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ORIGINAL ARTICLE

Characteristics of fracture and related factors in patients with rheumatoid arthritis

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Abstract To examine the clinical features of vertebral and non-vertebral fractures in patients with rheumatoid arthritis (RA), including insufficiency fractures, and to assess the risk factors for fracture, we prospectively studied 209 outpatients with rheumatoid arthritis for 1 year. The age, gender, Steinbrocker's functional class, glucocorticoid use, history of lower limb surgery, serum C-reactive protein (CRP), and use of bisphosphonates were evaluated. Examination for fractures was performed by radiography, computed tomography (CT), magnetic resonance imaging (MRI), and bone scanning. Thirty-three fractures occurred in 24 patients over the 1-year study period, and the incidence was 15.8 fractures per 100 patient-years. Fractures occurred at various sites. The majority (70%) was insufficiency fracture, and more than 50% caused ambulatory dysfunction. Radiographic findings were absent in 39% of the fractures at the onset of pain. The functional class and glucocorticoid dose were significantly associated with fracture development. This prospective study showed that the incidence of fractures, especially insufficiency fractures, was very high in patients with rheumatoid arthritis and that most of their fractures caused gait disturbance. Early intervention to prevent secondary osteoporosis is recommended to maintain the quality of life in patients with rheumatoid arthritis, especially those with functional impairment or undergoing glucocorticoid therapy.

Keywords Fracture · Incidence · Insufficiency fracture · Rheumatoid arthritis · Risk factor

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Introduction

Patients with rheumatoid arthritis (RA) have a greater risk of osteoporosis and fracture than the general population, because of impaired walking ability, inflammation due to their disease, and glucocorticoid use. Fractures influence the morbidity and mortality of patients with primary osteoporosis, and some reports have demonstrated that the risk of vertebral or hip fractures is higher in patients with RA than in those with primary osteoporosis [1–5]. Several previous studies have revealed that many osteoporotic fractures occur at sites other than the spine and proximal femur in patients with RA [6–10]. Although those studies assessed the incidence of fractures, including fractures other than those of the spine and hips, most of them were performed retrospectively. On the other hand, several reports have emphasized the occurrence of pelvic or juxta-articular insufficiency fractures in patients with RA [11–15], but no study has yet clarified the incidence of insufficiency fracture in this disease. Insufficiency fractures are difficult to detect clinically without careful examination and appropriate radiological investigation based on a suspected diagnosis [16], making a retrospective study design not adequate to evaluate the occurrence of such fractures. Recently, a large-scale, population-based, cohort study

performed in the UK [9] and a large-scale, prospective, observational study performed in Japan [10] were reported, but those studies were based on medical records and self-reporting by questionnaire, in which insufficiency fractures might also have been overlooked. A prospective study design and detection of fractures through careful clinical assessment by orthopaedic surgeons are recommended to clarify the actual characteristics of fractures affecting RA patients.

Therefore, we conducted a prospective study to investigate the actual incidence of fracture for all skeletal sites, including insufficiency fractures, and to assess the clinical presentation and risk factors for fracture in RA patients.

Materials and methods

Patients and study protocol

This prospective study enrolled subjects undergoing regular outpatient review at intervals of less than 3 months to avoid missing fractures. A total of 209 outpatients (31 men and 178 women) with RA according to the American College of Rheumatology 1987 revised classification criteria [17] were entered into the study at Osaka University Hospital. The prospective follow-up period was 1 year from October 2003 to September 2004. The baseline characteristics of the patients are shown in Table 1 and include age, gender, duration of RA, serum C-reactive protein (CRP) level for assessment of disease activity, functional class (Steinbrocker's criteria [18]), surgical treatment of gait disturbance, glucocorticoid use (daily dose and duration), and bisphosphonate therapy for more than 2 years. Surgery for gait disturbance included total hip arthroplasty in 28 patients (nine bilateral), total knee arthroplasty in 64 patients (41 bilateral), total ankle arthroplasty in eight patients (two bilateral), hindfoot surgery in 14 patients (five bilateral), and forefoot surgery in 34 patients (14 bilateral). Ninety-two patients underwent lower limb surgery, and 61 patients had more than two operations. The sites of fracture, incidence of fracture, circumstances under which fractures occurred, and radiographic findings were evaluated. When more than two vertebral fractures occurred on one occasion, they were counted as one fracture. Circumstances were classified as no trauma (insufficiency fracture), minor trauma (such as a fall), or major trauma (such as a traffic accident). Insufficiency fracture was defined as fracture occurring in abnormal bone already weakened by decreased mineralization and with insufficient elastic resistance [16, 19].

Table 1 Baseline characteristics of 209 patients with RA

Variables	Value	Number of patients
Age (years) (mean ± SD)	60.4 ± 11.5	
Gender		
Male (%)	14.8	31
Female (%)	85.2	178
Disease duration (years) (mean ± SD)	14.5 ± 10.2	
Disease activity (CRP) (mean ± SD)	2.0 ± 2.4	
Functional class		
I (%)	43.5	91
II (%)	41.6	87
III (%)	14.8	31
IV (%)	0	0
Surgical treatment of lower legs (%)	45.9	96
Glucocorticoid use		
Current users (%)	74.2	155
Daily dose (mg/day) (mean ± SD)	5.94 ± 3.45	
Duration (years)		
None (%)	23.4	49
0–2 years (%)	14.8	31
2–5 years (%)	19.6	41
5–10 years (%)	29.2	61
10 years ≤ (%)	12.9	27
Bisphosphonate use		
None or ≤ 2 years (%)	77.5	162
> 2 years (%)	22.5	47

RA rheumatoid arthritis, *CRP* C-reactive protein

Functional classes according to Steinbrocker criteria

Fractures that occurred during normal daily activities without any trauma were considered to be insufficiency fractures.

Diagnosis of fracture

When patients presented to the hospital with pain, a single orthopaedic surgeon (J.H.) examined them for fractures. Detection of fractures was initially performed by careful clinical examination and plain radiography. In the absence of radiographic findings, magnetic resonance imaging (MRI), computed tomography (CT), bone scanning, and longitudinal radiographic changes were used for diagnosis.

Statistical analysis

Risk factors associated with the occurrence of fracture were evaluated. Those shown in Table 1 were assessed by univariate analysis using SPSS version 11.5 J software (SPSS Inc., Chicago, IL, USA), and the odds ratios and 95% confidence intervals (CI) were calculated. Subsequently, multivariate logistic regression analysis was performed for the factors that were significant according to univariate analysis. The level of significance was set at $P < 0.05$ for all tests.

Results

Incidence and characteristics of fractures

We detected 33 fractures in 24 patients during the 1 year follow-up period. The incidence of fractures was 15.8 per 100 patient-years, and the incidence of patients with new fractures was 11.5 per 100 patient-years. Fractures occurred at various sites, including the rib, vertebra, pelvis, humerus, elbow, femur, knee, and foot (Table 2). All fractures affecting the pelvis and lower extremities, as well as one of four fractures involving the lumbar spine, resulted in ambulatory dysfunction (54.5%). The fracture occurred with no trauma (insufficiency fracture) in 23 cases (69.7%), and with minor trauma (fall) in ten cases (30.3%). There were no fractures due to major trauma (Table 2). Twenty fractures (60.6%) could be diagnosed by plain radiography at the onset of pain, while 13 fractures (39.4%) could not be detected on plain radiographs at that time (Table 2). MRI, CT, bone scanning, and longitudinal radiography were able to detect these 13 fractures. Among the 24 patients suffering fractures, nine had not received any prior surgical treatment, 15 had undergone lower limb surgery (two had total hip arthroplasty, nine had total knee arthroplasty, one had total ankle arthroplasty, four had hindfoot surgery, and seven had forefoot surgery), and eight patients had undergone more than two lower limb operations.

Table 2 Fracture sites, circumstances, and radiographic change at onset of pain

Fracture site	Total number	Circumstance		Radiographic change	
		Minor trauma	Insufficiency fracture	Present	Absent
Ribs	2	0	2	2	0
Shoulder	4	4	0	4	0
Elbow	1	1	0	1	0
Spine	9	1	8	7	2
Sacrum	3	0	3	0	3
Pubis	5	0	5	2	3
Ilium	1	1	0	0	1
Hip	1	1	0	0	1
Femur	2	2	0	2	0
Knee	3	0	3	0	3
Foot, ankle	2	0	2	2	0
Total	33	10	23	20	13

Risk factors for fracture

Univariate analysis comparing patients with and without fractures was performed as an aid to selecting variables for inclusion in subsequent multivariate analysis. Among the variables investigated, functional class and glucocorticoid use (daily dose and duration) showed a significant difference ($P < 0.05$), while age and lower limb surgery showed borderline significance ($P < 0.1$) (Table 3). Variables that showed $P < 0.1$ on univariate analysis were included in the subsequent multivariate analysis. The results of multivariate logistic regression analysis showed that daily glucocorticoid use and functional class were significantly associated with the risk of fracture in RA patients (Table 3). Using the same process, we found that the only significant risk factor for vertebral fractures was the daily glucocorticoid dose according to multivariate analysis (Table 3). On the other hand, analysis of the risk factors for pelvis/lower limb fractures revealed that only functional class was a significant predictor by multivariate analysis (Table 3). No risk factor was significantly correlated with fracture of the upper limbs (data not shown). Figure 1 shows the incidence of fractures stratified according to functional impairment. The incidence of all fractures tended to increase as the functional status deteriorated. Fractures of the upper limbs occurred in only stage I and

