morphology and function of multinucleated bone-resorbing giant cells, which were derived from CD14-positive monocyte-like cells in their SF.

Patients and methods

Patients

Seven patients with RA (seven women) and five patients with OA (two men and three women) participated in this study. All of the patients were treated at Osaka University Hospital or affiliated facilities. The average ages of the RA and OA patients were 52.8 ± 6.1 and 66.0 ± 6.0 yr, respectively. The diagnosis of RA was based on the 1987 revised criteria of the American College of Rheumatology (formerly, the American Rheumatism Association) [33], and that of OA was based on clinical and radiological features.

Cell numbers and surface antigen analysis

Synovial fluid was obtained from the knee joints of RA and OA patients by aspiration with an 18-gauge needle under aseptic conditions. Full informed consent was obtained from the patients for sample aspiration and all of the subsequent procedures. The joint-infiltrating cells in the SF of the RA and OA patients were collected by centrifugation at 1900 *g*. The cells were counted using a haemocytometer, whereby dead cells that were stained with trypan blue were excluded.

The surface markers of the cells in the SF samples were examined by staining with monoclonal antibodies (mAbs). In this study, we used fluorescein isothiocyanate (FITC)-conjugated anti-human mAbs that were specific for CD4, CD8, CD15 or CD19 (all from Becton Dickinson, Franklin Lakes, NJ), and phycoerythrin (PE)-conjugated anti-human mAbs that were specific for CD14, CD16 or HLA-DR (all from Becton Dickinson). The mononuclear cells (100 000) were incubated at 4°C for 30 min with 1 mg/ml of the FITC- or PE-conjugated mAbs. After washing twice with phosphate-buffered saline (PBS), the cells were analysed by flow cytometry using FACScan (Becton Dickinson), and the individual cell surface antigens were quantified. Dead cells were eliminated by propidium iodide staining, and excluded from the analysis by setting the scatter gates. The data were analysed using the CellQuest software (Becton Dickinson).

Mononuclear cell culture from the synovial fluid

The joint-infiltrating cells in the SF samples from RA and OA patients were collected as described previously [32]. Briefly, the cells were collected by centrifugation at 1900 *g*, and cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Gaithersburg, MD) that was supplemented with 10% heat-inactivated fetal calf serum (FCS; Gibco BRL) and 100 U/ml of penicillin-streptomycin (Gibco BRL) (Maintenance Medium; MM), and seeded into 6-well flat-bottomed culture plates (Becton Dickinson, Mountain View, CA). The cells were maintained at 37°C in humidified air that contained 7% CO_2, and half of the medium was changed weekly. After 3 to 5 weeks of culture, most of the lymphocytes and granulocytes had disappeared, and the monocyte-like cells that floated on the fibroblast-like cells, which adhered to the bottom of the culture plate, predominated. The non-adherent cells were harvested and the CD14-positive monocyte-like cells were purified from these non-adherent cells using the magnetic-activated cell sorter (MACS; Miltenyi Biotec GmbH, Germany) and magnetic beads that were conjugated with the anti-CD14 antibody, according to the manufacturer's instruction.

Formation of multinucleated bone-resorbing giant cells from CD14-positive monocyte-like cells

A total of 50 000 CD14-positive monocyte-like cells were cultured in MM for 96 h at 37°C and 7% CO_2 in 4-well chamber slides (Lab-Tek Chamber Slide System; Nalge Nunc International, IL), in the presence or absence of the following reagents: recombinant human (rh) interleukin (IL)-3, IL-5 and IL-7; granulocyte–macrophage colony-stimulating factor (GM-CSF); a combination of macrophage colony-stimulating factor (M-CSF) and the receptor activator of nuclear-factor-κB ligand (RANKL); or in the presence of 10% conditioned medium (CM). The optimal concentration of each cytokine was determined in preliminary experiments.

The frequency of multinucleated giant cell formation was calculated as the fusion index, which has been described previously [34]. Briefly, a minimum of 1000 nuclei within TRAP-positive multinucleated giant cells (>4 nuclei/cell) were counted. The fusion indices of the cells were calculated according to the following formula:

Fusion index (%) = [total number of nuclei within the multinucleated (>4 nuclei/cell) cells/total number of nuclei counted] $\times 100$

Cytochemical and immunocytochemical staining

At the end of culture period, the cells were stained with May-Grunwald–Giemsa and for the tartrate-resistant acid phosphatase (TRAP). TRAP staining was performed with a staining kit (Sigma Chemical Co., St. Louis, MO) in accordance with the manufacturer's instruction. May-Grunwald–Giemsa staining involved a 5-min incubation with a 1:1 dilution of May-Grunwald solution (Merck, Darmstadt, Germany), followed by a 10-min incubation with 1:20 dilution of Giemsa solution (Merck).

The multinucleated giant bone-resorbing cells, which differentiated from CD14-positive monocyte-like cells, were fixed with cold acetone, and stained immunocytochemically with rabbit polyclonal antibodies that were specific for actin (Santa Cruz Biotechnology Inc., Santa Cruz, CA), carbonic anhydrase II (Rockland, Gilbertsville, PA) or the vitronectin receptor (Chemicon International Inc., Temecula, CA), or with a goat polyclonal antibody that was specific for the calcitonin receptor (Santa Cruz Biotechnology).

Cytokines and reagents

rhIL-3, IL-5, GM-CSF and M-CSF were purchased from R&D Systems (Minneapolis, MN). rhIL-7 was obtained from Genzyme Corporation (Cambridge, MA), and the receptor activator of nuclear-factor-κB ligand (RANKL) was purchased from Peprotech (London, UK). CM were prepared as reported previously [35]. Briefly, a mixture of peripheral blood mononuclear cells (PBMC) from 10 healthy donors was stimulated with phytohaemagglutinin (PHA; Sigma) at 37°C for 72 h. The culture supernatant fluids were collected, filtered and used as conditioned media.

Bone resorption assays

To determine the resorption activities of the TRAP-positive giant cells, 70 000 CD14-positive monocyte-like cells were cultured on dentin slices that were placed in 4-well chamber slides in medium with different cytokines or CM for 14 days. The cells on the dentin slices were removed by brushing in distilled water, cleaned by ultrasonication to remove adherent cells, and stained with haematoxylin (Sigma). The resorption pits were counted under a microscope. As an alternative method for analysing bone resorption, the CD14-positive monocyte-like cells were cultured on

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calcium phosphate-coated discs (Osteologic; Millenium Biologix Inc., Ontario, Canada) using the culture conditions described above. After 14 days of incubation, the discs were washed in 6% NaClO and 5.2% NaCl to remove the cells, dried, and examined by phase-contrast microscopy. The resorbed area on each disc was measured using the MacSCOPE image analyser (Mitani Corp., Fukui, Japan).

Statistical analysis

The values are presented as the means ± standard deviation (S.D.). Statistical analysis was performed using the non-parametric Mann–Whitney U-test. P values of >0.05 were considered to be statistically significant.

Results

Numbers and cell surface markers of joint-infiltrating cells in the RA and OA patients

In order to evaluate the absolute number of joint-infiltrating cells in the SF, the cells were collected and counted. The average number of cells in the SF of the RA patients was $11 \pm 7.8 \times 10^5$/ml, and that of the OA patients was $7.3 \pm 3.9 \times 10^4$/ml. There was a significant increase in the average number of cells in the SF of RA patients, as compared with the SF of OA patients ($P < 0.01$). FACScan analysis was performed to examine the cell surface phenotype of the joint-infiltrating cells. The cells in the RA and OA SF samples were positive for HLA-DR, and weakly positive for CD4, CD8 and CD16. The percentage of CD4-positive cells in the RA-SF was significantly higher than in the OA-SF (RA-SF, 39.2 ± 8.6% *vs* OA-SF, 18.1 ± 9.3%; $P < 0.05$). The only significant difference between the RA and OA patients was in the levels of CD4-positive cells in their SF.

Differentiation of cultured CD14-positive monocyte-like cells into multinucleated bone-resorbing giant cells

The non-adherent cells were harvested after 4 weeks of primary culture, and the CD14-positive cells were collected using MACS with magnetic beads that were conjugated to the anti-CD14 antibody. The purity of the CD14-positive cells was >98%, as assessed by FACS analysis (data not shown). The CD14-positive monocyte-like cells from the RA-SF and OA-SF samples were cultured with IL-3 in the absence of fibroblast-like cells. These cells differentiated into multinucleated bone-resorbing giant cells (Fig. 1A and C). However, the numbers of cell nuclei differed among the two groups of patients. We counted the nuclei in all of the multinucleated cells that had five nuclei or more. The average number of nuclei was significantly higher in the RA group (RA, 24.66 ± 6.06 nuclei *vs* OA, 11.2 ± 2.61 nuclei; $P < 0.05$) (Fig. 2). These multinucleated cells were positive for TRAP (Fig. 1B and 1D), and resorption pits were observed on dentin slices (Fig. 5A and B) and on Osteologic discs (Fig. 7A and B). The multinucleated cells were positive for carbonic anhydrase II, actin, vitronectin receptor and calcitonin receptor (Fig. 4). The average positive percentage of calciton receptor in TRAP-positive multinucleated cells was 90.69 ± 7.2%, and TRAP-positive mononuclear cells were calcitonin receptor negative. IL-5, IL-7, GM-CSF and a combination of RANKL and M-CSF also induced the differentiation of CD14-positive monocyte-like cells into multinucleated cells. These cells were also positive for TRAP, and had the same functions and characteristics (data not shown). In the presence of each of the cytokines, the fusion index of the multinucleated cells, which were derived from the CD14-positive monocyte-like cells from the RA-SF, was significantly higher than that of the OA-SF ($P < 0.05$). Interestingly, the CD14-positive cells that were cultured with a mixture of RANKL and M-CSF exhibited lower fusion indices than cells that were treated with IL-3 (Fig. 3). These results were confirmed in three separate experiments, using various concentrations of the cytokines. The

