48 h. The culture supernatant of each RA-BM or HD-BM line was collected and the amount of each cytokine in the culture supernatant was measured with an ELISA kit.
–, not detectable; ND, not done.

TABLE 4. Production of cytokines from the complex of RA-BM stromal cell lines and lymphocytes[a]

Cell lines	Cytokines in culture supernatant (pg/ml)							
	IL-1α	IL-1β	IL-6	IL-7	IL-8	G-CSF	GM-CSF	TNF-α
Medium control	–	–	–	–	–	–	–	–
MC/car	–	–	–	–	–	–	–	–
Molt-17	–	–	–	–	–	–	–	–
RA-BMNC-1	–	–	38 250	–	1480	–	150	–
+ MC/car	–	320	89 015	–	33 510	755	915	275
+ Molt-17	–	235	78 750	–	10 615	540	355	255
RA-BMNC-2	–	–	23 810	–		–	220	–
+ MC/car	–	540	65 240	–	18 950	515	450	860
+ Molt-17	–	475	54 430	–	14 500	475	725	575

[a]3×10^4 cells/well of RA-BMNC were inoculated and cultured overnight, and 1×10^6 cells of MC/car of Molt-17 were added to the culture. After 5 day incubation, the culture supernatants were collected and the amount of each cytokine in the culture supernatant was measured with an ELISA kit.
–, not detectable.

evaluated the production of HA by RA-BMNC and examined the effect of co-culture with lymphoma cell line. RA-BMNC secreted significantly higher levels of HA (1515 ± 313 ng/ml) when compared with BM stromal cells derived from healthy donors (533 ± 195 ng/ml) ($P < 0.01$). The levels of HA of RA-BMNC co-culture with MC/car or Molt-17 were 2515 ± 381 and 2215 ± 352 ng/ml, respectively. RA-BMNC produced higher levels of HA when co-cultured with lymphoma cell lines ($P < 0.05$ for both MC/car and Molt-17). Neither MC/car nor Molt-17 produced detectable levels of HA (Fig. 4). Pre-treatment of lymphoma cell lines with mitomycin C did not change the HA levels (data not shown); thus, HA may be produced primarily by RA-BMNC. Interestingly, HA production by RA-BMNC was correlated with pseudoemperipolesis activity ($r = 0.819$) (Fig. 5).

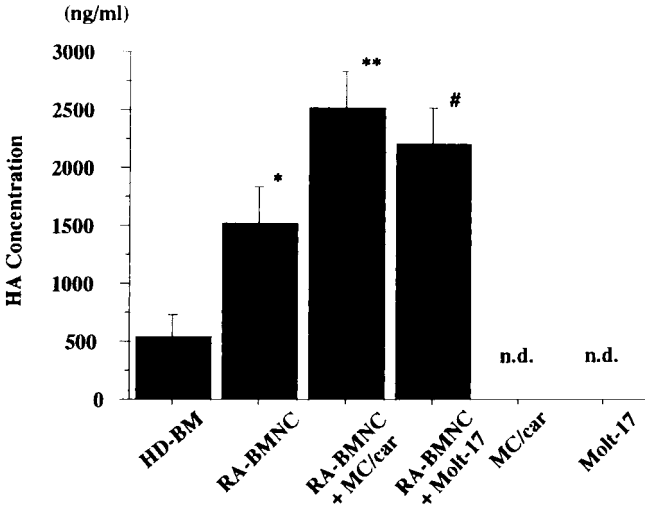

FIG. 4. Production of hyaluronan from the complex of RA-BM stromal cell lines and lymphocytes. 1×10^4 cells/well of RA-BMNC were inoculated and cultured overnight, and 5×10^5 cells of MC/car or Molt-17 were added to the culture. Following 48 h incubation, the culture supernatants were collected and the amount of hyaluronan in the culture supernatant measured using a hyaluronan assay kit. HD-BM, healthy donor BM stromal cells; $*P < 0.01$ *vs* HD-BM; $**P < 0.01$ *vs* RA-BMNC; $\#P < 0.01$ *vs* RA-BMNC.

Induction and maintenance of CD14+ myeloid cells unique to severe RA patients by RA-BMNC

We previously reported accumulation of unusual (di-Le^x+, CD14+) myeloid cells in the iliac and epiphyseal BM of patients with severe RA [4, 22]. No accumulation of these myeloid cells was observed in non-RA controls or in patients with mild RA. Furthermore, CD14+ myeloid cells were differentiated from CD14 − myeloid cells obtained from iliac BM of severe RA patients [22, 40]. The above myeloid cells with unusual phenotype could not be maintained in ordinary culture