Table 3 Possible risk factors for fracture in RA patients

Variables	*P*	Odds ratio	95% CI
All fractures			
Univariate logistic regression			
Age	0.057	1.040/year	0.999–1.084
Functional class	0.001	2.963/ class	1.598–5.496
Glucocorticoid use			
Daily dose	0.003	1.174/mg	1.054–1.306
Duration	0.019	1.095/year	1.015–1.181
Surgical treatment of lower legs	0.089	2.140/site	0.891–5.138
Multivariate logistic regression			
Functional class	0.020	2.277/ class	1.136–4.562
Glucocorticoid use			
Daily dose	0.034	1.141/mg	1.010–1.290
Vertebral fractures			
Univariate logistic regression			
Age	0.056	1.084/year	0.998–1.177
Glucocorticoid use			
Daily use	0.034	1.212/mg	1.014–1.449
Multivariate logistic regression			
Glucocorticoid use			
Daily use	0.032	1.232/mg	1.018–1.492
Fractures of pelvis and lower legs			
Univariate logistic regression			
Functional class	0.001>	4.987/ class	2.032–12.237
Glucocorticoid use			
Daily use	0.010	1.195/mg	1.043–1.370
Duration	0.008	1.131/year	1.033–1.239
Surgical treatment of lower legs	0.093	2.819/site	0.840–9.463
Multivariate logistic regression			
Functional class	0.007	3.924/ class	1.462–10.535

CI confidence interval

stage II patients, while fractures of the pelvis/lower limbs and vertebra occurred in stage II and stage III patients. The incidence of pelvic/lower limb fractures increased along with functional deterioration.

Discussion

We performed our prospective study of 209 outpatients with RA to investigate the characteristics of fractures in this disease. We found that the incidence of fracture, including non-vertebral and insufficiency fractures, was very high in RA, being 15.8 fractures per 100 patient-years and 11.5 patients per 100 patient-years. These incidence

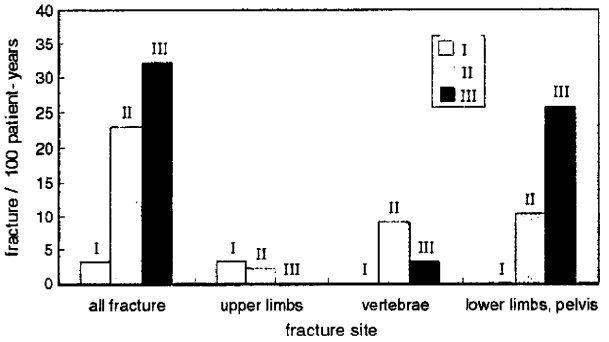

Fig. 1 Fracture incidence stratified by functional impairment. The incidence of all fractures increased along with the severity of functional impairment. Upper limb fractures tended to occur in relatively mild RA, but the association was not significant, and vertebral fractures were not associated with functional status. On the other hand, fractures of the lower limbs and pelvis were significantly more common as the functional status deteriorated. *I*, *II*, and *III* refer to Steinbrocker's functional classes

rates were higher than those reported previously, which were 1.57 fractures per 100 patient-years [7], 1.62 fractures per 100 patient-years (non-vertebral fractures) [8], 1.07 fractures per 100 patient-years [9], 1.68 fractures per 100 patient-years [10], and 3.59 fractures per 100 patient-years [20]. However, all of those previous studies were retrospective and depended on medical records, radiology reports, telephone interviews, or questionnaires. In our prospective study, fracture diagnosis was made by careful assessment of symptoms and signs by a single experienced rheumasurgeon along with adequate diagnostic imaging, because insufficiency fractures or juxta-articular fractures are difficult to detect in RA patients without careful examination and appropriate radiological investigation based on a high index of suspicion [16]. Thus, misdiagnosis and failure to detect insufficiency fractures or juxta-articular fractures were thought to be minimized in our study. This difference in methodology probably explains a large part of the difference in the incidence of fractures between our study and previous studies.

Another possible reason for the discrepancy may be differences in the background factors of the subjects. Our patients all had relatively severe RA and regularly attended a university hospital; 74.2% used oral corticosteroids, and approximately half of them had undergone major surgery on the lower limbs (total hip arthroplasty and/or total knee arthroplasty). Steroid use was varied in previous studies (58% [7], 47.8% [8], 25% [9], 61% [10]), but was lower than in our series, and it has been reported that 71% of patients who develop fractures have taken steroids [20]. In the most recent study [10], 22.5% of patients had a history of orthopedic surgery and 8.9% had a history of total knee arthroplasty, while 44% of our patients had received lower

limb surgery (excluding those with upper limb or spinal surgery) and 30.6% had undergone total knee replacement. Differences in the severity of RA, and different levels of corticosteroid use, might have influenced the incidence of fracture, making it inappropriate for our findings to be compared directly with those of other general cohort studies.

Our study showed four characteristic features of fractures occurring in patients with RA. The first was, as previously reported, that fractures may occur at various sites throughout the entire skeleton [6–10], including the ribs, upper and lower limbs, vertebrae, and pelvis, while hip and vertebral fractures predominate in patients with primary osteoporosis. This finding indicates that RA patients may suffer from both generalized osteoporosis and juxta-articular osteoporosis. The second point was that insufficiency fractures, which are caused by normal physiological stress, are the most common type of fracture (69.7%) in patients with RA. It has been reported that RA is one of the most common underlying diseases in patients with insufficiency fracture [11–15], and there have been many case reports of insufficiency fractures in RA patients [21–23], but the frequency of these fractures has been unclear. In our study, misdiagnosis and the overlooking of insufficiency fractures or juxta-articular fractures were presumably minimized. The third point was that fractures undetectable by plain radiography at the onset of pain are very common (39.4%). Among the insufficiency fractures, 47.8% could not be seen on the first plain radiograph obtained at the onset of pain (data not shown). Fractures of the upper extremity were caused by trauma and were radiologically apparent. Most of the vertebral and pelvic fractures were insufficiency fractures, but the former were relatively apparent on plain radiographs, and the latter were mostly undetectable by radiography. Long-bone fractures of the lower limbs were apparent on plain radiographs, but fractures of the knee and hip joints were frequently undetectable. According to previous reports, pelvic fractures are frequently insufficiency fractures [13, 24–26], with CT and MRI being more reliable methods for diagnosis than plain radiography [19, 26, 27]. Bone scanning is also useful for detection of sacral insufficiency fractures [28]. In our series, CT, MRI, and bone scanning were all useful, especially for pelvic fractures and peri-articular fractures. Thus, it is important to suspect the presence of an insufficiency fracture based on the results of meticulous physical examination, and such fractures will often be undetectable on plain radiographs in patients with RA. The fourth point was that the 54.5% of the fractures resulted in ambulatory dysfunction. This shows that prevention of fractures is important in patients with RA to maintain their mobility, just as prevention of joint destruction by anti-rheumatic therapy is important.

According to prior reports, the risk factors for fracture in RA patients are age, gender, low body mass index (BMI), ambulatory dysfunction, corticosteroid use [6, 7], bone mineral density [8], and joint deformity [15]. Our multivariate analysis showed that daily glucocorticoid use and functional class were significant overall risk factors for fracture in RA patients (Table 3). Analyzing the risk factors by site, we found that daily glucocorticoid use was a significant risk factor for vertebral fractures, but not for pelvic and lower limb fractures. This result is consistent with previous reports that vertebral fractures are significantly associated with daily glucocorticoid use [3, 29]. Our study also showed that functional class was a significant risk factor for pelvic and lower limb fractures, but not for vertebral fractures. Corticosteroid use, severe osteoporosis, and joint deformity have also been reported as risk factors of pelvic and lower limb fractures [15, 30]. Relationships between functional disturbance and hip fracture [31], wrist and hip fractures [32], and any type of fracture [33] have all been reported previously. Our findings did not contradict those previous reports and, additionally, showed that functional impairment is a stronger risk factor than glucocorticoid use for pelvic and lower limb fractures. Figure 1 shows the fracture incidence in relation to Steinbrocker's functional classification. The incidence of all fractures, as well as fractures of the pelvis and lower limbs, increased along with functional impairment. However, the incidence of upper limb and vertebral fractures was not correlated with the functional class. This suggests that greater mechanical stress due to weight bearing or ambulation is quite important to protect the pelvis and lower limbs from bone fragility.

Our study had several limitations. First, our subjects had relatively severe disease, with their physical function being worse than in others and half of them had undergone major lower limb surgery, and the rate of oral glucocorticoid use was high. Also, some possible risk factors for fracture were not evaluated in this study, such as bone mineral density, body mass index, fracture history, other medications for osteoporosis and RA, alcohol, and smoking. Third, we only evaluated painful fractures, without counting asymptomatic fractures (especially vertebral fractures). Since asymptomatic vertebral fractures and deformities are common in patients with primary osteoporosis [29, 34–37] as well as RA [38], our study would have underestimated the total fracture incidence by excluding painless vertebral fractures.

In conclusion, we prospectively investigated the characteristics of fractures, the overall fracture incidence, and risk factors for fracture (including insufficiency fracture) in RA patients over a 1-year period. The incidence of fracture was relatively high, and any site could be affected. In addition, 55% of the fractures caused gait impairment, 70%

were insufficiency fractures, and 39% were not detectable by plain radiography at the onset of pain. Significant risk factors for fracture were functional disability and glucocorticoid use. Early intervention to prevent secondary osteoporosis is important to avoid fractures and maintain the quality of life in patients with RA, especially those with functional impairment or glucocorticoid use.