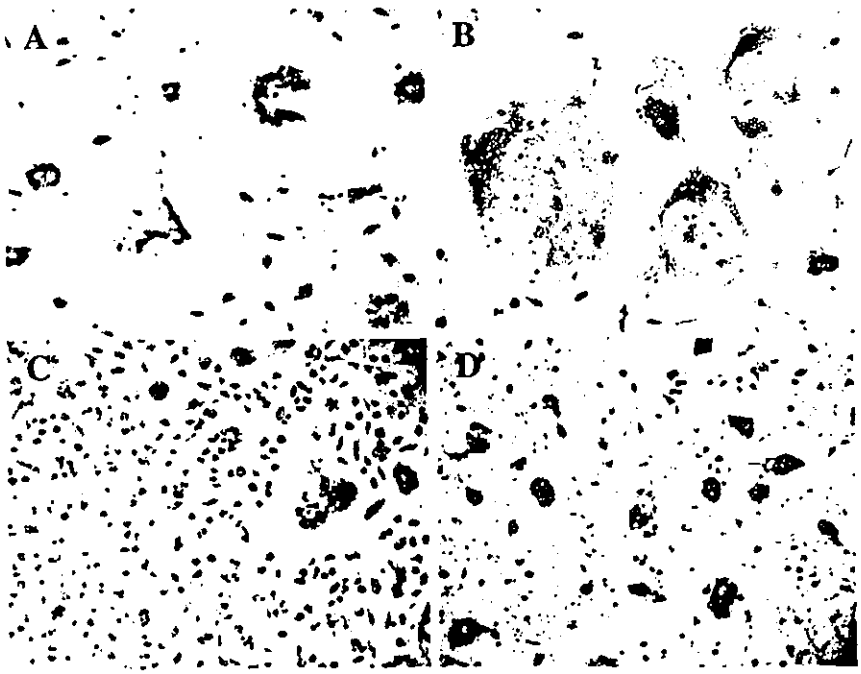

FIG. 1. Morphological examination of multinucleated giant cells that were derived from CD14-positive cells from the RA-SF or OA-SF. The CD14-positive monocyte-like cells were cultured with IL-3 (1 ng/ml). (A, C) May-Grunwald–Giemsa staining and (B, D) TRAP staining of multunucleated giant cells. Multinucleated giant cells from the RA-SF were bigger and have more nuclei compared with those from the OA-SF. Original magnification ×100.

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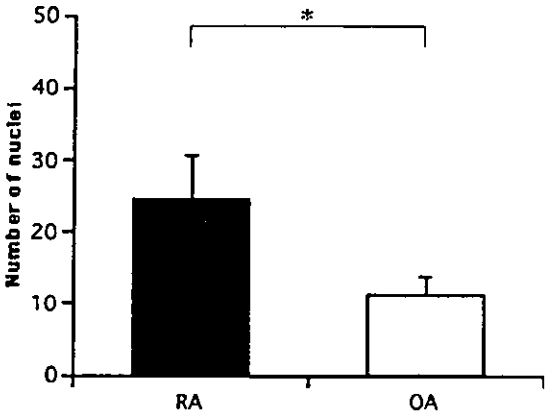

FIG. 2. The number of the multinucleated giant cells was counted. The nuclei in all of the multinucleated cells with five nuclei or more were counted. The average number of nuclei was significantly higher in the RA group. $^*P < 0.05$ *vs* OA.

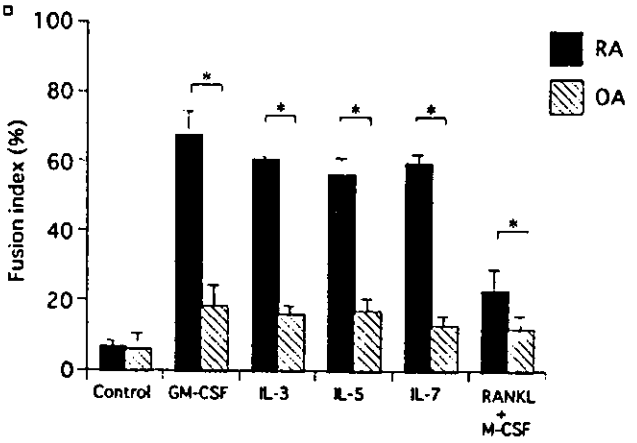

FIG. 3. IL-3, IL-5, IL-7, GM-CSF and a combination of RANKL and M-CSF induced the differentiation of CD14-positive monocyte-like cells into multinucleated cells. $^*P < 0.05$ *vs* OA.

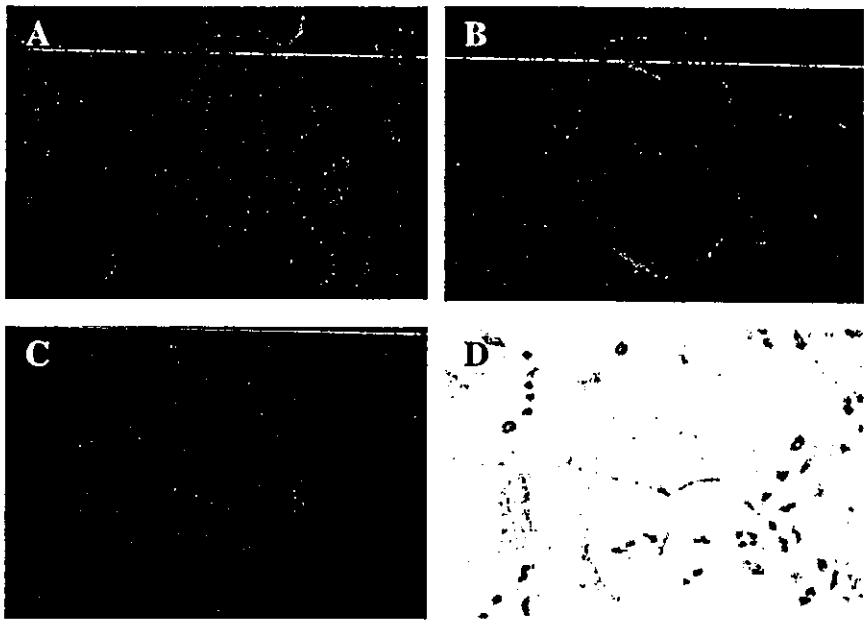

FIG. 4. Immunocytochemical examination of multinucleated giant cells which were derived from CD14-positive cells from the RA-SF. The CD14-positive monocyte-like cells were cultured with IL-3 (1 ng/ml). Multinucleated giant cells were positive for (A) carbonic anhydrase II, (B) actin, (C) vitronectin receptor, (D) calcitonin receptor. Original magnification ×200.

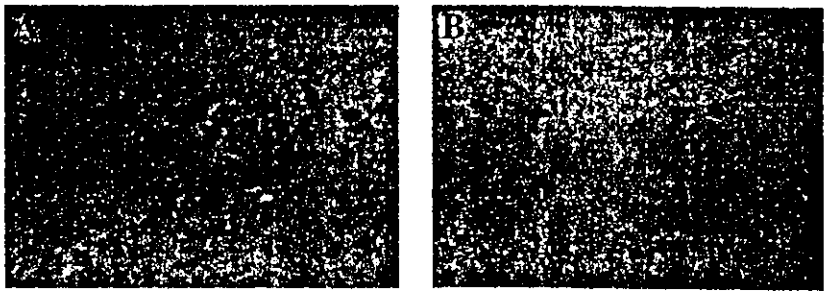

FIG. 5. Examination of resorption pits formed by multinucleated bone-resorbing giant cells on dentin slices. The CD14-positive monocyte-like cells were cultured with IL-3 (1 ng/ml) for 14 days on dentin slices. (A) Resorption pits were observed on dentin slices by bone-resorbing giant cells which were derived from CD14-positive cells from the RA-SF, but (B) not observed from the OA-SF. Original magnification ×200.

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multinucleated cells were induced by those cytokines in a dose-dependent manner and then the effect became fixed both in RA and OA.

Number of resorption pits on the dentin slices, and the percentage resorption on Osteologic discs

In order to determine the ability of multinucleated cells to absorb bone, the CD14-positive monocyte-like cells were cultured in medium with IL-3 for 14 days on either dentin slices or Osteologic discs. After the incubation period, the multinucleated giant cells from CD14-positive monocyte-like cells in the IL-3-stimulated RA-SF and OA-SF samples formed resorption pits on the dentin slices. The number of resorption pits formed by the RA-SF was significantly higher (143.0 ± 19.52; $P < 0.05$) than that formed by the OA-SF (9.0 ± 2.0) (Fig. 6). Since all of the cultures formed resorption pits on the Osteologic discs, the percentage resorption was calculated as the area of resorption relative to the total surface area of the disc. A marked increase in lacunar resorption was noted for the RA-SF cultures, in which the extent of resorption was $46.04 \pm 6.39\%$, as compared with that of the OA-SF cultures ($12.38 \pm 2.18\%$; $P < 0.05$) (Fig. 8). When the CD14-positive cells were purified from RA-SF and OA-SF which does not pre-culture for 4 weeks, they did not form resorption pits on either dentin slices or Osteologic discs (data not shown).

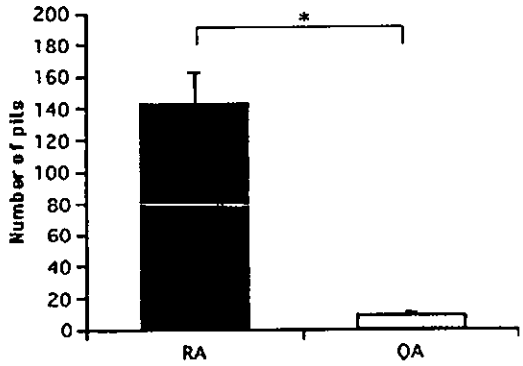

FIG. 6. Comparison of capability of resorption pit formation on dentin slices between bone-resorbing giant cells which were derived from CD14-positive cells from the RA-SF and OA-SF. The number of resorption pits was counted under microscopic examination. The number of resorption pits formed by the RA-SF was significantly higher than that formed by the OA-SF. *$P < 0.05$ *vs* OA.

Discussion

In this study, we demonstrated that multinucleated bone-resorbing giant cells were induced from CD14-positive monocyte-like cells in both RA-SF and OA-SF. However, the fusion indices and functional parameters of multinucleated cells that were derived from CD14-positive monocyte-like cells were increased in the RA-SF. While the percentages of monocyte/macrophage cells were similar, the absolute numbers of those cells were significantly higher in RA-SF than in OA-SF. These results suggest that the RA-SF contains many more cells with the ability to differentiate into TRAP-positive preosteoclasts than are found in the OA-SF. The resorption pits on either dentin slices or Osteologic discs of multinucleated cells that were derived from CD14-positive monocyte-like cells were increased in the RA-SF. In these functional parameters, high bone-resorbing activity of multinucleated cells in the RA-SF may be related to the survival rate or the number of multinucleated cells.