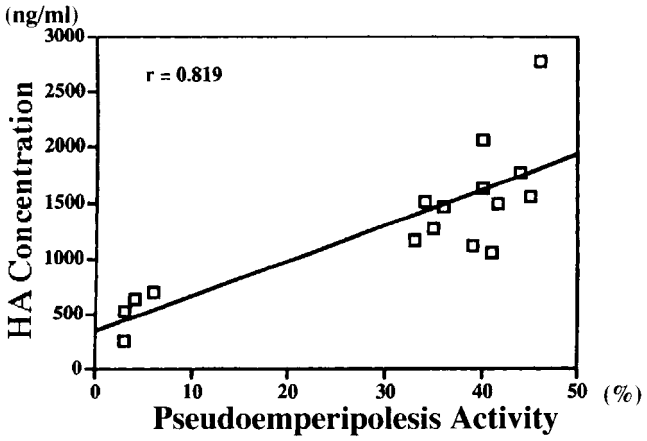

FIG. 5. Relationship between pseudoemperipolesis and production of hyaluronan by bone marrow stromal cell lines. 1×10^4 cells/well of RA bone marrow stromal cells were cultured overnight, and 5×10^5 cells of human B-lymphoma cell line (MC/car) were added. More than 200 stromal cells were counted after 6 h incubation. Stromal cells that had more than three MC/car cells were counted as positive cells for pseudoemperipolesis. 1×10^4 cells/well of RA bone marrow stromal cells were cultured for 48 h. The culture supernatant of each bone marrow stromal cell line was collected and the amount of hyaluronan in the culture supernatant was measured using a hyaluronan assay kit. Correlation was analysed with Spearman's test of rank correlation.

conditions. Survival of these CD14+ myeloid cells may suggest the presence of a unique BM microenvironment such as nurse cells. Thus, we evaluated the ability of RA-BMNC to support CD14+ myeloid cells. CD14– myeloid cells derived from iliac BM of severe RA patients were cultured in the presence of RA-BMNC for 5 days. The number of CD14+ myeloid cells was increased to significantly higher levels under conditions where CD14− myeloid cells and RA-BMNC were cultured together, compared to the number seen under conditions where CD14− myeloid cells and RA-BMNC were cultured in the same wells without direct cell–cell contact ($P < 0.01$). We attempted to maintain CD14+ myeloid cells, but found it was difficult to maintain them for >2 weeks in culture medium alone or even with some growth factor added. However, CD14+ myeloid cells were maintained for longer periods of time when co-cultured with RA-BMNC lines. The number of CD14+ myeloid cells was significantly higher on day 14 when co-cultured with RA-BMNC than when co-cultured with BM stromal cell lines derived from a healthy donor or cultured in medium alone ($P < 0.05$) (Fig. 6).

Discussion

Stromal cell lines established from BM of patients with RA were large in size compared with those derived from BM of healthy donors. RA-BMNC were determined to be of mesenchymal origin, given that they were positive for vimentin, which is a mesenchymal or fibroblastic marker, and negative for cytokeratin. Under the transmission electron microscope, RA-BMNC had thin filaments, but did not show any tonofilaments. RA-BMNC did not show any desmosomes or classical junctional complex, both of which are characteristic features of epithelial cells. Elongated and branching mitochondria were present in cytoplasm of RA-BMNC, and caveolae, which are unique to the cells of mesenchymal origin, were present on the surface.

One of the important features of RA-BMNC was their pseudoemperipolesis activity. In the present study, we showed pseudoemperipolesis between RA-BMNC and human B lymphocytes, T lymphocytes and myeloid lineage cells. Wekerle and Ketelsen [28, 29] first described this phenomenon using thymic nurse cells and thymocytes. They concluded that thymic nurse cells played an important role in the differentiation of thymocytes. Nurse cell-like clones from human skin and RA synovial tissue have recently been reported [33, 34]. The mesenchymal cell lines established in the present study were nurse cells which proliferate pathologically in the BM of RA patients. As the BM stromal cells derived from healthy donors showed very little pseudoemperipolesis activity, nurse cell activity is seen to be a unique feature of BM stromal cells derived from RA.

RA-BMNC produced IL-6, IL-7, IL-8 and GM-CSF. Levels of IL-6 and IL-8 produced by RA-BMNC were higher compared to those produced by BM stromal cell lines derived from healthy donors. Moreover,