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Inflammatory osteoclastogenesis can be induced by GM-CSF and activated under TNF immunity

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Abstract

In inflammatory arthritis such as RA, osteoclastic activity is severely enhanced. GM-CSF was reportedly elevated in synovial fluid, but is a strong inhibitor of osteoclastogenesis; here lies a contradiction. Our objective was to examine what type of osteoclasts generate and resorb bone with resistance to GM-CSF in an inflammatory joint. Monocyte-derived cells generated in GM-CSF were morphologically and immunophenotypically different from both the conventional DC and macrophage. They could differentiate into osteoclasts in the presence of RANKL + M-CSF, acquiring a stronger osteoclastic activity under TNF treatment. Furthermore, their differentiation was not inhibited by GM-CSF, while monocyte-derived osteoclast differentiation was completely inhibited. The resorption was suppressed by GM-CSF, and the existence of another osteoclastic pathway has been suggested. Our findings indicate another type of osteoclast exists in inflammatory arthritis.
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Keywords: Osteoclastogenesis; Inflammation; GM-CSF; TNFα; Rheumatoid arthritis

Osteoclasts are the multinuclear cells specialized for bone resorption, playing a crucial role in bone remodeling [1,2]. Osteoclasts have been known to differentiate from hematopoietic precursors of the monocyte/macrophage lineage in the presence of macrophage-colony stimulating factor (M-CSF or CSF-1) and receptor activator of NF-κB ligand (RANKL) [3–6]. However, the accurate lineage of osteoclast precursors remains uncertain, and it is unclear whether there are any osteoclast varieties.

Both rheumatoid arthritis (RA) and osteoporosis are major adult skeletal diseases that are due to excessive osteoclastic activity, leading to an imbalance of bone remodeling in favor of bone resorption [7,8]. There are big differences among these diseases and their pathogeneses. The environment where osteoclasts generate and work may partially explain theses discrepancies; RA involves inflammation while osteoporosis does not. It is unknown, however, whether inflammation will affect osteoclastogenesis in RA.

Tumor necrosis factor-alpha (TNFα), Interleukin-1 (IL-1), M-CSF, GM-CSF, interferon-gamma (IFNγ) and IL-18 are the representative pro-inflammatory cytokines that are characteristic of RA [7,9]. Though TNFα and IL-1 have been known to stimulate osteoclastogenesis, GM-CSF, IFNγ and IL-18 reportedly have an inhibitory effect on osteoclastogenesis [10–15]. This is a contradiction in 'inflammatory' osteoclastogenesis. Recently it has reported that IL-17-producing helper T cell subset (Th17) is responsible for bone destruction in RA [16,17]. Th17 immunity may well explain this contradiction. However Th17 immunity in RA is controversial and the actual elevation of GM-CSF and IL-18 in RA synovial fluid cannot be neglected [18,19].

GM-CSF was initially defined by its ability to generate granulocyte and macrophage colonies from precursor cells in vitro [20]. But GM-CSF is difficult to detect in steady state circulation and GM-CSF-deficient mice showed no obvious deficiency in the numbers of myeloid cells.

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Therefore, GM-CSF seems to have another function at the non-steady state, particularly in inflammation [21,22]. In fact, upon appropriate stimulation and other pro-inflammatory cytokines, much of the production and action of GM-CSF occurs locally at the site of inflammation [23].

What role does GM-CSF play in 'inflammatory' osteoclastogenesis? Is there truly an inhibitory effect towards osteoclastogenesis?

Considering anti-GM-CSF therapy decreased the disease severity and reduced the levels of TNFα and IL-1 in inflammatory joint or block post-onset collagen induced arthritis, GM-CSF is not expected to have such an inhibitory property in 'inflammatory' osteoclastogenesis [24,25]. Considering IL-18 induced GM-CSF, not IFNγ, production in primary osteoblasts, GM-CSF must have some promotive function in 'inflammatory' osteoclastogenesis, resulting in severe bone destruction [26].

We hypothesized that osteoclasts in the inflammatory state are different from those charged in bone remodeling in the non-inflammatory state. Therefore, GM-CSF can be a potential cytokine for 'inflammatory' osteoclastogenesis in place of M-CSF, which can generate the monocyte/macrophage populations into osteoclast precursors.

In this study, we investigated the property of monocyte-derived osteoclast precursors generated in GM-CSF and analyzed their osteoclastic activity under TNF immunity. Then we found that their differentiation could not be inhibited by GM-CSF. These cells acquire a stronger osteoclastic activity by TNFα treatment.

Materials and methods

Cell purification, culture, and reagents. Mononuclear cells (PBMCs) were isolated from peripheral blood of healthy adult volunteers by Ficoll-Paque (Amersham Biosciences, Tokyo, Japan) density gradient centrifugation. Monocytes were purified using CD14 Microbeads (Miltenyi Biotec, Auburn, CA) and subsequently cultured during 7 days in 24-well plates ($5–6 \times 10^5$ cells/ml) in RPMI1640 (Gibco/BRL, Gaithersburg, MD) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 IU/ml penicillin and 100 μg/ml streptomycin (Gibco/BRL) in the presence of 50 ng/ml recombinant human (rh)GM-CSF, 10 ng/ml rhTNFα + 50 ng/ml rhGM-CSF, 50 ng/ml rhIL-4 + 50 ng/ml rhGM-CSF, or 50 ng/ml rhM-CSF, respectively. Recombinant human IL-4, TNFα, GM-CSF and M-CSF were purchased from PeproTech (London, UK).

Flow cytometry. The phenotype of cells was analyzed by flow cytometry using a FACS Calibur (Becton Dickinson, San Jose, CA). For monocyte-derived cells, the following mAbs were used; fluorescein isothiocyanate (FITC)-labeled anti-CD80, anti-CD11b, anti-CD14, and PE-Cy5 (PC5)-labeled anti-CD86 (Biolegend, San Diego, CA). Results were expressed in mean fluorescence intensity (MFI) values of total positive cells. At least 10,000 cells per sample were analyzed.

Osteoclast differentiation and TRAP assay. Monocyte-derived cells were seeded at 1×10^5 cells/ml per well in Lab-Tek 16 well chamber slides (Nalge Nunc, Rochester, NY) in α-minimum essential medium (α-MEM) (Gibco/BRL) supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin in the presence of 25 ng/ml M-CSF and 100 ng/ml sRANKL (PeproTech) for 14 days. For some experiments, 0.1 ng/ml or 1 ng/ml GM-CSF was co-treated in the culture media during the assay. Media were changed every three days. Then the cells were washed and stained with TRAP stain kit (Cell Garage, Tokyo, Japan). The number of TRAP positive multinuclear (three or more nuclei) cells was counted.

Bone resorption assay. To assess resorption ability, monocyte-derived cells were cultured on carbonated calcium phosphate coated plate (OAAS Osteoclast Activity Assay Substrate; OCT, Choongnam, Korea) or on

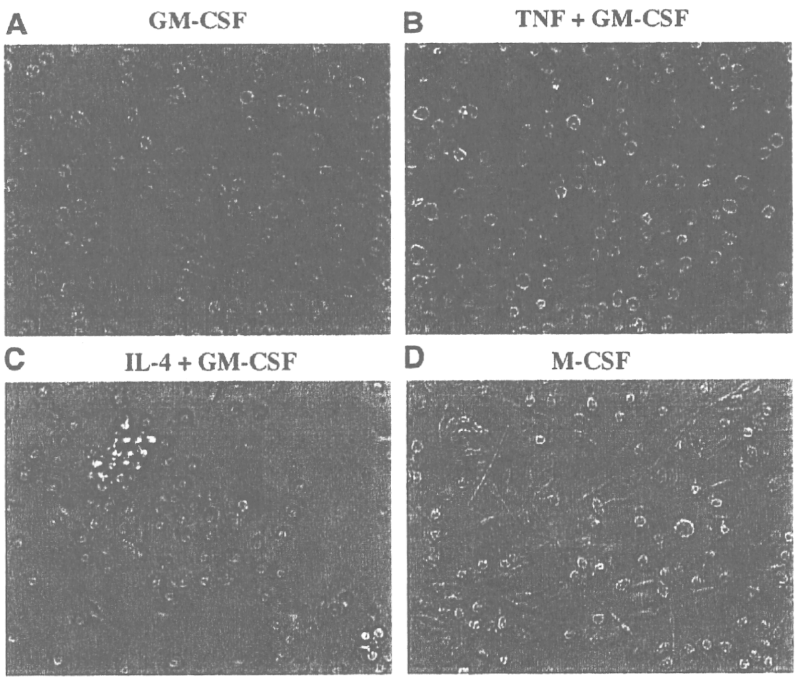

Fig. 1. The characterization of myeloid cells generated in GM-CSF or TNFα + GM-CSF from CD14 + monocytes. Inverted phase contrast microscopic pictures of monocytes cultured in the presence of (A) GM-CSF, (B) TNFα + GM-CSF, (C) IL-4 + GM-CSF (cDC), or (D) M-CSF (macrophage) for 7 days. (original magnifications: ×400).

dentine slices (Wako, Osaka, Japan). The technique of osteoclast differentiation was described above. Finally cells were washed vigorously and the calcified matrix resorption on each well was measured with a Win Roof image analyzer (Mitani, Fukui, Japan). Dentine slices were stained with Mayer's hematoxylin solution (Wako) to detect resorption pits under light microscopy.