We reported previously that CD14-positive monocyte-like cells could be induced and maintained in the presence of nurse-like cells (RA-NLC), which were isolated from RA synovial tissue and bone marrow [32]. These cells expressed TRAP activity, and differentiated into multinucleated bone-resorbing giant cells when stimulated with IL-3, IL-5, IL-7 or GM-CSF in the absence of accessory cells. The RA-NLCs were shown to play an important role in the differentiation and maturation of lymphocytes through pseudoemperipolesis in RA joints [29–31]. In the primary cultures of SF mononuclear cells, the relatively large, round-shaped, non-adherent cells and adherent cells predominated after 3 weeks, and some of these adherent cells had nursing activities (H. Takano, personal communication), which suggests that adherent cells with nursing activities may play a role in the osteoclastogenesis observed in this study.

The multinucleated bone-resorbing cells shown in this study differentiated in the presence of IL-3, IL-5, IL-7 and GM-CSF. These cytokines and growth factors are known to promote the proliferation and differentiation of primitive hacmatopoietic cells. IL-3, IL-5 and GM-CSF are produced by activated T cells, and their receptors contain a common beta subunit [36]. In the present study, we found that CD4-positivity was more prevalent in the RA-SF than in the OA-SF, which suggests that T cells accumulate and produce IL-3, IL-5 and GM-CSF in the RA synovial fluid. GM-CSF is often detected in the joints of RA patients [37, 38], and synovial RA-NLCs produce GM-CSF *in vitro* [29, 30]. Matayoshi *et al.* [3] reported that both IL-3 and GM-CSF induced the differentiation of haematopoietic precursor cells into osteoclasts in the absence of stromal cells [3].

RANKL has been reported as a potent inducer of osteoclast development from monocytes, and is a key molecule in osteoclastogenesis [39, 40]. However, it was very interesting to note that a

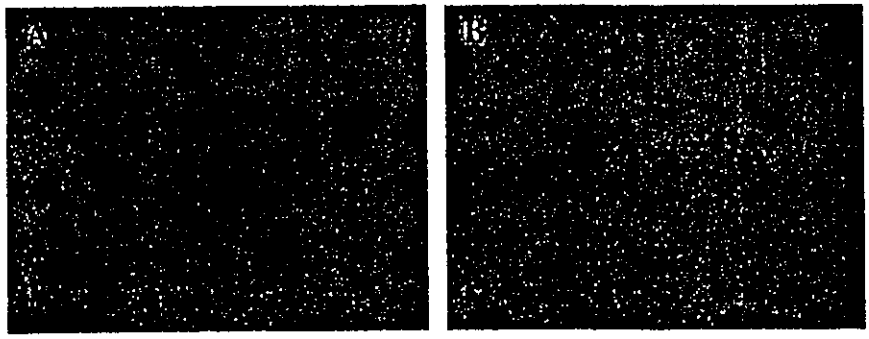

FIG. 7. Examination of resorption area formed by multinucleated bone-resorbing giant cells on Osteologic discs by using phase-contrast microscopy. The CD14-positive monocyte-like cells were cultured with IL-3 (1 ng/ml) for 14 days on Osteologic discs. (A) A wide-ranging area was formed by bone-resorbing giant cells which were derived from CD14-positive cells from the RA-SF, but (B) not formed in OA-SF. Original magnification ×100.

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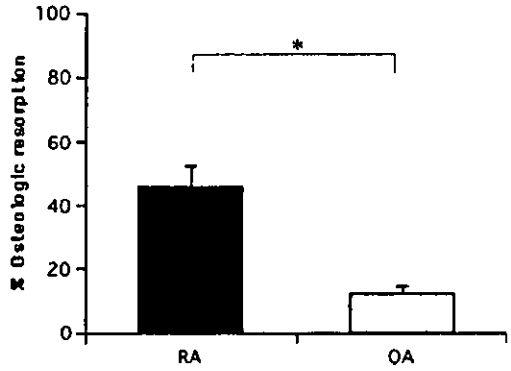

FIG. 8. Comparison of capability of resorbing area on Osteologic discs between bone-resorbing giant cells which were derived from CD14-positive cells from the RA-SF and OA-SF. The resorbed area on each disc was measured using the MacSCOPE image analyser. A marked increase in lacunar resorption was noted for the RA-SF cultures as compared with that of the OA-SF cultures. $*P < 0.05$ *vs* OA.

mixture of RANKL and M-CSF exhibited weaker induction of multinucleated cells than IL-3, IL-5, IL-7 and GM-CSF in our experiments. This finding suggests that stimulation of IL-3, IL-5, IL-7 and GM-CSF at the step of fusion of the pre-cultured CD14-positive monocyte-like cells is more dominant than RANKL. However, it is likely that RANKL is participating in differentiation of CD14-positive monocyte-like cells into preosteoclasts during the co-culture with fibroblast-like cells.

In this study, the fusion indices and functional parameters of the multinucleated cells, which were derived from CD14-positive monocyte-like cells, were much higher in the RA-SF than in the OA-SF. This result concurs with the conclusions of previous reports, in which histochemical studies indicated that TRAP-positive multinucleated cells were more numerous in the RA synovium than in the OA synovium [21, 41, 42]. In this study, similar numbers of CD14-positive cells were cultured from the SF of RA and OA patients, but there were significant differences between the RA-SF and OA-SF in terms of fusion indices and bone resorption activities. Our results suggest that the functions of CD14-positive cells may be enhanced in RA due to the enhanced ability of RA-SF stromal cells with nursing activity to support the differentiation of monocyte-like cells into TRAP-positive preosteoclasts. A detailed investigation of the differences in CD14-positive cell populations between RA and OA patients is underway.

In conclusion, CD14-positive cells and activated T cells in the RA-SF may play important roles in RA pathogenesis, which is characterized by progressive bone destruction and the enhanced function of haematopoietic cells, such as preosteoclast-like cells. These discoveries may provide a tool for understanding the mechanisms of bone destruction in RA, and for the development of effective treatments for joint destruction in RA patients.

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[21.11.2003–9:05pm] [FIRST PROOFS] [1–7] [Page No. 7] {OUP}Keg/KEH077.3d (RHE) Paper: KEH077

Synergistic Effect on the Attenuation of Collagen Induced Arthritis in Tumor Necrosis Factor Receptor I (TNFRI) and Interleukin 6 Double Knockout Mice

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ABSTRACT. ***Objective.*** To evaluate any additive effect on attenuation of collagen induced arthritis (CIA) in tumor necrosis factor receptor I (TNFRI) and interleukin 6 (IL-6) double knockout (DKO) mice.

Methods. CIA was induced in wild-type (Wt), TNFRI knockout (TNFRIKO), IL-6 knockout (IL-6KO), and DKO mice. Comparative studies were performed among these different mouse genotypes observing clinical (incidence, arthritis score), histological, radiologic, and immunological aspects.

Results. More than 90% of the Wt, TNFRIKO, and IL-6KO mice developed definite CIA, while only 20% of the DKO mice did so. Severity of arthritis, indicated by the arthritis score, was significantly reduced in both the TNFRIKO and IL-6KO mice compared with the Wt mice. Moreover, the severity of arthritis in the DKO mice was significantly reduced compared with each single KO mouse (by arthritis scores; DKO vs TNFRIKO, IL-6KO mice, $p < 0.05$). In addition, histological and radiologic changes were also significantly reduced in the DKO mice compared with each single KO mouse (by histological and radiologic scores; DKO vs TNFRIKO, IL-6KO mice, $p < 0.05$ and $p < 0.01$ respectively). In immunological studies, serum anti-type II collagen (anti-CII) antibody concentrations were significantly decreased in the DKO mice compared with each single KO mouse (DKO vs TNFRIKO, IL-6KO mice, $p < 0.01$).

Conclusion. Simultaneous blockade of TNFRI and IL-6 showed synergistic rather than additive effects on the attenuation of CIA. Combinations of anti-TNF-α and anti-IL-6 therapy may provide clinical benefits for treatment of rheumatoid arthritis compared with therapy against each single cytokine. (J Rheumatol 2003;30:22–7)

Key Indexing Terms:
TUMOR NECROSIS FACTOR RECEPTOR I
DOUBLE KNOCKOUT MICE
INTERLEUKIN 6
COLLAGEN INDUCED ARTHRITIS

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease characterized by bone destructive polyarthritis. Although its etiology remains unknown, cumulative evidence suggests that proinflammatory cytokines such as interleukin 1 (IL-1), tumor necrosis factor (TNF), and IL-6 play a pivotal role in the pathology of RA[1-3]. These proinflammatory cytokines are suggested to be optimal therapeutic targets for RA. Indeed, the outcome of recent clinical trials of anticytokine therapies using neutralizing antibodies, soluble receptors, and other inhibitors encouraged us[4-7]. Among them, anti-TNF therapy using neutralizing antibodies or soluble TNF receptors (TNFR) showed excellent efficacy and is currently accepted as a promising new therapy for RA[5,6]. In addition, a recent study showed that IL-6 blockade by anti-IL-6 receptor (IL-6R) antibody also showed some clinical efficacy for RA, and multicenter clinical trials are proceeding in Japan[7]. However, despite the excellent efficacy, these single cytokine blockade therapies do not always attenuate disease activity sufficiently, and there are still some populations of non-optimal responders for each therapy directed against a single cytokine[8]. In such cases of non-optimal responders for each single cytokine therapy, combination with other types of therapy was suggested to be necessary. Indeed, the combination of anti-TNF-α and anti-T cell (anti-CD4) therapies showed enhanced efficacy in a mouse arthritis model[9].

There may be another potential problem in inhibiting a single cytokine as a therapeutic approach for RA, which relates to the overlapping activities of different cytokines. This problem can possibly be addressed by simultaneous blockade of different cytokines. Partial attenuation of

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collagen induced arthritis (CIA), a mouse model for RA, was reported in TNF-receptor I (TNFRI)[10] and IL-6 deficient mice[11], suggesting that single cytokine blockade may not ameliorate CIA sufficiently.