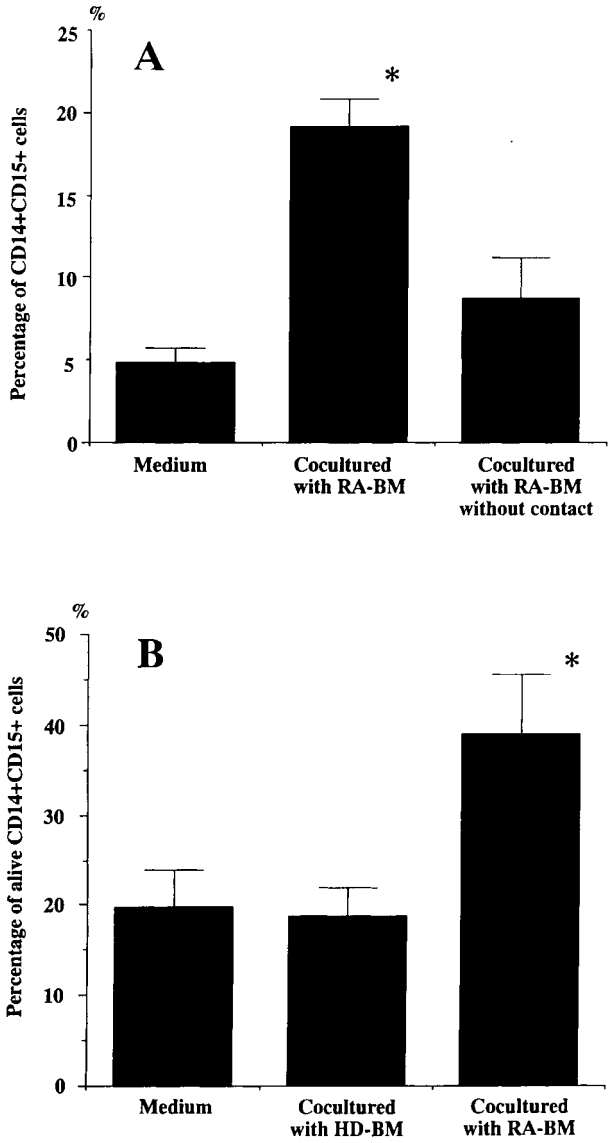

FIG. 6. Induction and maintenance of unusual myeloid lineage cells by RA-BMNC. (A) CD14− CD15+ cells were isolated from mononuclear cell fraction derived from severe RA patients. 3×10^4 cells/well of RA-BMNC were incubated overnight, then 1×10^5 of CD14− CD15+ cells were added to the well. After a 5 day incubation, cells were collected and analysed by FACScan. RA-BM, RA-BMNC; $*P < 0.01$ *vs* medium and co-culture with RA-BM without direct cell–cell contact. (B) 3×10^4 cells/well of RA-BMNC were incubated overnight, then 1×10^5 of CD14+ CD15+ cells were added to the well. After a 14 day incubation, the numbers of living cells were counted. HD-BM, healthy donor BM stromal cells; $*P < 0.05$ *vs* medium and co-culture with HD-BM.

RA-BMNC produced IL-1, G-CSF and TNF-α, and significantly higher levels of IL-6, IL-8 and GM-CSF, after co-culture with lymphocytes. We previously reported that the levels of IL-6 and IL-8 were high in the BM sera of patients with RA, but low in peripheral blood sera, and that IL-1 was sometimes detected in the BM sera of patients with RA, but not in peripheral

blood sera [42]. These results may suggest that RA-BMNC forming pathological lesions in BM produced cytokines, possibly by forming a complex with lymphocytes. Production of inflammatory cytokines in BM of patients with RA may reflect the contribution of RA-BMNC to the pathogenesis of RA.

Bone marrow stromal cell lines derived from healthy donors produced HA. RA-BMNC produced significantly higher levels of HA, and HA production by BM stromal cells correlated with pseudoemperipolesis activity. Although in this study we were not able to show any precise findings related to HA production, the activation of RA-BMNC by direct cell–cell contact may be essential. Some studies have shown high levels of HA in the peripheral blood sera of patients with RA, and a correlation between HA levels and the severity of RA [43–45]. Although it may be released by the destruction of articular cartilage, our study showed that RA-BMNC is one of the major sources of HA. A high HA concentration in peripheral blood of RA patients may be due to its production by RA-BMNC.

We reported an abnormality of BM in patients with RA involving unique myeloid cells in the epiphyseal BM adjacent to joints affected with an active severe subset of RA [4]. In the present study, we induced differentiation of CD14+ myeloid cells from CD14− myeloid cells, and then maintained these CD14+ myeloid cells in the presence of RA-BMNC. RA-BMNC supported the growth of pre-B lymphocytes, T lymphocytes and myeloid lineage cells. It is likely that RA-BMNC have the ability to support a wide range of haematopoietic cells and, as a result, may contribute to the pathogenesis of RA. Differentiation and survival of these unique myeloid lineage cells may be one of the characteristic features in severe RA. In addition, these unique myeloid cells are probably induced in haematopoietic BM. In the epiphyseal BM adjacent to affected joints, only unique CD14+ myeloid cells were observed. Polymorphonuclear cells, functionally developed cells of myeloid lineage, were detected in the epiphyseal BM adjacent to affected joints and joint synovium. These observations suggest that haematopoietic BM is the site where unique myeloid cells are produced. Based on the above results, we conclude that the differentiated unique myeloid cells are generated in the epiphyseal BM, migrate into the joint cavity and contribute to the pathogenesis in arthritic joints.

We also established nurse cell-like clones from synovial tissue in affected joints of RA patients. Stromal cell clones established from RA synovial tissue have characteristics and functions similar to RA-BMNC [34]. The presence of stromal cells with nursing activity in BM and joint synovium of RA patients may lead to pathogenesis by inducing growth and differentiation of haematopoietic cells with abnormal functions. It remains unknown why similar stromal cells exist in synovium and BM. To answer this question, we employed a collagen-induced experimental arthritis model and found that BM stromal cells migrated into the joint cavity and contributed to the proliferation of synovial cells [36]. We hypothesize that the same stromal cells exist in BM and joint synovium, and these stromal cells may play a pivotal role in the pathogenic microenvironment both in BM and inflamed synovial tissue.

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