Immunofluorescence staining. Cells cultured on glass chamber slides were fixed for 15 min with 4% paraformaldehyde in PBS and then permeabilized with TBST for 15 min. After blocking for 60 min with 5% skim milk in TBST, cells were incubated with anti-cathepsin K (goat polyclonal; Santa Cruz Biotechnology, CA) or rhodamine-labeled phalloidin (Sigma–Aldrich, St. Louis, MO). Slides were then treated with the appropriate conjugated secondary antibodies (Invitrogen Molecular Probes, Carlsbad, CA). Primary antibodies were applied overnight at 4 °C and secondary antibodies were applied for 60 min at room temperature in a humidified chamber. DNA nuclei were stained with DAPI. Between each step, slides were washed three times for 5 min in TBST. Samples were examined under a confocal laser-scanning microscope (Eclipse E800M; Nikon).

Statistical analysis. Data were expressed as means ± SD. Sample values below the detection limit for the assay were regarded as negative and assigned a value of 0. Statistical analysis was performed using the Student's *t*-test or the Mann–Whitney U test, where appropriate according to the distribution data. $p < 0.05$ was considered statistically significant.

Table 1
Immunophenotypical analysis of cells

	IL4 + GM-CSF	GM-CSF	TNF + GM-CSF	M-CSF
CD80	95.7 ± 29.6	82.3 ± 7.4	59.3 ± 9.5	268.0 ± 52.5
CD86	107.7 ± 7.8	131.0 ± 39.6	167.0 ± 33.4	232.0 ± 41.4
CD14	79.0 ± 20.9	914.0 ± 264.0	370.0 ± 170.0	1350.0 ± 80.2
CD11b	89.0 ± 21.0	104.0 ± 18.6	72.0 ± 18.7	279.0 ± 43.5

Table summarizes the phenotype of myeloid cells generated in IL-4 + GM-CSF, in GM-CSF, in TNFα + GM-CSF, and in M-CSF after 7 days culture. MFI of all the positive cells is shown as means ± SD from three independent experiments.

Results

Osteoclast precursors generated in GM-CSF or TNFα + GM-CSF were different from cDC or macrophage

We first examined how myeloid cells were generated in GM-CSF or TNFα + GM-CSF. Exposure of CD14 positive monocytes to GM-CSF or TNFα + GM-CSF in seven days lead to adherent cells with small projections (Figs. 1A and B). These cells displayed a distinguishable morphology while the conventional dendritic cell (cDC) treated with IL-4 + GM-CSF displayed typical non-adherent dendritic morphology (Fig. 1C) and macrophage treated with M-CSF displayed an adherent various morphology (Fig. 1D).

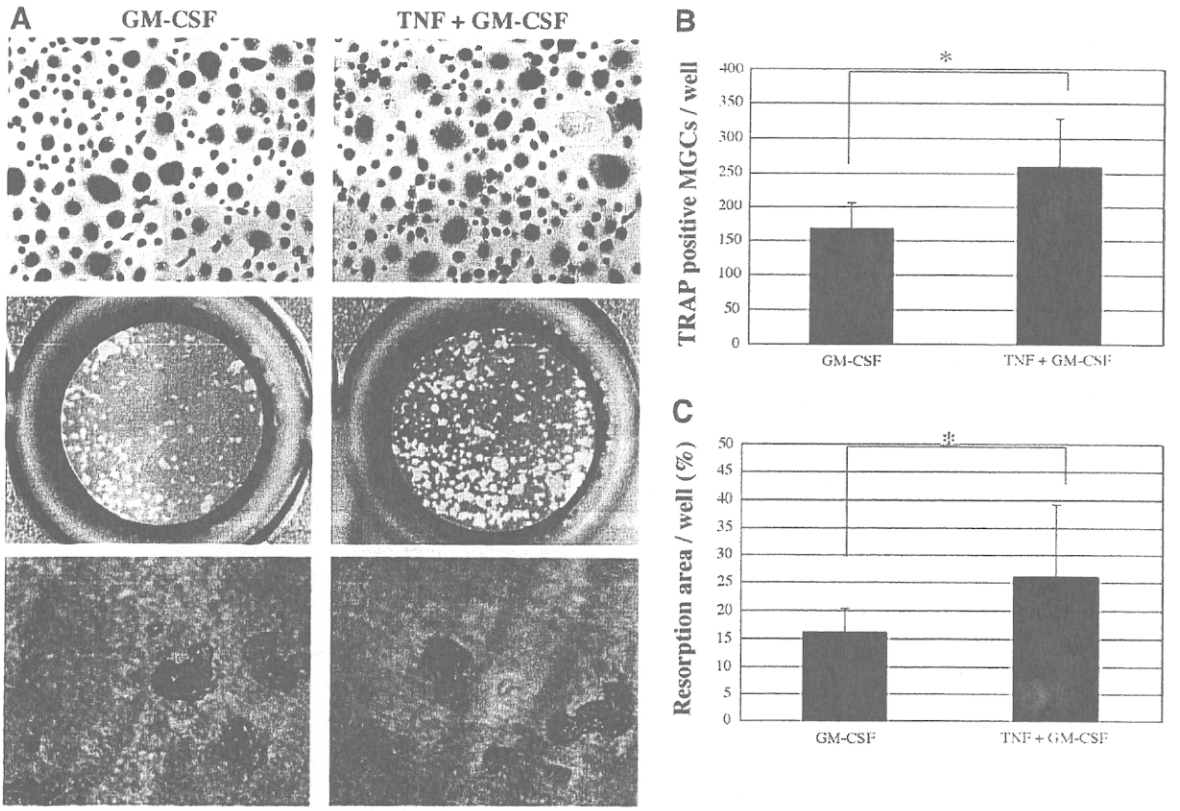

Fig. 2. RANKL + M-CSF induce differentiation of GM-OP and TNF-OP into osteoclasts. Osteoclastogenesis cultures treated with sRANKL (100 ng/ml) and M-CSF (25 ng/ml) for 14 days. (A) Representative figures of GM-OP-derived osteoclasts and TNF-OP-derived osteoclasts were shown in TRAP stain (top), on carbonated calcium phosphate coated plate (middle), and on dentine slices (bottom). (B) After 14 days the number of TRAP-positive MGCs (three or more nuclei) per well was counted. Results are expressed as means ± SD ($^*p < 0.005$). (C) After 14 days the resorption area on carbonated calcium phosphate coated plate was calculated. Results are expressed as means ± SD ($^*p < 0.05$).

These cells expressed CD80+CD86+CD11b+, similar to cDC (Table 1). However, CD14 expression was much higher than cDC and no less than the macrophage. They were morphologically and immunophenotypically distinguished from both cDC and macrophage, and thought to be another lineage of myeloid cells.

Two myeloid cells could differentiate into osteoclasts

To examine how myeloid cells can differentiate into osteoclasts, we treated these cells with RANKL and M-CSF. After 14 days treatment, multinucleated giant cells (MGCs) exhibiting intense TRAP activity were formed (Fig. 2A). Interestingly the sizes of osteoclasts were smaller than monocyte-derived osteoclast, with easily distinguishable morphology (Fig. 4A).

The number of TRAP positive MGCs derived from TNFα + GM-CSF treated cells (TNF-OP) was significantly larger than that of MGCs from GM-CSF treated cells (GM-OP). (TNF-OP 259 ± 68/well; GM-OP 167 ± 38/well: $p < 0.005$) (Fig. 2B). They also formed typical resorption pits and tracks on dentine slices, indicating that they were functional mobile osteoclasts (Fig. 2A). The resorption area of calcium phosphate coated plate on TNF-OP derived OC and GM-OP derived OC was 26.1 ± 13.0% and 16.1 ± 4.13%, respectively (Fig. 2C). The resorption area of TNF-OP derived OC was significantly larger than GM-OP derived OC ($p < 0.05$).

From immunofluorescence staining, both GM-OP derived OC and TNF-OP derived OC exhibited characteristic osteoclastic belts of actin podosomes and Cathepsin-K (CATK) expression (Fig. 3).

In this way under RANKL + M-CSF treatment, both TNF-OP and GM-OP could differentiate functionally and phenotypically into osteoclasts.

GM-CSF did not inhibit their osteoclast differentiation, but inhibited resorption ability

To clarify whether TNF-OP and GM-OP are not inhibited in their osteoclastogenesis, they were co-treated with GM-CSF (0.1 or 1 ng/ml) in addition to RANKL + M-CSF. Certainly, GM-CSF inhibited monocyte-derived osteoclast differentiation completely (Fig. 4B). Interestingly or as expected, the osteoclast differentiation of GM-OP and TNF-OP was not inhibited and they formed TRAP positive MGCs. Alternatively, additional GM-CSF treatment significantly increased the number of TRAP positive MGCs compared with no GM-CSF treatment (Fig. 4C).

Through comparison of TNF-OP derived OC and GM-OP derived OC, TNFα treatment always increased the number of TRAP positive MGCs in spite of additional GM-CSF.