To investigate whether simultaneous blockade of TNF and IL-6 shows any additive effect on the attenuation of CIA, we carried out a comparative study in wild-type (Wt), TNFRI knockout (TNFRIKO), IL-6 knockout (IL-6KO), and double knockout (DKO) mice, observing clinical, histological, radiologic, and immunological aspects.

MATERIALS AND METHODS

Mice. Animal experiments in this study were approved by the Animal Experimentation Committee of Osaka University and performed in accord with their guidelines. IL-6KO mice were provided by Dr. M. Kopf, Max-Planck-Institut für Immunbiologie, Freiburg, Germany[12]. TNFRIKO mice were provided by Dr. T.W. Mak, Amgen Institute, Ontario Cancer Institute, Toronto, Canada[13]. IL-6KO mice and TNFRIKO mice were backcrossed with DBA/1 mice (Nippon Charles River, Kanagawa, Japan) for 8 generations to introduce CIA susceptibility. Then TNFRI and IL-6 DKO mice were generated by matching these mice. Mice were screened for IL-6 and TNFRI genotype by polymerase chain reaction (PCR) using DNA prepared from tail biopsies as described[12,13]. The Wt, IL-6KO, TNFRIKO, and DKO mice were between 8 and 12 weeks old at the time of first immunization. The ratio between male and female was kept equal in each group. Mice were bred under standard pathogen-free conditions in the Experimental Animal Center of Osaka University Medical School.

Induction of CIA. CIA was induced by established methods[14]. Briefly, mice were immunized by intradermal injection at the base of the tail with 100 μg of bovine type II collagen (CII) (Cosmo Bio, Tokyo, Japan) in 0.1 M acetic acid, emulsified with an equal volume of complete Freund's adjuvant (CFA; Difco, Detroit, MI, USA). Twenty-one days later, mice were boosted by the same method.

Clinical assessments of arthritis. Every week after the first immunization, during the entire followup period (15 weeks), mice were assessed by 2 independent observers for signs of arthritis. The severity of arthritis was graded on a 0–4 scale as follows: 0 = normal; 1 = swelling and/or redness in only 1 finger; 2 = swelling and/or redness in more than 1 finger; 3 = swelling and/or redness in the entire paw; 4 = deformity and/or ankylosis. Each paw was graded, and the 4 scores were summed, so the maximum possible score per mouse was 16. The group arthritis score was calculated by dividing the total score by the total number of mice that developed definite arthritis. The incidence was expressed as the percentage of the mice that showed visible arthritis.

Histological assessment of arthritis. Histological examinations were carried out as follows. Seven mice were randomly selected from the Wt, TNFRIKO, and IL-6KO groups. In the DKO group, 3 mice that developed definite arthritis were selected. They were killed at the end of the experiment. Both hind paws were removed, fixed in 4% paraformaldehyde in phosphate buffered saline (PBS), decalcified in hydrochloric acid and formic acid, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Microscopic evaluation was performed under blinded conditions. The severity of arthritis in each joint was classified as described[15] and graded 0–3 as follows: 0 = normal; 1 = mild; 2 = moderate; 3 = severe.

Radiologic assessment of arthritis. Mice were killed at the end of the experiment. Anteroposterior radiographs of the 4 limbs were obtained using a cabinet soft X-ray apparatus (CMB-2; Softex, Tokyo, Japan). Radiologic changes were evaluated under blinded conditions using a described scoring system[16], with some modification. Each limb was assessed for osteopenia and bone erosion and graded 0–3 as follows: 0 = normal; 1 = mild change; 2 = moderate change; 3 = severe change. The 4 scores were summed, for a maximum possible score per mouse of 12.

Measurements of serum anti-CII antibodies and nonspecific total IgG. Sera from mice of each genotype were obtained at the end of the experiment. Serum anti-CII antibody and nonspecific total IgG concentrations were measured by ELISA as described with some modification[17]. Briefly, 96 well plates were coated with bovine CII antigen solution (2 μg/ml) or anti-mouse IgG (Vector, Burlingame, CA, USA) solution (1 μg/ml) and incubated overnight at 4°C. Nonspecific binding was blocked with PBS containing 1% bovine serum albumin for 1 h at room temperature. Serially diluted serum samples (1:10 to 1:10.000) were added for 2 h at room temperature. Alkaline phosphatase conjugated horse anti-mouse IgG (Vector) or alkaline phosphatase conjugated goat anti-mouse IgG1 or IgG2a (Vector) was also added for 2 h at room temperature. Color development with p-nitrophenylphosphate (Kirkegaard and Perry, Gaithersburg, MD, USA) was monitored at 405 nm with an immunoreader NJ-2300 (Nihon InterMed, Tokyo, Japan). Values from each mouse genotype were shown as relative values compared with the mean value of the Wt group as 100 units.

Statistical analysis. Values were expressed as means ± SEM and variance analyses were performed before further statistical analyses. Using a Statview 5.0 statistical package for Macintosh (Abacus, Berkeley, CA, USA), the Mann-Whitney U test was used to assess the significance of the relative arthritis score, incidence, radiologic assessments, and antibody levels. Student t test was used to assess the significance of the histological findings. P values < 0.05 were considered significant.

RESULTS

Incidence and severity of CIA. CIA was induced in each different mouse genotype as described in Materials and Methods. As shown in Figure 1A, mice started to develop arthritis 2 weeks after the first immunization in the Wt, one week later in the TNFRIKO, 3 weeks later in the IL-6KO, and 4 weeks later in the DKO group. At the end of the experiment, all 19 mice had developed arthritis in the Wt, 23 of 24 in the TNFRIKO, 15 of 17 in the IL-6KO group, while CIA was observed in only 4 of 21 DKO mice. Thus, in the DKO mice, the incidence of arthritis was significantly lower compared with the Wt, TNFRIKO, and IL-6KO mice (DKO vs Wt, TNFRI, IL-6KO mice, $p < 0.01$). As shown in Figure 1B, the arthritis score on each single cytokine KO mouse was lower compared with the Wt mice, as previously reported[10,11]. In addition, the arthritis score of the DKO mice that developed arthritis was significantly lower compared with each single cytokine KO mouse (DKO vs TNFRIKO, IL-6KO mice, $p < 0.05$).

Histological changes. Both hind paws were removed at the end of the experiment, and histological changes were evaluated and scored as described in Materials and Methods. As a representative photograph shows (Figure 2), the ankle joint was almost completely destroyed with severe synovitis in the Wt mice (Figure 2, panels A and E). Marked joint destruction and synovitis were detected in the TNFRIKO mice, but were less severe compared with the Wt mice (panels B and F). In the IL-6KO mice, only moderate synovitis was observed (panels C and G), as reported[11]. In contrast, in the DKO mice, the ankle joints were almost completely preserved (panels D and H). The overall histological changes of each mouse genotype are summarized in Table 1. The histological score on each single cytokine KO

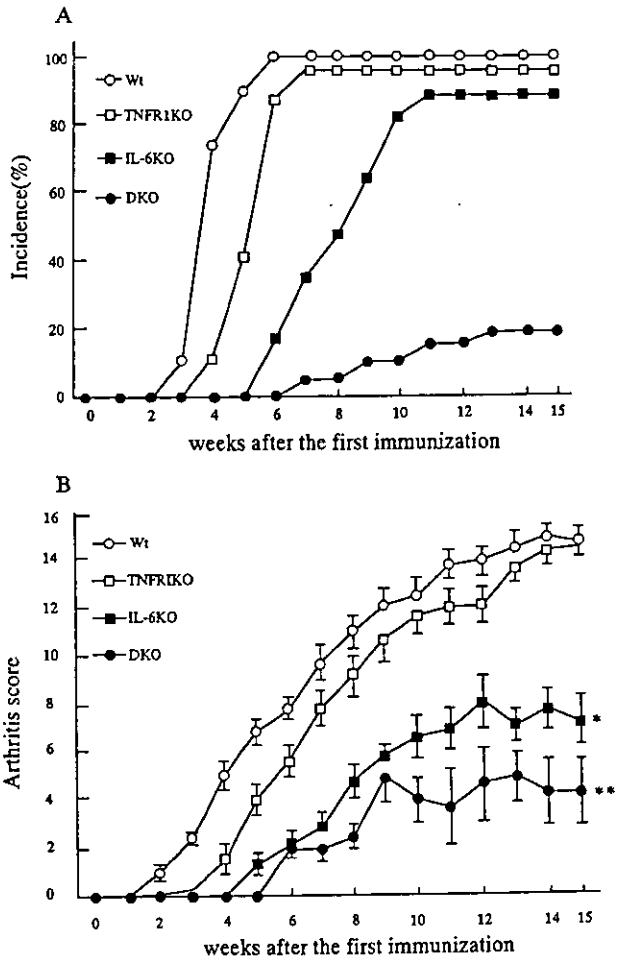

Figure 1. Development of CIA in Wt, IL-6KO, TNFRIKO, and DKO mice. DBA/1 mice of Wt, TNFRIKO, IL-6KO, and DKO mice were immunized with type II collagen in adjuvant, as described in Materials and Methods. Signs of arthritis were recorded every week from the first immunization through the entire followup period (15 weeks). A. Incidence of arthritis. B. Arthritis scores (means ± SEM). *p < 0.05 versus Wt, TNFRIKO mice. **p < 0.05 versus Wt, TNFRIKO, IL-6KO mice at the end of the experiment.

mouse was lower compared with the Wt mice, as reported[10,11]. In addition, the histological score of the DKO mice that developed arthritis was significantly lower compared with each single cytokine KO mouse (DKO vs TNFRIKO, IL-6KO mice, $p < 0.05$).

Table 1. Histological scores in each mouse genotype. Values are expressed as means ± SEM.

Genotype (No. of Mice Studied)	Histological Score
Wt (7)	2.63 ± 0.28
TNFRIKO (7)	2.00 ± 0.20*
IL-6K0 (7)	1.43 ± 0.37**
DKO (3)	0.67 ± 0.33***

* $p < 0.05$ versus Wt mice. ** $p < 0.05$ versus Wt, TNFRIKO mice. *** $p < 0.05$ versus Wt, TNFRIKO, and IL-6KO mice.

Radiologic changes. The summary of radiologic changes (osteopenia and bone erosion) of each mouse genotype is shown in Table 2. Each single cytokine KO mouse showed significant reductions in the radiologic scores compared with the Wt mice. The DKO mice showed significantly lower radiologic scores than those of each single cytokine KO mouse (DKO vs TNFRIKO, IL-6KO, $p < 0.01$), concordant with their histological changes.