Surprisingly, additional GM-CSF treatment suppressed their resorption ability, not only in monocyte-derived osteoclasts but also in GM-OP and TNF-OP derived osteoclasts (Fig. 4D). It was revealed that both GM-OP and

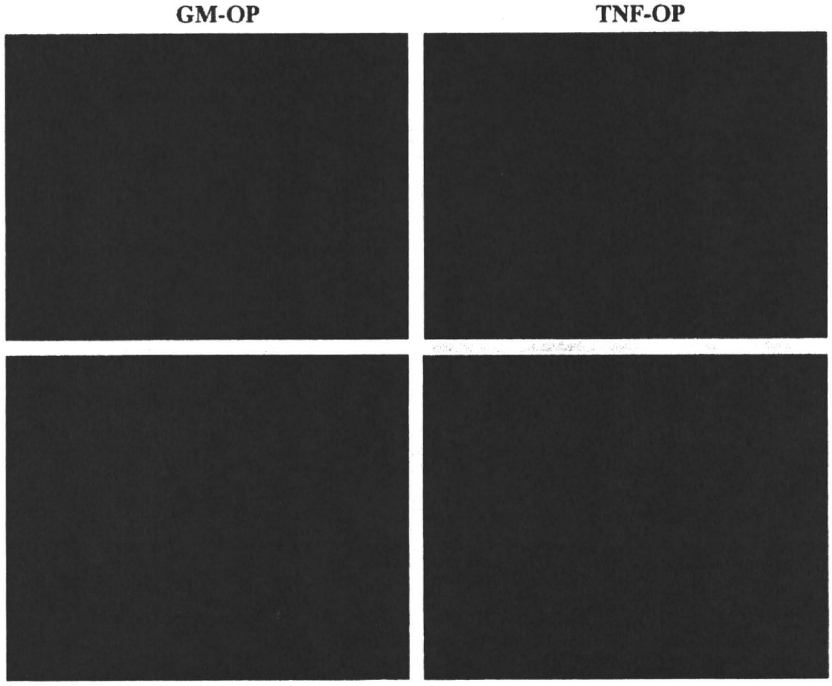

Fig. 3. Both osteoclasts expressed actin-ring and CATK. Representative figures of permeabilized GM-OP-derived osteoclasts (left) and TNF-OP-derived osteoclasts (right) were shown in rhodamine-labeled phalloidin (red) (upper) and in CATK staining (green)/DAPI (blue) (lower) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

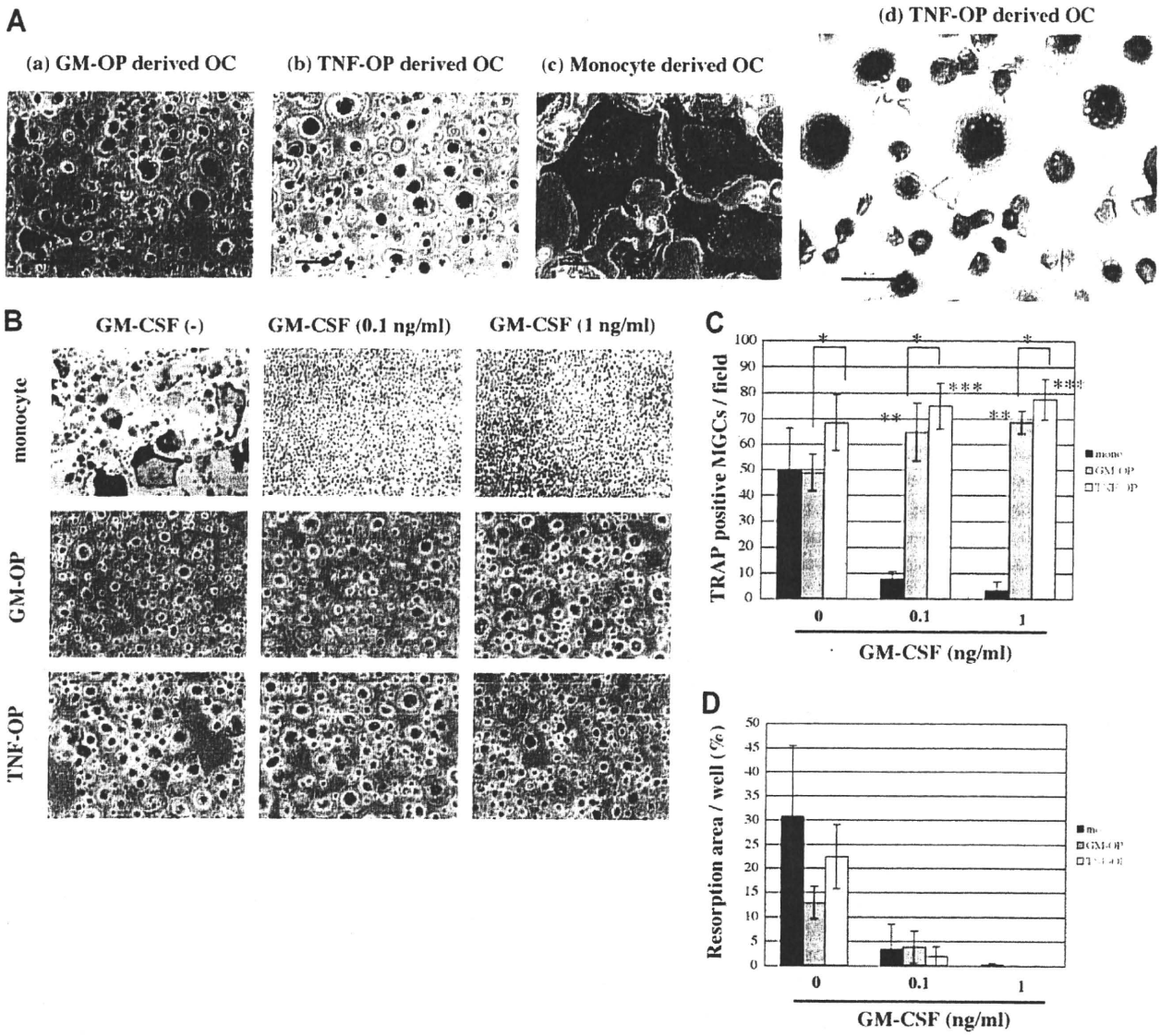

Fig. 4. Their osteoclast differentiation was not inhibited by GM-CSF. (A) Representative TRAP stain figures of (a) GM-OP-derived, (b) TNF-OP-derived or (c) monocyte-derived osteoclasts were shown: Bar = 100 μm. (d) Magnified figures of TNF-OP-derived osteoclasts: Bar = 50 μm. (B) Representative TRAP stain figures of monocyte-derived (top), GM-OP-derived (middle) or TNF-OP-derived (bottom) osteoclasts were shown, co-treated with GM-CSF 0 ng/ml (left), 0.1 ng/ml (center), and 1 ng/ml (right). (C) The number of TRAP-positive MGCs per field was counted. GM-CSF 0 ng/ml (left), 0.1 ng/ml (center), and 1 ng/ml (right). Results are expressed as means ± SD ($^{*}p < 0.05$; GM-OP v.s. TNF-OP, $^{**}p$, $^{***}p < 0.05$; 0 ng/ml GM-CSF v.s. 0.1 or 1 ng/ml GM-CSF). (D) The resorption area on carbonated calcium phosphate coated plate was calculated. GM-CSF 0 ng/ml (left), 0.1 ng/ml (center), and 1 ng/ml (right). Results are expressed as means ± SD.

TNF-OP derived osteoclasts were not inhibited the differentiation, rather the resorption by GM-CSF.

Discussion

The osteoclasts have been considered as the sole bone resorbing cell, derived from monocyte/macrophage. Recently immature DC has demonstrated transdifferentiation into osteoclasts in vitro [27]. This has noticed us the unknown origin of osteoclast precursors except for monocyte/macrophage.

We have shown that monocyte-derived cells generated in GM-CSF (GM-OP) could be another lineage of osteoclast precursor cells that are distinguishable from cDC and monocyte/macrophage. They expressed CD11b, compatible with osteoclast precursors induced by TNFα in vivo [28].

These cells could differentiate into osteoclasts in the presence of RANKL and M-CSF. They possessed about 3–10 nuclei and expressed a characteristic actin ring and CATK. The resorption area of monocyte-derived osteoclasts was considerably larger than that of GM-OP derived

osteoclasts, though the numbers of TRAP positive MGCs were almost the same. This inconsistence was probably because of the larger size of monocyte-derived osteoclasts. Furthermore by TNFα treatment, GM-OP became TNF-OP and then acquired much stronger osteoclastic activity. It was noteworthy that the early action of TNFα not only increased the number of osteoclast precursors, but also activated the resorption ability at the late stage.

As GM-CSF is detected abundantly in RA synovial fluid and scarcely detected in non-inflammatory synovial fluid, these osteoclast precursors seem to appear only in inflammatory arthritis [19]. In fact, we confirmed that GM-CSF was rarely detected in osteoarthritis synovial fluid and elevated in RA by about thirty times (data not shown). This elevation was equal to TNFα. In inflammatory arthritis such as RA, TNFα is always focused by the therapeutic effectiveness, but attention to GM-CSF should also be paid due to its elevation and specificity in the inflammatory environment [29]. Moreover, GM-CSF has been detected in the erosive inflammatory reaction around orthopedic implants or in the osteolytic bone metastasis by malignant tumor [30,31]. GM-CSF must have a pivotal function in bone destruction accompanied with inflammation.

GM-CSF, however, has an inhibitory effect against osteoclastogenesis [12,13]. During response to inflammation, GM-CSF may be released so as to inhibit severe bone destruction. Though it can surely block severe bone destruction, a marked number of neutrophils and macrophages will accumulate in the site, resulting in severe inflammation. GM-CSF can block osteoclastogenesis, but cannot block inflammation and arthritis. Taken that bone destruction always sympathizes with inflammation in arthritis, this incidence is impossible.

Even under such a strong inhibitory effect, GM-OP and TNF-OP could differentiate into osteoclasts. GM-OP and TNF-OP may elucidate this contradiction. This implies the existence of some different differentiation pathway and crosstalk from monocyte/macrophage-derived osteoclastogenesis.

In monocyte/macrophage-derived osteoclastogenesis, M-CSF is known for its crucial role of the survival and proliferation in osteoclast precursors [3]. RANKL is essential for osteoclast development and the determinant of the level of bone resorption [4]. TNFα increases osteoclast precursors, stimulates RANKL and M-CSF production by stromal cells and induces osteoclastogenesis in the presence of M-CSF [5].

On the other hand, in this 'inflammatory' osteoclastogenesis TNFα increased osteoclast precursors and endowed osteoclast precursors (GM-OP) with strong osteoclastic activity in spite of GM-CSF interference. GM-CSF gave rise to osteoclast precursors and promoted the proliferation of osteoclast precursors. Taking this into account, GM-CSF was almost substituted for M-CSF. This is contradictory to osteoclastogenesis at steady state [32]. Therefore, considering osteoclastogenesis, we should distinguish between inflammatory from non-inflammatory states.

In this current study GM-OP and TNF-OP proved to be able to survive as osteoclast precursors without the resorption ability. In other words, they were thought to wait for other signal than RANKL or M-CSF and be ready for resorbing and destroying the bone. Moreover, only RANKL could give the resorption ability to these precursors (data not shown). This suggested M-CSF-independent bone resorption might occur in inflammation. We could not find another inflammatory signal that gave resorption ability. IL-1, which promotes multinucleation of osteoclast precursors and enhances the capacity of the mature osteoclasts to resorb bone, or an unknown ligand, which stimulates DAP12, may be a good candidate [10,33]. Further study is necessary.

Therefore, when we think about 'inflammatory' osteoclastogenesis, we never neglect to consider GM-CSF in addition to TNFα. In a RA cytokine environment, it should be called not only TNF immunity but also GM-CSF immunity. The importance of GM-CSF in inflammation and autoimmunity has been often pointed out [23]. Some RA patients whom anti-TNF therapy did not work might get better by anti-GM-CSF therapy. Many reports that indicated anti-GM-CSF therapy effectiveness on inflammatory arthritis support this notion [24,25].

In conclusion, our study revealed that myeloid cells induced by GM-CSF could differentiate into osteoclasts and acquire stronger osteoclastic activity in the presence of TNFα. Considering GM-CSF is detectable only at the inflammation site and their osteoclast differentiation is not inhibited by GM-CSF, these osteoclast precursor cells are specialized for inflammatory arthritis. There is another lineage distinguishable from cDC and monocyte/macrophage. We are the first to suggest the existence of 'inflammatory' osteoclastogenesis and the mechanism involved. Clarification of the mechanism of 'inflammatory' osteoclastogenesis will undoubtedly lead us to a new therapy for inflammatory arthritis, such as RA.

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Circulating Bone Marrow-Derived Osteoblast Progenitor Cells Are Recruited to the Bone-Forming Site by the CXCR4/Stromal Cell-Derived Factor-1 Pathway

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ABSTRACT

Previous studies demonstrated the existence of osteoblastic cells in circulating blood. Recently, we reported that osteoblast progenitor cells (OPCs) in circulation originated from bone marrow and contributed to the formation of ectopic bone induced by implantation of a bone morphogenetic protein (BMP)-2-containing collagen pellet in mouse muscular tissue. However, the character of circulating bone marrow-derived osteoblast progenitor cells (MOPCs) and the precise mechanisms involving the circulating MOPCs in the osteogenic processes, such as signals that recruit the circulating MOPCs to the osseous tissues, have been obscure. In this report, we demonstrated for the first time that the MOPCs were mobilized from intact bones to transiently occupy approximately 80% of the mononuclear cell population in the circulating blood by BMP-2-pellet implantation. The mobilized MOPCs in the circulation did not express the hematopoietic marker CD45 on their surface, but they expressed CD44 and CXCR4, receptors of osteopontin and stromal cell-derived factor-1 (SDF-1), respectively. The MOPCs isolated from the mouse peripheral blood showed the ability to be osteoblasts in vitro and in vivo. Furthermore, the MOPCs in the circulation efficiently migrated to the region of bone formation by chemoattraction of SDF-1 expressed in vascular endothelial cells and the de novo osteoblasts of the region. These data may provide a novel insight into the mechanism of bone formation involving MOPCs in circulating blood, as well as perspective on the use of circulating MOPCs to accelerate bone regeneration in the future. STEM CELLS *2008;26:223–234*

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

Bone marrow contains hematopoietic stem cells and mesenchymal stem/progenitor cells (MPCs) that can differentiate into various mesenchymal tissues, such as bone, cartilage, fat, and muscle [1–3]. These cells are also found in various mesenchymal tissues [4], although the relationship between the marrow mesenchymal stem/progenitor cells (MMPCs) and extramarrow MPCs has not been fully understood. Marrow MPCs have been shown to engraft not only in bone marrow but also in multiple mesenchymal tissues after systemic infusion [3, 5], suggesting that circulating blood might be a natural route for MMPC migration to the mesenchymal tissues in vivo.

Previous studies have shown the existence of osteoblast-lineage cells in the circulating blood of various mammals, including humans [6–8]. The circulating osteoblast-lineage cells were shown to form bone in culture and in transplanted animals [6]. Studies have also reported more circulating osteoblast-lineage cells during the adolescent growth spurt than in adulthood [6]. However, the origin and the functional role of those osteoblastic cells in human circulation are unclear.

Bone morphogenetic protein (BMP)-2 and other members of the BMP family are well-known inducers of bone formation in vitro and in vivo [9]. In the process of a fracture healing, BMP stimulation recruits MPCs to the fracture lesion and induces their differentiation into osteoblasts. An experimental model has also indicated that BMP-2 stimulation is essential for ectopic bone formation when BMP-2 is transplanted in the back muscles of mice [10]. Recently, we reported that marrow-derived osteoblast progenitor cells (MOPCs) in circulating blood participated in BMP-2-induced ectopic bone formation [11]. If circulating MOPCs play a major role in bone regeneration in vivo, efficient recruitment of MOPCs from bone marrow to the lesion seems to be critical to obtain mature and sufficient regeneration. However, the character of circulating MOPCs and precise mechanisms involving the MOPCs in the osteogenic processes, such as signals that recruit circulating MOPCs to the osseous tissues, have been obscure.

In this study, we characterized MOPCs in the circulating blood without expansion in culture and showed that MOPCs were mobilized in the circulation after stimulation with tissue injury, migrated to damaged tissues by chemoattraction of stromal cell-derived factor-1 (SDF-1), and provided a significant number of mature osteoblasts with BMP-2 stimulation during bone formation. We believe these findings provide novel insights into bone regeneration involving circulating MOPCs.

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MATERIALS AND METHODS

Bone Marrow Transplantation

Under sterile conditions, bone marrow cells were isolated from 8- to 10-week-old male C57BL/6 transgenic mice that ubiquitously expressed enhanced green fluorescent protein (GFP) [12]. Eight- to 10-week-old female C57BL/6 mice were lethally irradiated with 10 Gy. For total bone marrow transplantation (BMT), each irradiated recipient received 5×10^6 bone marrow cells from GFP transgenic mice. For CD45/GFP double-positive BMT, the CD45-positive bone marrow cells of GFP transgenic mice were sorted using the magnetic cell sorting (MACS) system (Miltenyi Biotec, Bergisch Gladbach, Germany, http://www.miltenyibiotec.com). Each irradiated recipient received 4.5×10^6 CD45-positive marrow cells of GFP transgenic mice in combination with 0.5×10^6 CD45-negative marrow cells of wild-type mice. All BMT mice were used at least 6 weeks after BMT. All animals were handled according to approved protocols and the guidelines of the Animal Committee of Osaka University.

Parabiotic Mouse Model

The parabiotic mouse model was generated as previously described [13]. A total BMT mouse and a wild-type mouse (C57/BL6) were sutured from the olecranon to the knee joint on the corresponding lateral aspects.

Preparation and Implantation of BMP-2-Containing Collagen Pellets

Recombinant human BMP-2 was provided by Astellas Pharma Inc. (Tokyo, http://www.astellas.com). The BMP-2 was suspended in buffer solution (5 mmol/l glutamic acid, 2.5% glycine, 0.5% sucrose, and 0.01% Tween 80, pH 4.5) at a concentration of 1 μg/μl. Next, 3 μl (3 μg of BMP-2) of the BMP-2 solution was diluted in 22 μl of phosphate-buffered saline (PBS) and blotted into a porous collagen disc (6 mm diameter, 1 mm thickness), freeze-dried, and stored at −20°C. All procedures were carried out under sterile conditions. BMP-2-containing or control PBS-containing collagen pellets were implanted on the backs of BMT mice, parabiotic mice, C57BL/6 mice, or nude mice. Three weeks later, fluorescent photos of ectopic bones were taken using a digital microscope (Multiviewer system VB-S20; Keyence, Osaka, Japan, http://www.keyence.com).

Immunohistochemistry and Analysis

The ectopic bones were removed and fixed with 4% paraformaldehyde at 4°C for 48 hours. After soft x-ray photos were taken, bones were decalcified with EDTA solution at 4°C for 6 days. The EDTA solution was changed every other day. After decalcification, the pellets were equilibrated in PBS containing 15% sucrose for 12 hours and then in PBS containing 30% sucrose for 12 hours, embedded in Tissue-Tek OCT Compound (Sakura Finetek, Tokyo, http://www.sakuraeu.com), frozen on dry ice, and stored at −20°C.