Serum anti-CII antibody and nonspecific total IgG levels. Serum anti-CII antibody and nonspecific total IgG levels were measured by ELISA. As shown in Table 3, each single cytokine KO mouse showed significantly lower anti-CII antibody levels in total IgG, IgG1, and IgG2a than those of the Wt mice ($p < 0.01$). Serum anti-CII antibody levels of the DKO mice in total IgG, IgG1, and IgG2a were significantly lower compared with each single cytokine KO mouse (DKO vs TNFRIKO, IL-6KO, $p < 0.01$). However, nonspecific total IgG levels were not significantly decreased in each single cytokine KO and DKO mouse.

DISCUSSION

We performed a comparative study in Wt, TNFRIKO, IL-6KO, and DKO mice induced with CIA, observing clinical, histological, radiologic, and immunological findings. Partial attenuation of CIA was observed in the TNFRIKO mice as described[10]. In addition, more obvious partial attenuation of CIA was also observed in the IL-6 KO mice, as we reported previously[11]. However, we could not observe complete protection from CIA in the IL-6KO mice as Alonzi, *et al* reported[18]. Although we cannot provide a definite reason for the difference between their results and ours, 2 points are worth consideration. First, the immunization protocols were different. We used complete Freund's adjuvant (CFA) for both immunizations, while they used CFA for the first immunization but incomplete Freund's adjuvant for the second. Second, the genetic background of the IL-6KO mice might be different. The mice used by Alonzi, *et al* were backcrossed with DBA/1J for 5 generations, while our mice were backcrossed for 8 generations. Therefore, the induction of CIA (or immunization) in our experimental system might be stronger than in theirs, and our mice might have been more susceptible to CIA. Indeed, almost all the littermate Wt mice used in the present study developed CIA as severe as DBA/1J mice, whereas a lower incidence and milder severity of CIA were reported by Alonzo, *et al*.

We also observed enhanced attenuation of CIA in the DKO mice compared with each single cytokine KO mouse. Interestingly, almost all the mice of each single cytokine KO group developed definite CIA, as reported[10,11], while only 20% of the mice in the DKO group developed CIA. Moreover, only marginal changes were observed in the histologic and radiologic examinations of the DKO mice. Thus, the effect on the attenuation of CIA in the DKO mice appears to be synergistic rather than additive. Although it is

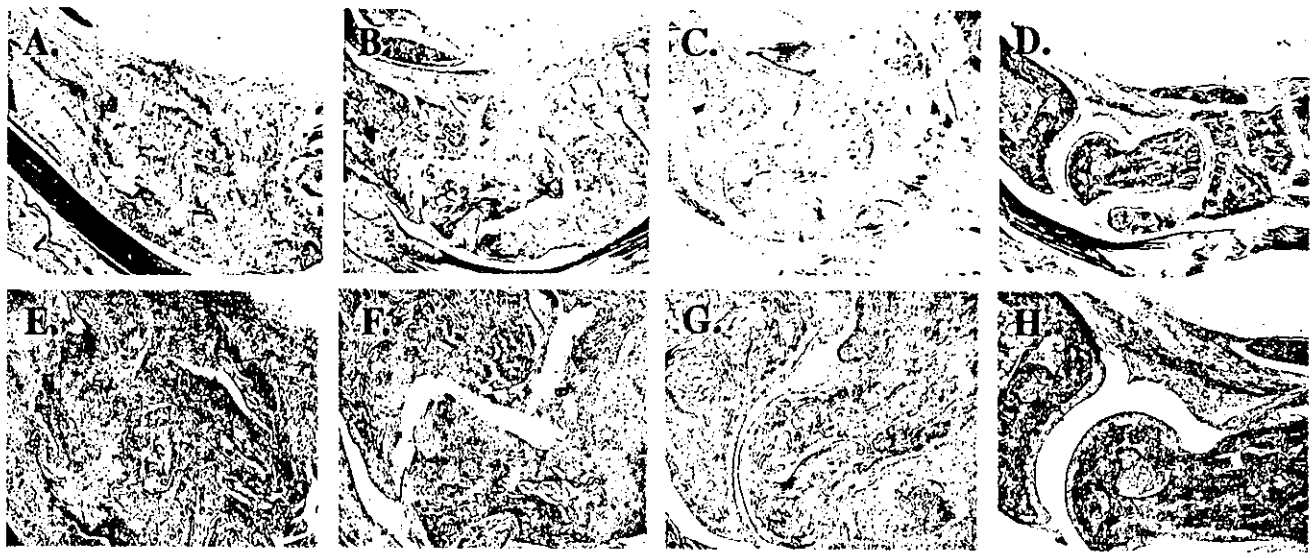

Figure 2. Histopathological evaluations of CIA in Wt, IL-6KO, TNFRIKO, and DKO mice were performed at the end of the experiment. Each photograph shows an ankle joint of a hind limb stained with hematoxylin-eosin. A and E show paws of Wt mice with CIA (A, original magnification ×20; E, original magnification ×40). In A and E, ankle joints are almost completely destroyed by severe synovitis in Wt mice. B and F show paws of TNFRIKO mice with CIA (B, original magnification ×20; F, original magnification ×40). In B and F, marked joint destruction and synovitis are shown, but are less severe compared with Wt mice. C and G show paws of IL-6KO mice with CIA (C, original magnification ×20; G, original magnification ×40). In C and G, only moderate synovitis is shown. D and H show paws of DKO mice with CIA (D, original magnification ×20; H, original magnification ×40). In G and H, almost normal joint organization is preserved.

Table 2. Radiologic changes in each mouse genotype. Values are expressed as means ± SEM.

Genotype (No. of Mice Studied)	Osteopenia	Bone Erosion
Wt (19)	9.21 ± 0.56	10.07 ± 0.38
TNFRIKO (24)	7.04 ± 0.66*	8.83 ± 0.62*
IL-6KO (17)	3.82 ± 0.78**	4.88 ± 1.04**
DKO (21)	0.68 ± 0.25***	0.95 ± 0.49***

* $p < 0.05$ versus Wt mice. **$p < 0.05$ versus Wt, TNFRIKO mice.
*** $p < 0.01$ versus Wt, TNFRIKO, and IL-6KO mice.

not clear, there is a possible explanation for such synergistic effects on the attenuation of CIA in the DKO mice. Proinflammatory cytokines such as TNF, IL-1, and IL-6 are well known to show redundancy in several biologic activities. In addition, since the individual cytokines do not act independently, but interact with each other, synergistic effects are often observed in *in vitro* studies. For example, the synergistic effect on acute phase protein production in combinations of IL-6 and TNF-α has been reported. TNF-α alone does not induce serum amyloid A (SAA), but in combination with IL-6 shows enhanced production of SAA in human hepatoma cell lines[19]. In contrast, IL-6 alone does not induce plasminogen activator inhibitor type 1 in Hep G2 cells, but markedly enhances its induction in combination with TNF-α[20]. Thus, simultaneous blockade of TNF and IL-6 may turn off such synergistic biologic activities, which relate to the pathogenesis of CIA, resulting in some synergistic effect on the attenuation of CIA.

Our findings also suggest that the combination of anti-TNF and anti-IL-6 therapy may have some clinical benefits for the treatment of RA in some cases. The outcome of recent clinical trials of therapies directed against single cytokines, including anti-TNF and anti-IL-6 therapies, appears to be excellent, and some are currently accepted as

Table 3. Serum anti-type II collagen (anti-CII) antibody titers and nonspecific total IgG levels in each mouse genotype. Values from each mouse genotype are shown as relative values compared with the mean value of the Wt group as 100 units.

Genotype (No. of Mice Studied)	Total IgG	IgG1	IgG2a	Nonspecific IgG
Wt (16)	100 ± 11.80	100 ± 20.56	100 ± 14.19	100 ± 17.45
TNFRIKO (17)	42.71 ± 10.26†	48.01 ± 13.28†	43.89 ± 10.11†	111.36 ± 23.07
IL-6KO (14)	65.35 ± 11.84†	52.68 ± 15.08†	76.57 ± 14.33†	88.41 ± 23.16
DKO (16)	13.29 ± 4.22††	9.58 ± 2.21††	13.58 ± 4.45††	95.56 ± 9.95

† $p < 0.01$ versus Wt mice. †† $p < 0.01$ versus Wt, TNFRIKO, and IL-6KO mice.

promising new therapy for RA[5-7]. However, despite the excellent efficacy, these single cytokine blockade therapies do not always sufficiently attenuate disease activity, and there are still some populations of non-optimal responders for each single cytokine therapy[8]. In such cases of non-optimal response for single cytokine therapy, combination with other types of therapy is suggested to be necessary. Indeed, the combination of anti-TNF-α and anti-T cell (anti-CD4) therapies showed enhanced efficacy in a mouse arthritis model[9].

There may be another potential problem in inhibiting a single cytokine as a therapeutic approach for RA, which relates to the overlapping activities of different cytokines. For example, we described a patient with severe RA who did not respond optimally to anti-TNF-α antibody therapy[21]. The patient showed lymphadenopathy and underwent a biopsy of the swollen lymph nodes to eliminate the possibility of malignancy. When we assessed TNF-α and IL-6 mRNA expression in the lymph nodes by reverse transcription PCR, only enhanced IL-6 but not TNF-α mRNA expression was observed. In addition, anti-TNF-α antibody could not inhibit spontaneous *in vitro* IL-6 production of the lymph node block culture, indicating IL-6 production in the lymph node might be independent of TNF-α. In such a case of a non-optimal response, it would be necessary to block not only TNF-α but also IL-6 simultaneously.

As for the immunological aspects, serum anti-CII antibody concentrations in the DKO mice were significantly decreased compared with each single cytokine KO mouse and were less than 10% of the Wt mice. This suggests that the DKO mice might be in an immunocompromised state. However, nonspecific total IgG concentrations in serum were not significantly decreased in the DKO mice compared with each single KO mouse. As well, we observed no infections in DKO mice or single KO mice during the whole experiment period. Thus the risk of infection in the DKO mice may not be higher than in each single KO mouse, although they were bred under standard pathogen-free conditions. However, since recent reports note that infections, particularly opportunistic infections including tuberculosis, are the most serious adverse effects of anti-TNF-α therapy[22,23], we should observe great caution in the use of anticytokine combination therapy.

We observed synergistic effects on the attenuation of CIA in TNFRI and IL-6KO mice, and CIA was almost completely prevented in the DKO mice. Recently, Bendele, *et al* reported synergistic effects of IL-1 receptor antagonist and PEG soluble TNFRI therapy on the amelioration of CIA[24]. Thus our findings suggest a clinical benefit of the combination of anti-TNF-α and anti-IL-6 therapies compared with therapy against each single cytokine in some cases of non-optimal responses to single cytokine therapy, although treatment experiments using combination of neutralizing antibodies to TNF-α and IL-6 must still be carried out in mice with established CIA.

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Possibility of Preventive Treatment for EBV-associated NK Cell-lineage Proliferative Disorders

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Possibility of Preventive Treatment for EBV-associated NK Cell-lineage Proliferative Disorders

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Abstract

Objective **Chronic active EBV infections (CAEBV) are often causative of malignant lymphoproliferative disorders, such as natural killer (NK) cell-lineage granular lymphocyte proliferative disorders (NK-GLPD), which are refractory to several conventional chemotherapies and usually show a poor prognosis. To explore the possibility of preventive treatment for Epstein-Barr virus (EBV)-infected NK-GLPD, we examined the effect of antiviral drugs on EBV-infected pre-malignant NK cells.**

Methods **EBV-infected pre-malignant NK cells (P-NK cells) were isolated from the periphery of a patient suffering from severe hypersensitivity to mosquito bites (SHMB). Abnormal oligoclonal expansion of EBV-infected CD56(+)/CD3(−) NK cells was observed in her periphery. Effects of several antiviral drugs were examined both on the proliferation and on EBV-replication of P-NK cells.**

Results **Vidarabine and foscarnet, but not acyclovir nor gancyclovir, significantly suppressed both the proliferation and EBV-DNA replication of P-NK cells in a dose-dependent manner, whereas these drugs did not suppress the proliferation of YT, which is an EBV-infected malignant NK cell line. The combination of vidarabine and foscarnet had an additive effect and almost completely suppressed the proliferation of P-NK cells.**

Conclusion **The present results indicate that vidarabine and/or foscarnet may be effective for preventive treatment of EBV-associated NK-GLPD.**

(Internal Medicine 42: 250–254, 2003)

Key words: severe hypersensitivity to mosquito bites (SHMB), vidarabine, foscarnet

Introduction

Severe hypersensitivity to mosquito bites (SHMB) is a rare disease that mostly affects Asians, including Japanese. When an individual with SHMB is bitten by a mosquito, they soon develop a high-grade fever and the skin at bitten sites forms deep ulcers. Another important clinical feature of SHMB is the high mortality because of complications, such as malignant NK cell-lineage granular lymphoproliferative disorder (NK-GLPD). Recent reports have revealed that chronic active Epstein-Barr virus infection (CAEBV) is closely associated with the pathogenesis of SHMB and its malignant complications (1, 2). Conventional chemotherapies are not effective and therefore the prognosis is poor (3).

Recently we treated a young female suffering from SHMB and CAEBV (4). In her periphery, we observed a marked oligoclonal expansion of EBV-infected NK cells. Considering the close association between malignant NK-GLPD and CAEBV/SHMB, it is highly suggested that the NK cells in the patient (P-NK cells) represent a preneoplastic population. In order to rescue not only this patient but also other individuals suffering from CAEBV from a desperate clinical course, we explored the possibility of preventive treatment for NK-GLPD. Some antiviral drugs, such as foscarnet and vidarabine, are emerging as good candidates for possible preventive treatment.

Patient and Methods

All experiments were done with both the informed consent of the patient and the approval of the Osaka University Human Research Committee.

Patient

The patient was an 18-year-old female. When she was 14

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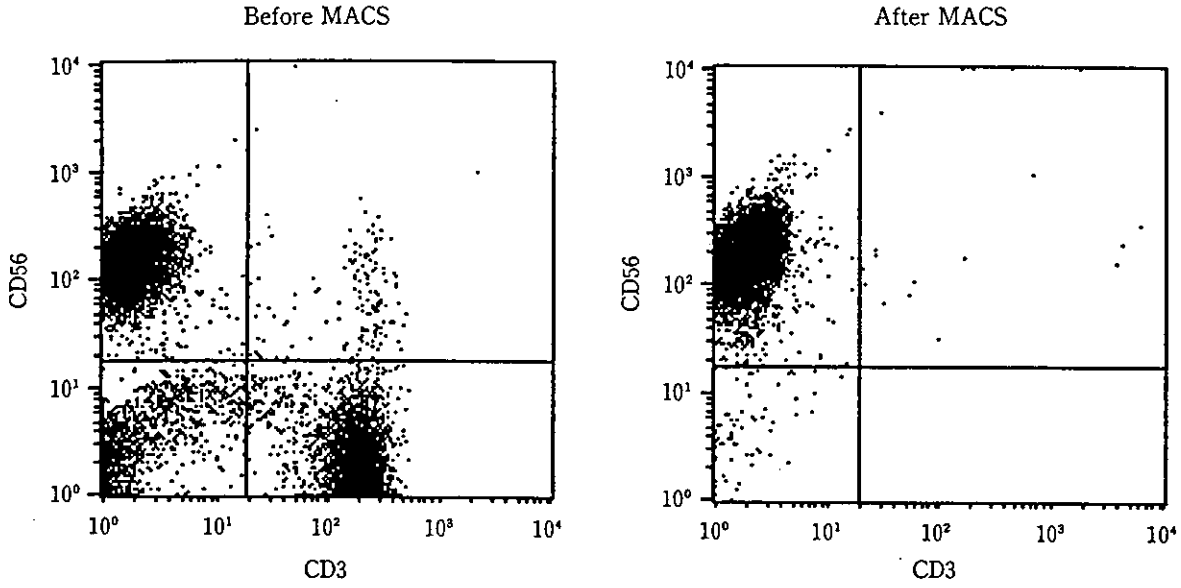

Figure 1. Highly purified NK cells (CD56(+), CD3(–)) obtained using MACS. The expression of CD3 and CD56 in mononuclear cells (MNC) from the patient with SHMB and CAEBV was assessed before (*left* panel) and after (*right* panel) MACS by two-color FACS analysis.

years old, she first experienced severe hypersensitivity to mosquito bites. The mosquito-bitten sites developed into deep ulcers, and she also had high grade fever as a systemic symptom. She was diagnosed as having a severe hypersensitivity to mosquito bites (SHMB). Because her symptoms did not improve with corticosteroid therapy, she was admitted to our hospital for further examinations. On admission, no remarkable physical findings were noted except multiple scarring lesions on her face and extremities. Laboratory studies showed a normal white blood cell count ($4.6\times10^3/\mu l$), whereas the proportion of lymphocytes was increased (54.4%). Flow cytometric analysis revealed a marked increase in the CD56+/CD3– (NK cell) population (51.1%). In addition, the pattern of serum EBV-specific antibody was compatible with CAEBV as anti-EB-VCA IgG markedly was increased at ×2,560 whereas anti-EBNA was not increased at ×80. Bone marrow aspiration and gallium scintillation examinations showed the absence of hematological malignant disorders.

Purification of P-NK cells and their characteristics

Peripheral blood mononuclear cells from the patient were isolated by density-gradient centrifugation with Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden). NK cells were purified by magnetic cell sorting (MACS) using a StemSep Kit (Stem Cell Technologies Inc, British Columbia, Canada) (P-NK cells). After the MACS, more than 99% of collected cells were CD56-positive but CD3-negative (Fig. 1). As a control, NK cells from a healthy female were purified using the same protocol (C-NK cells). All experiments were carried out using these highly purified NK cells. The characteristics of the highly purified NK cells were analyzed by flow cytometry (FACScan Cytometer, Becton Dickinson Immunocytometry System, Mountain View, CA, USA). Compared to C-NK cells, P-NK cells showed an enhanced expression of high-affinity IL-2 receptor (CD25), Bcl-2 and Fas-ligand. In addition, P-NK cells showed augmentation of the proliferative response to IL-2 and resistance to Fas-induced apoptosis (4). The further characterization of cell surface markers by flow cytometric analysis showed CD94(+), NKG2A(+), CD161(–), CD69(–), C1.7(+), CD158a(+)~(±), CD158b(+), CD159(+), CD9(+), Vα24(–), and MDR(+). The NK activity of P-NK cells was highly enhanced, suggesting a high risk of malignancy according to a previous report (5).

Detection of EBV

The detection of EBV in P-NK cells was performed by polymerase chain reaction (PCR) analysis using primers specific for EBV DNA as described previously (6). The oligonucleotide sequences of primers are: 5′-CCAGAGGTAAG TGGACTT-3′ and 5′-GACCGGTGCCTTAGG-3′. As a positive control, we used genomic DNA from the EBV-positive Burkitt's lymphoma line, Raji.

Clonality of P-NK cells

The clonality of P-NK cells was assessed by Southern blot analysis using a terminal repeat EBV probe as described previously (7). Briefly, 10 μg of genomic DNA extracted from P-NK cells was digested with *Bam*HI, separated on a 0.7% agarose gel and transferred to a nitrocellulose membrane. The membrane was hybridized with a ^{32}P-labeled DNA

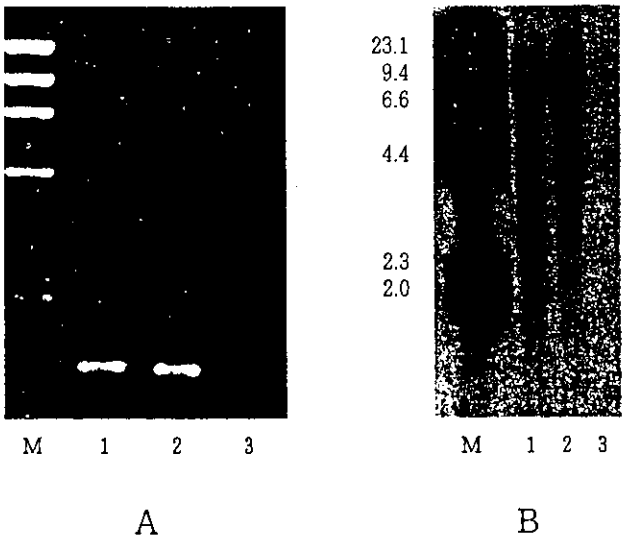

Figure 2. (A) Detection of EBV DNA in P-NK cells by PCR analysis. Lane 1; P-NK cells (the patient), Lane 2; Raji cells (a positive control), Lane 3; C-NK cells (healthy control). (B) Clonality of P-NK cells. *Bam*HI-digested DNA from P-NK cells showed two distinct bands, indicating P-NK cells were oligoclonal. Lane 1; P-NK cells (the patient), Lane 2; Raji cells (a positive control), Lane 3; C-NK cells (healthy control).

fragment that contains the LMP-1 open reading frame and detects the right terminal repeats. DNA from Raji cells was used as a positive control.