For immunofluorescence staining, 6-μm-thick sections were cut with a cryostat (Leica Microsystems AG, Wetzlar, Germany, http://www.leica.com). After washing, the sections were treated with 0.1% trypsin (Difco Laboratories, Detroit, MI, http://www.bd.com/ds) in PBS for 30 minutes at 37°C to activate antigens. Then, those sections were blocked with normal goat serum for 1 hour before incubation with polyclonal anti-mouse osteocalcin antibody (1:250; Takara Bio, Shiga, Japan, http://www.takara-bio.com) or polyclonal anti-mouse SDF-1a antibody (1:250; eBioscience Inc., San Diego, http://www.ebioscience.com). Subsequently, sections were stained with Alexa Fluor 546 goat anti-rabbit IgG secondary antibody (Molecular Probes, Eugene, OR, http://probes.invitrogen.com) for 2 hours. Then, sections were stained with 4′,6-diamidino-2-phenylindole (DAPI) for 10 minutes at room temperature and mounted with the antifade solution Vectashield (Vector Laboratories, Burlingame, CA, http://www.vectorlabs.com).

For staining endothelial progenitor cells and tissues around the pellets, the pellets were removed daily until day 7 after implantation. After removal, pellets were embedded in Tissue-Tek OCT Compound and frozen on dry ice. Six-micrometer-thick sections were blocked with normal goat serum for 1 hour before incubation with monoclonal anti-mouse CD31 antibody (1:250; BD Pharmingen, San Jose, CA, http://www.bdbiosciences.com/index_us.shtml), monoclonal anti-mouse CD34 antibody (1:100; BD Pharmingen), monoclonal anti-smooth-muscle actin antibody (1:250; Sigma-Aldrich, St. Louis, http://www.sigmaaldrich.com), or polyclonal anti-mouse SDF-1a antibody (1:250; eBioscience). Subsequently, sections were stained with Alexa Fluor 546 goat anti-rat IgG secondary antibody, Alexa Fluor 488 anti-rat IgG secondary antibody, or Alexa Fluor 488 anti-mouse IgG secondary antibody (Molecular Probes) with M.O.M. Kit (Vector Laboratories) for 2 hours. The sections were mounted with antifade solution Vectashield after 10 minutes of DAPI staining. All pictures were taken with a confocal laser microscope, model Radiance 2100 using LaserSharp 2000 software (Bio-Rad Japan, Tokyo, http://www.bio-rad.com). To assess the frequency of MOPCs for osteoblast differentiation, we counted the number of GFP-positive cells in the osteocalcin-positive osteoblasts lining the trabecular bone. The ratio was quantitatively calculated in at least five low-power visual fields.

Peripheral Blood Mononuclear Cell Isolation

Peripheral blood was taken from the heart with a 24-gauge needle and 1-ml syringe containing heparin and enriched for low-density mononuclear cells by Ficoll-Paque (Amersham Biosciences, Uppsala, Sweden, http://www.amersham.com) centrifugation. Red blood cells were removed by resuspending in 0.125% Tris-NH_4Cl buffer and sieving through a nylon mesh. Isolated peripheral blood mononuclear cells (PBMNCs) from BMP-2 pellet-implanted mice were reacted with anti-mouse CD45 microbeads (Miltenyi Biotec), and the CD45-negative cells dominantly containing MOPCs were sorted using the MidiMACS system (Miltenyi Biotec) according to the manufacturer's protocol.

In Vitro Differentiation

For induction of osteoblast differentiation in culture, MACS-sorted CD45-negative PBMNCs from BMP-2-implanted GFP transgenic mice were plated on a 24-well plate. The sorted cells were then inoculated in basal medium consisting of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 100 U/ml streptomycin/penicillin, and 50% conditioned culture medium (DMEM with 10% FCS) of mouse bone marrow mesenchymal cells as a growth factor supplement (S. Otsuru and K. Tamai, unpublished data). To induce osteoblast differentiation, 300 ng/ml BMP-2 was added to the culture medium for 3 weeks. In some experiments, the sorted CD45-negative PBMNCs were cultured in the osteogenic medium consisting of Iscove's modified Dulbecco's medium supplemented with 0.1 μM dexamethasone (Nacalai Tesque Inc., Kyoto, Japan, http://www.nacalai.co.jp/en), 10 mM β-glycerol phosphate (Sigma-Aldrich), and 0.05 mM ascorbic acid 2-phosphate (Sigma-Aldrich) for 3–4 weeks.

Alizarin Red S Staining

To observe calcium deposition, cells were fixed with 4% paraformaldehyde and stained with 2% alizarin red S (Nacalai Tesque) solution in water for 10 minutes. Excess stain was removed by several washes with distilled water.

Alkaline Phosphatase Assay

Alkaline phosphatase (ALP) activity was assessed as previously described [14]. Cell lysates were centrifuged, and supernatants were used for the enzyme assay. Alkaline phosphatase activity was measured according to the methods of Kind-King, using a test kit (Wako Chemical, Osaka, Japan, http://www.wako-chem.co.jp/english)

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with phenylphosphate as a substrate. Enzyme activity was expressed in King-Armstrong units, normalized to protein concentration.

In Vivo Bone Forming Assay

Fully open interconnected porous calcium hydroxyapatite ceramics were synthesized by adopting a "foam-gel" technique from a slurry of hydroxyapatite (60% wt/wt) with a cross-linking substrate (polyethyleneimine, 40% wt/wt) as previously reported [15]. Blocks of the ceramics were cut and shaped into 5-mm-diameter disks that were 2 mm thick. Cell culturing in the pores of the ceramics was performed as previously reported [16, 17]. The ceramic disk was soaked in 200 μl of CD45-negative cultured PBMNC suspension from GFP-transgenic mice in normal medium (10^6 cells per milliliter). After overnight incubation in a 96-well plate, normal medium was changed to osteogenic medium. The medium was renewed three times a week, and the cultures were maintained for 2 weeks. After a wash with PBS, the disks were implanted under the muscular fascia in the backs of nude mice. Disks without cells were also implanted as controls. Eight weeks later, the disks were harvested and fixed in 4% paraformaldehyde. After decalcification with K-CX (Falma Co., Osaka, Japan; http://www.falma.co.jp), the disks were embedded in paraffin, and the sections were stained with hematoxylin and eosin.

For immunofluorescence staining, the sections were treated with 0.1% trypsin (Difco Laboratories) in PBS for 30 minutes at 37°C to activate antigens. Next, those sections were blocked with normal goat serum for 1 hour before incubation with polyclonal anti-GFP antibody (1:250; MBL International Corp., Nagoya, Japan, http://www.mblintl.com). Subsequently, sections were stained with Alexa Fluor 488 goat anti-rabbit IgG secondary antibody (Molecular Probes) for 2 hours. Sections were then stained with DAPI for 10 minutes at room temperature and mounted with the antifade solution Vectashield (Vector Laboratories).

RNA Extraction and Reverse Transcription-Polymerase Chain Reaction

Total RNA was prepared with an RNeasy Kit (Qiagen, Tokyo, http://www1.qiagen.com) according to the manufacturer's protocol. Reverse transcription was performed by conventional protocols with Superscript reverse transcriptase (Invitrogen, Carlsbad, CA, http://www.invitrogen.com), and polymerase chain reaction (PCR) amplification was performed using the following primer sets: Cbfa1 (NM_009820, 289 base pairs [bp]), 5′-CCGCACGACAACCGCACCAT-3′ (forward) and 5′-CGCTCCGGCCCACAAATCTC-3′ (reverse); osteopontin (NM_009263, 437 bp), 5′-TCACCATTCGGATGAGTCTG-3′ (forward) and 5′-ACTTGTGGCTCTGATGTTCC-3′ (reverse); ALP (NM_007431, 180 bp), 5′-CGCCAGAGTACGCTCCCGCC-3′ (forward) and 5′-TGTACCCTGAGATTCGT-3′ (reverse); osteocalcin (X04142, 350 bp), 5′-CTGACCTCACAGATCCCAAG-3′ (forward) and 5′-GGAGCTGCTGTGACATCC-3′ (reverse); and SDF-1 (NM_021704, 538 bp), 5′-ACGCCAAGGTGGTCGCCGTGCTGG-3′ (forward) and 5′-GTTAGGGTAATACAATTCCTTAGA-3′ (reverse).

Flow Cytometry

Isolated PBMNCs were suspended in 100 μl of PBS containing fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD45; phycoerythrin (PE)-conjugated anti-mouse CD11b, CD31, CD34, CD44, Flk-1, and Sca-1 (BD Pharmingen); and biotin-conjugated anti-mouse Gr-1 and CXCR4 (BD Pharmingen). Cells were then incubated for 30 minutes at 4°C in the dark. Subsequently, cells were stained with Streptavidin PE or Streptavidin PerCP (BD Pharmingen) or anti-rat IgG secondary antibody or anti-goat IgG secondary antibody (Molecular Probes) for 30 minutes at 4°C in the dark.

For the time-course analysis of the CD45-negative population in PBMNCs, BMP-2 pellets were implanted on the backs of 8- to 10-week-old female C57BL/6 mice daily, one mouse per day, for 7 days. At day 7, blood samples were taken, and hemolyzed PBMNCs were harvested. The harvested PBMNCs were reacted with FITC-conjugated anti-mouse CD45. Flow cytometry analysis was performed with a FACScan instrument using CellQuest software (Becton, Dickinson and Company, San Diego, http://www.bd.com).