Cell culture and the effects of anti-viral drugs on the proliferative response to IL-2

Highly purified NK cells (5×10^5 cells/wel) were cultured with 0.2 ml of RPMI (Gibco BRL, Rockville, MD, USA) supplemented with 10% fetal calf serum (Sigma-Aldrich, St. Louis, MO, USA) in the presence of 100 U/ml of IL-2 (Shionogi Pharmaceutical Co., Osaka, Japan). Vidarabine (Mochida Pharmaceutical Co., Tokyo, Japan), foscarnet (Astra Japan Pharmaceutical Co., Osaka, Japan), acyclovir (Nippon Welcome Pharmaceutical Co., Tokyo, Japan), gancyclovir (Tanabe Pharmaceutical Co., Osaka, Japan), or a combination with vidarabine and foscarnet were added to the NK cells cultured with the IL-2. YT, a cell line derived from a patient with EBV-associated NK lymphoma (8) was kindly provided by Dr. Kanegane (Toyama Medical and Pharmaceutical University, Toyama, Japan) and cultured under the same conditions. The quantification of cell proliferation was measured by [^{3}H]-thymidine incorporation on day 3. All experiments were done in triplicate cultures.

Quantitative assay for EBV-DNA

Genomic DNA was extracted from NK cells cultured with the individual anti-viral drugs containing medium for 5 days, using a QIAamp Blood Kit (QIAGEN Inc., Chasworth, CA, USA). Quantitative PCR for EBV was performed by precisely following a method described previously (9).

Statistical analysis

All results are shown as mean values and error bars represent the s.e.m. Statistical differences were evaluated by Stu dent's unpaired *t* test. A statistical probability of $p<0.05$ was taken as a significant difference.

Results

Detection of EBV and clonality of P-NK cells

To examine whether P-NK cells were infected with EBV, PCR amplification for EBV-DNA was performed. As shown in Fig. 2A, a distinct band was observed in the first lane of P-NK cells (lane 1) at the same position as the positive control (lane 2), while no bands were observed in the lane of purified NK cells from the healthy control, C-NK cells (lane 3). In addition, *in situ* hybridization assay for EBV-encoded small nuclear RNA 1 (EBER 1) showed that more than 90% of P-NK cells were positive for EBER 1 (data not shown). These results indicated that P-NK cells were infected with EBV. The clonality of P-NK cells was examined by Southern blot hybridization using a terminal repeat probe. In the results, *Bam*HI-digested DNA from P-NK cells showed two distinct bands, indicating that P-NK cells were oligoclonal (Fig. 2B).

Effects of anti-viral drugs on in-vitro cell proliferation of P-NK cells and YT cells

To explore the possible treatment of EBV-associated NK-GLPD, we examined the effect of several anti-viral drugs on the proliferation of P-NK cells. Cells were cultured for 3 days in the absence or presence of antiviral drugs (vidarabine, foscarnet, acyclovir and gancyclovir). The doses of individual drugs were based on a previous report (10) and could be used clinically. As shown in Fig. 3A, both vidarabine and foscarnet suppressed the proliferation of P-NK cells in a dose-dependent manner, while the proliferation of YT, a cell line derived from EBV-infected NK-lymphoma (8), was not sensitive to these drugs. The combination of vidarabine and foscarnet had a significant additive effect, such that the highest doses of these two drugs suppressed cell proliferation almost completely (>90%). The doses of vidarabine and foscarnet used in this assay showed no cytotoxicity against normal NK cells under the same conditions (data not shown), suggesting that the effects were not brought about by non-specific cytotoxic activity. On the other hand, neither acyclovir nor gancyclovir affected cell proliferation even at the highest clinical doses.

Effects of anti-viral drugs on EBV-DNA replication of P-NK cells

To confirm that these anti-viral drugs suppress the proliferation of P-NK cells by inhibiting viral DNA-replication, we examined the effects of these drugs on EBV-DNA

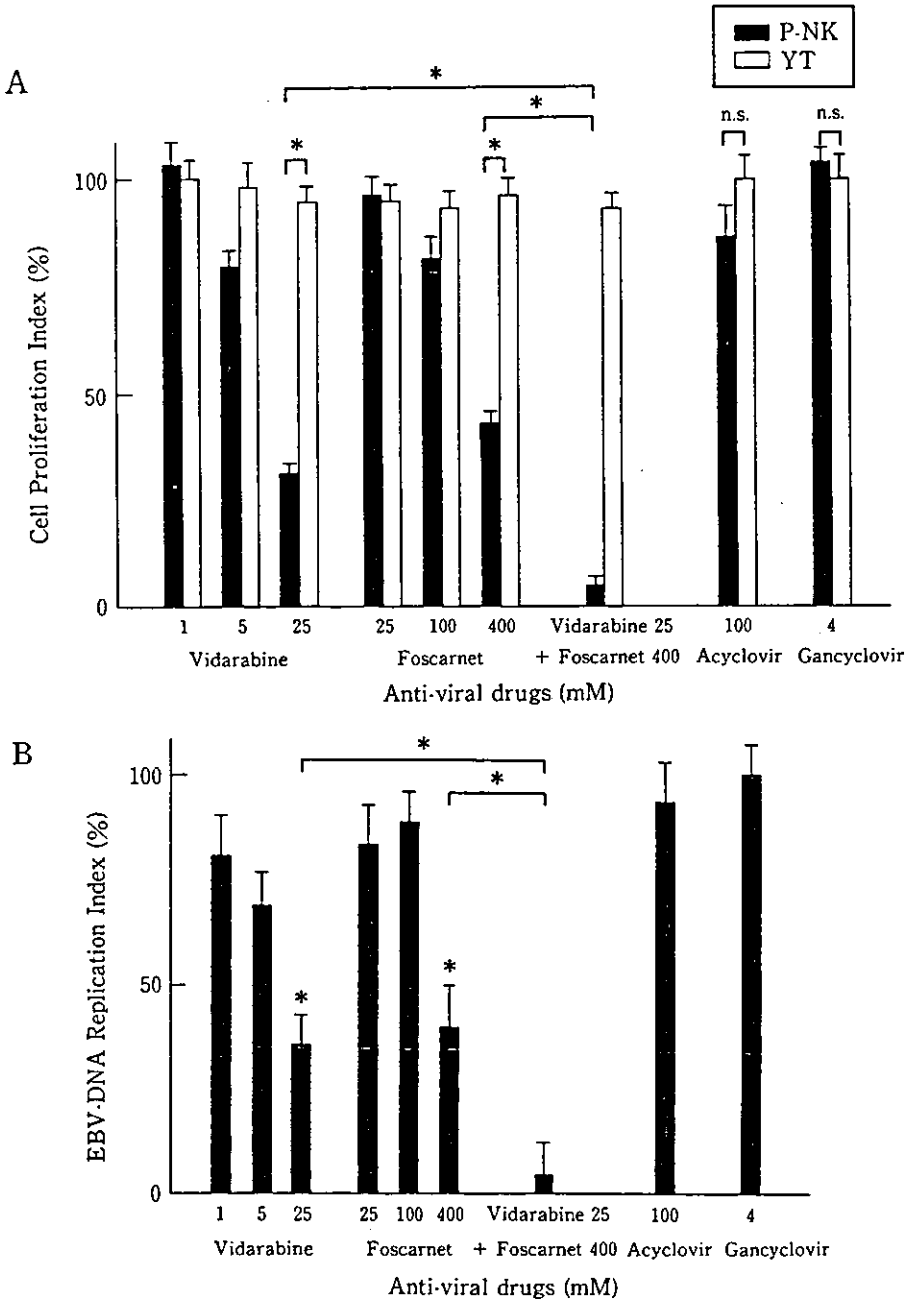

Figure 3. (A) Effect of anti-viral drugs on the proliferation of P-NK cells and YT cells. The cell proliferation index is a ratio of cell proliferation in the presence of several drugs (shown below) relative to that in the control condition. Filled bars indicate the cell proliferation index of P-NK cells, while open bars indicate that of YT cells. Results are shown as mean values obtained from three independent experiments and error bars represent the s.e.m. Asterisk: $p<0.05$. n.s.: not significant. (B) Effect of anti-viral drugs on DNA-replication of P-NK cells. EBV-DNA replication index exhibits the ratio of viral DNA replication in the presence of several drugs (shown below) relative to that in the control condition.

replication in P-NK cells. Genomic DNA was extracted from the cells after 5-day culture, and then EBV-DNA was quantified by PCR assay using EBV-specific primers. As shown in Fig. 3B, both vidarabine and foscarnet reduced EBV-DNA replication significantly in a dose-dependent manner. Also in this assay, the additive effect of a combination of vidarabine and foscarnet was detected. Neither acyclovir nor gancyclovir affected the EBV-DNA replication of cells. These results were completely consistent with the results shown in Fig. 3A.

Discussion

Regarding treatment for CAEBV, several trials have been reported (11, 12). However, most reports have been anecdotal, and no definite treatments have been established to date. In the present study, we revealed that two anti-viral drugs, vidarabine and foscarnet, had suppressive effects on the proliferation of P-NK cells, which were infected with EBV and were oligoclonally expanding still at the preneoplastic stage. On the other hand, these drugs were ineffective in YT cells, which were also infected by EBV and had completely transformed into malignant stages. These results indicate that the proliferation of P-NK cells is dependent on the EBV activity, whereas that of YT is independent of EBV. Therefore, these anti-viral drugs might be useful for preventing CAEBV in patients at the preneoplastic stages from the progression of the clinical course.

Whereas acyclovir and gancyclovir need to be phosphorylated intracellularly by viral thymidine kinase for drug activity (13), vidarabine and foscarnet are not required for viral kinase activity. Previous reports revealed that the activity of viral thymidine kinase isolated from acyclovir-resistant herpesviruses was remarkably decreased or absent, and the acyclovir-resistant viruses were still susceptible to both vidarabine and foscarnet (14). Vidarabine (9-β-D-ribofuranosyladenine) is an adenosine analogue and is supposed to inhibit viral DNA synthesis by competing with endogenous deoxyadenosine (15). Foscarnet (trisodium phosphonoformate) is an inorganic pyrophosphate analogue and inhibits viral nucleic acid synthesis by directly interacting with herpesvirus DNA polymerase and blocking its activity (16). Considering the difference between the mode of action of vidarabine and that of foscarnet, the additive effect achieved by the combination of these two drugs seems to be reasonable.

Vidarabine is often used for the treatment of infections of herpes symplex virus (HSV) and varicella-zoster virus (VZV), and foscarnet is used for cytomegalovirus (CMV)-infection, while both drugs have not commonly been utilized for the treatment of EBV-infection (17). Nevertheless, some recent reports demonstrate the effectiveness of these drugs against EBV-related lymphoproliferative disorders (18, 19). Systemic symptoms of four severe CAEBV patients were shown to be improved by the administration of vidarabine (18), and treatment with foscarnet was revealed to induce prolonged remission in EBV-associated post-transplantational lymphoproliferations, which were refractory to acyclovir and gancyclovir (19). The present study and these recent reports suggest that vidarabine, foscarnet and their combination are emerging as good candidates for possible treatment of EBV-related lymphoproliferative disorders, such as NK-GLPD.

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Enhanced production of osteopontin in multiple myeloma: clinical and pathogenic implications

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Summary. In this study, we examined osteopontin (OPN) production in myeloma cells and plasma OPN levels in multiple myeloma (MM) patients. We assessed OPN production in bone marrow cells (BMCs) by immunocytochemistry and enzyme-linked immunosorbent assay (ELISA). We also assessed OPN production in various B-cell malignant cell lines, including three myeloma cell lines by reverse transcription polymerase chain reaction (RT-PCR) and Western blotting. In addition, we measured plasma OPN concentrations by ELISA in 30 MM patients, 21 monoclonal gammopathy of undetermined significance (MGUS) patients and 30 healthy volunteers. As a result, in an immunocytochemical study, abundant OPN was detected in BMCs from overt MM patients, whereas no OPN was detected in BMCs from patients with other haematological diseases, including MGUS. Cultured BMCs from overt MM patients produced more OPN than those from patients with either smouldering MM or MGUS. Myeloma cell lines spontaneously produced OPN. Plasma OPN levels of MM patients were significantly higher than those of MGUS patients and healthy volunteers ($P < 0{\cdot}05$). Moreover, they correlated with both progression and bone destruction of the disease ($P < 0{\cdot}05$). These suggest that myeloma cells actively produce OPN, which possibly contributes to osteoclastic bone resorption in MM. Plasma OPN levels may be a useful biomarker for assessing bone destruction in MM and distinguishing MM from MGUS or smouldering MM.

Keywords: osteopontin, multiple myeloma, bone resorption, osteoclast, monoclonal gammopathy of undetermined significance (MGUS).

Multiple myeloma (MM) is a malignant disease that results from the uncontrolled proliferation of plasma cells derived from a single clone (Billadeau *et al*, 1996). The most common clinical problem in patients with MM is extensive bone destruction (or bone resorption), which occurs in almost all patients and is accompanied by severe and intractable pain and susceptibility to fracture (Roodman, 1997). As histological sections of bone are characterized by an increase in osteoclast activity occurring adjacent to the myeloma cells, the bone destruction in MM is suggested to be caused not only by the invasion (or proliferation) of myeloma cells but also by the activation of osteoclasts (Mundy *et al*, 1974; Bataille *et al*, 1992). It has been suggested that the osteoclasts can respond to a certain osteoclast activating factor (OAF) secreted by myeloma cells (Mundy *et al*, 1974; Durie *et al*, 1981; Bataille *et al*, 1992). Although recent cumulative evidence suggests that the OAF activity is mediated by several cytokines, including interkeukin (IL)-1β (Cozzolino *et al*, 1989), lymphotoxin (tumour necrosis factor beta: TNFβ) (Bertolini *et al*, 1986; Garret *et al*, 1987) and IL-6 (Bataille *et al*, 1989; Bataille &

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Klein, 1991), the factor (or molecule) directly involved in bone resorption of MM is still unknown.

Osteopontin (OPN) is a secreted phosphoglycoprotein, originally isolated from the bone extracellular matrix (Oldberg *et al*, 1986; Prince *et al*, 1987). OPN is expressed by various cell types (Denhardt & Gou, 1993), including osteoclasts, macrophages, activated T cells, smooth muscle cells and epithelial cells, and is present in several tissues including bone, kidney, placenta, smooth muscle and secretory epithelia. OPN contains an Arg-Gly-Asp (RGD) sequence that interacts with αvβ1, β3 and β5 integrins, and is capable of promoting cell attachment, chemotaxis and signal transduction in several different cell types (Miyauchi *et al*, 1991). OPN is now suggested to be involved in normal tissue remodelling processes such as bone resorption, angiogenesis, wound healing and tissue injury as well as certain diseases such as vascular restenosis, atherosclerosis, renal diseases and tumorigenesis. On the other hand, the mechanism for osteoclastic bone resorption has been clarified at the molecular level in studies using knockout mice (Soriano *et al*, 1991; Grigoriadis *et al*, 1994; Tondravi *et al*, 1997). The attachment of activated osteoclasts to the bone surface is a critical step in bone resorption by osteoclasts, and this step is mediated by the ligation of OPN with αvβ3 integrin (Reinholt *et al*, 1990). This suggests that OPN plays a crucial role in bone resorption by osteoclasts. Recently, we and other investigators demonstrated that OPN plays a significant role in certain bone-resorbing pathological states (or diseases) including rheumatoid arthritis (RA) (Petrow *et al*, 2000; Ohshima *et al*, 2002a,b; Yumoto *et al*, 2002) and post-menopausal osteoporosis (Yoshitake *et al*, 1999). As MM is an osteoclastic bone-resorbing disease, as mentioned earlier, OPN may play certain roles in bone resorption in MM. However, there have been no studies regarding OPN in MM.

In the present study, to investigate the involvement of OPN in bone resorption of MM, we examined OPN production in myeloma cells and plasma OPN levels in patients with MM.

PATIENTS AND METHODS

Patients/samples. This study was approved by the human ethics review committee of Osaka University Medical School. Written informed consent was obtained from each patient and volunteer. We obtained blood samples from 30 patients with MM. All patients fulfilled the diagnostic criteria of MM (Kyle, 1992). The staging of disease was performed according to the Durie–Salmon staging system based on the assessment of tumour mass (Durie & Salmon, 1975). Six patients were stage I (low tumour mass) MM patients who showed no bone lesions or osteoporosis. Stage I MM is referred to as smouldering MM. The remainder of the patients (24 patients) were stage III (high tumour mass) MM patients who showed one of the following abnormalities: (1) haemoglobin < 8·5 g/dl, haematocrit < 25%; (2) serum calcium > 3·012 mmol/l; (3) very high serum or urine myeloma protein production rates; (4) > three lytic bone lesions on bone survey. In addition, we classified the stage III patients into two groups according to the assessment of their clinical disease activity, such as their response to treatment. Twelve were inactive stage III patients, and the remainder were active. As a control, we used blood samples from 21 patients with monoclonal gammopathy of undetermined significance (MGUS) and 30 healthy volunteers. The patients with MGUS were diagnosed according to the criteria of Kyle & Lust (1989). The MGUS patients showed the following abnormalities: (1) serum monoclonal protein < 3 g/dl, either no or small amounts of Bence Jones proteinuria; (2) < 10% marrow plasma cells and no aggregates on biopsy; (3) no anaemia, renal failure or hypercalcaemia (bone lesions absent). We also obtained bone marrow cells (BMCs) from three patients with overt MM and five patients with different haematological diseases (MGUS, myelodysplastic syndrome, idiopathic thrombocytopenic purpura, acute myelocytic leukaemia, hereditary spherocytosis).

Immunocytochemistry for OPN. We examined OPN expression in BMCs freshly prepared from three patients with overt MM by immunocytochemistry using the avidin–biotinylate peroxidase complex method. As a control, BMCs from five patients with different haematological diseases, including MGUS, were used. BMCs were isolated by density gradient centrifugation. Freshly prepared 1×10^5 BMCs from each patient were spun down on a glass slide using Cytospin 2 (Shandon Southern Products, Cheshire, UK). The slides were kept at −80°C until used. A mouse monoclonal immunoglobulin (Ig) G anti-human OPN antibody, 4C1 generated by our laboratory (Kon *et al*, 2002), was used as the primary antibody. An equal concentration of irrelevant mouse IgG (Pharmingen, San Diego, CA, USA) was used as a negative control for the primary antibody. Biotinylated horse anti-mouse IgG antibody (Vector Laboratories, Burlingame, USA) was used as the secondary antibody. The cytospin slides were fixed with cold isopropanol for 2 min. After blocking with 10% normal horse serum, they were incubated with either 4C1 or the control antibody overnight at 4°C. Then, endogenous peroxidase activity was blocked by applying 0·3% hydrogen peroxidase dissolved in methanol for 30 min. After washing with phosphate-buffered saline (PBS), the slides were incubated with the biotinylated secondary antibody for 1 h at room temperature. After washing, they were incubated with the avidin–horseradish peroxidase complex (Vectastain Elite ABC kit; Vector Laboratories) for 1 h. Then, the colour reaction was developed using diaminobenzidine tetrahydrochloride (DAB) substrate solution, and the slides were counterstained with Giemsa's stain solution.

Reverse transcription polymerase chain reaction (RT-PCR) analysis for OPN. We examined OPN mRNA expression in various B-cell malignant cell lines [RPMI 8226, U266, ARH77: myeloma cell lines; Daudi, Ramos, Raji: lymphoblastic-like B cell lines from Burkitt's lymphoma; Kopn-8: a pre-B-cell line from human leukaemia (Matsuo & Drexler, 1998); NALM-16, REH: prepre-B-cell lines from human leukaemia (Matsuo & Drexler, 1998)] by RT-PCR using primers specific for human OPN (the sense primer 5′-GGACTCCATTGACTCGAACG-3′; the antisense primer