In Vitro Migration Assay

After different concentrations of SDF-1a (R&D Systems Inc., Minneapolis, http://www.rndsystems.com) were added to the lower chamber, the 5×10^5 isolated CD45-negative PBMNCs from BMP-2 pellet-implanted mice in 100 μl of DMEM without growth factors were applied to the upper chamber of the membrane of the 96-well cell migration kit (Chemicon, Temecula, CA, http://www.chemicon.com). Some cells were pretreated with a CXCR4-blocking antibody (2B11; BD Pharmingen). After 4 hours of incubation at 37°C, the migratory cells on the bottom of the insert membrane were dissociated from the membrane by incubation with cell detachment buffer. These cells were stained with CyQuant GR dye (Molecular Probes, Eugene, OR, http://probes.invitrogen.com), and the fluorescence was measured with a fluorescence plate reader.

In Vivo Migration Assay

For the transplantation experiment, nude mice implanted with BMP-2-containing collagen pellets were injected via a tail vein with sorted CD45-negative PBMNCs with or without CXCR4-blocking antibody (2B11; BD Pharmingen) pretreatment from the GFP-transgenic BMP-2-implanted mice for 7 days.

Real-Time PCR

Primers and probes for hypoxia inducible factor-1 (HIF-1), SDF-1, and glyceraldehyde-3-phosphate dehydrogenase were purchased from Applied Biosystems (Foster City, CA, http://www.appliedbiosystems.com). Real-time PCR was carried out and measured by the ABI Prism 7900HT Sequence Detection System using SDS 2.2 software (Applied Biosystems).

Statistical Analysis

All experiments were repeated four to seven times. Statistical analyses were performed with the unpaired t test or the paired Student t test. p values <0.05 were considered statistically significant.

RESULTS

Bone Marrow-Derived Osteoblast Progenitor Cells in Circulation Contribute to BMP-2-Induced Ectopic Bone Formation

We have already reported that osteoblast progenitor cells (OPCs) were recruited from bone marrow to the circulating blood and contributed to the BMP-2-induced ectopic bone formation [11]. To obtain more direct evidence that circulating MOPCs were recruited to the region of the BMP-2 pellet to generate ectopic bone, we established a mouse model with parabiotic pairings that shared a circulatory system between a wild-type mouse and a GFP-BMT mouse (Fig. 1A) [13]. In our parabiotic mouse model, a wild-type mouse was surgically connected with a GFP-BMT mouse whose bone marrow had been replaced by GFP-transgenic bone marrow cells. The wild-type mouse can receive bone marrow-derived GFP-positive circulating cells from the GFP-BMT mouse after they develop shared circulation in a few weeks. We implanted a BMP-2 pellet into the wild-type mouse of the parabiotic pairings (Fig. 1A). Three weeks after transplantation, GFP fluorescence was detected at the region where the ectopic bone had formed (Fig. 1B). Histologic analysis revealed that 24.5% ± 3.1% of the osteoblasts that aligned on the regenerating bone and expressed osteocalcin (OC) were GFP-positive cells that originated from the bone marrow of the GFP-BMT mouse (Fig. 1C; supplemental online Fig. 1).

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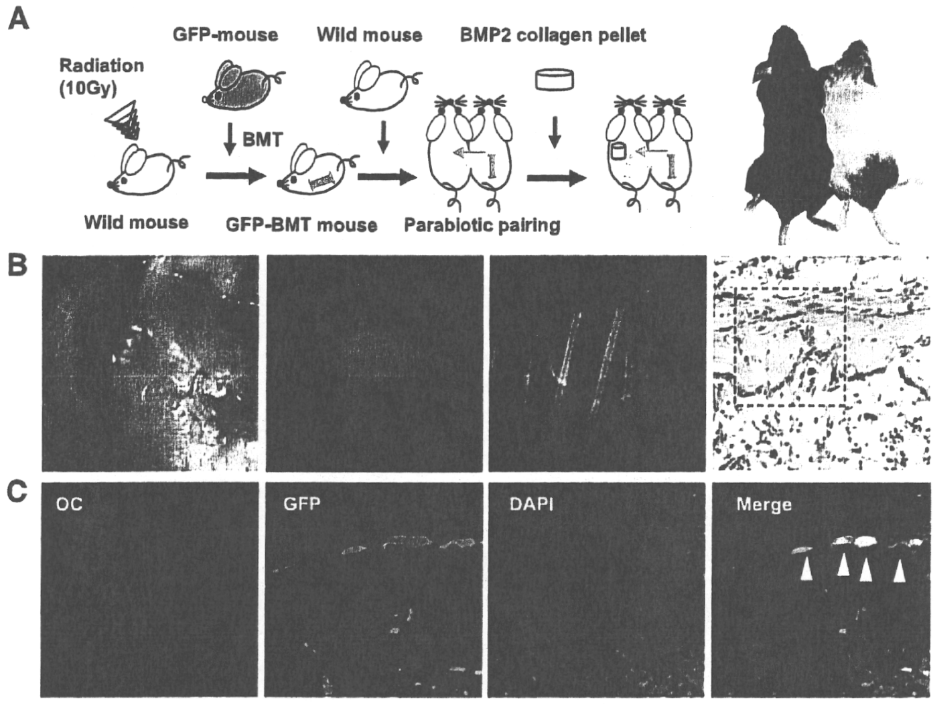

Figure 1. Bone marrow-derived osteoblast progenitor cells contribute to BMP-2-induced ectopic bone formation via circulation in a parabiotic mouse. **(A):** Parabiotic pairing between a GFP-BMT mouse and a wild-type mouse. A BMP-2 pellet was implanted into the wild-type mouse that could receive GFP-positive bone marrow cells from the GFP-BMT mouse through the circulation. **(B):** A BMP-2 pellet showed accumulation of GFP fluorescence 3 weeks after implantation under the muscular fascia of a wild-type parabiotic mouse. A soft x-ray photo of the BMP-2 pellet 3 weeks after the implantation demonstrated that ectopic bone formed in the BMP-2 pellet. Histologic section stained with H&E of the BMP-2 pellet 3 weeks after implantation also revealed bone formation in the BMP-2 pellet. Magnification, ×200. **(C):** Immunofluorescence staining of the boxed region in the H&E section showed that the cells lining the newly generated bone were osteoblasts expressing OC. Some of those osteoblasts expressing osteocalcin also exhibited GFP fluorescence (arrowheads). Magnification, ×600. Abbreviations: BMP, bone morphogenetic protein; BMT, bone marrow transplantation; DAPI, 4′,6-diamidino-2-phenylindole; GFP, green fluorescent protein; OC, osteocalcin.

If both mice provide the bone marrow cells equally, these data suggest that approximately 50% of the regenerating osteoblasts may be derived from endogenous circulating MOPCs in parabiotic mice.

CD45-Negative Fraction in Bone Marrow Is a Major Source of Circulating MOPCs

We next examined a particular population in bone marrow to determine the major source of the circulating MOPCs. To determine whether the major source of MOPCs in bone marrow is CD45-positive or CD45-negative, we transplanted two types of bone marrow cells in combination to a lethally irradiated mouse before BMP-2-pellet implantation: a combination of CD45-negative/GFP-negative bone marrow cells and CD45-positive/GFP-positive bone marrow cells to generate CD45/GFP-BMT (Fig. 2A). The ectopic bone formed in the CD45/GFP-BMT mouse showed less accumulation of GFP fluorescence than that in the GFP-BMT mouse (Fig. 2B, 2C). Histologic examination revealed that the transplanted cells with a reduced CD45-negative/GFP-positive fraction formed ectopic bone with significantly fewer GFP-positive osteoblasts (11.0% ± 3.4%) than the controls (43.4% ± 10.6%, $p = .00127$; Fig. 2D). These data suggested that CD45-negative cells in bone marrow might be the major source of circulating MOPCs in BMP-2-implanted mice, although the contribution of CD45-positive cells to ectopic bone formation can not be completely excluded.

Kinetic Analysis of Circulating MOPCs

The data obtained led us to further characterize the kinetics of CD45-negative cell migration from bone marrow to circulating blood. To view CD45-negative cells in the circulation, five sets of the experiment were performed independently. In each experiment, seven mice were serially implanted (i.e., one mouse per day) with a BMP-2 pellet, and at day 7, they were all at once subjected to flow cytometry analysis to evaluate the CD45-negative cell populations in the PBMNCs. Before the implantation, the basal population of the CD45-negative cells in PBMNCs was less than 20%, possibly containing a remnant fraction of red blood cells even after the conventional PBMNC isolation procedure. Surprisingly, large increases of the CD45-negative population in PBMNCs, up to 83% frequency, were observed within 7 days after BMP-2-pellet implantation, coinciding with a significant reduction in the CD45-negative population in bone marrow cells within 7 days after BMP-2 implantation (Fig. 3A). A similar increase in the CD45-negative population in PBMNCs was observed at least once within 7 days after BMP-2-implantation in the other four sets of experiments. The induction of CD45-negative population in PBMNCs at the peak in each set of experiments was significantly higher in the BMP-2-implanted mice than in the control mice ($p = .0000142$; Fig. 3B). Implantation of empty collagen pellets also showed a relatively smaller but significant induction of the CD45-negative population in PBMNCs at the peak ($p = .0198$; Fig. 3B).

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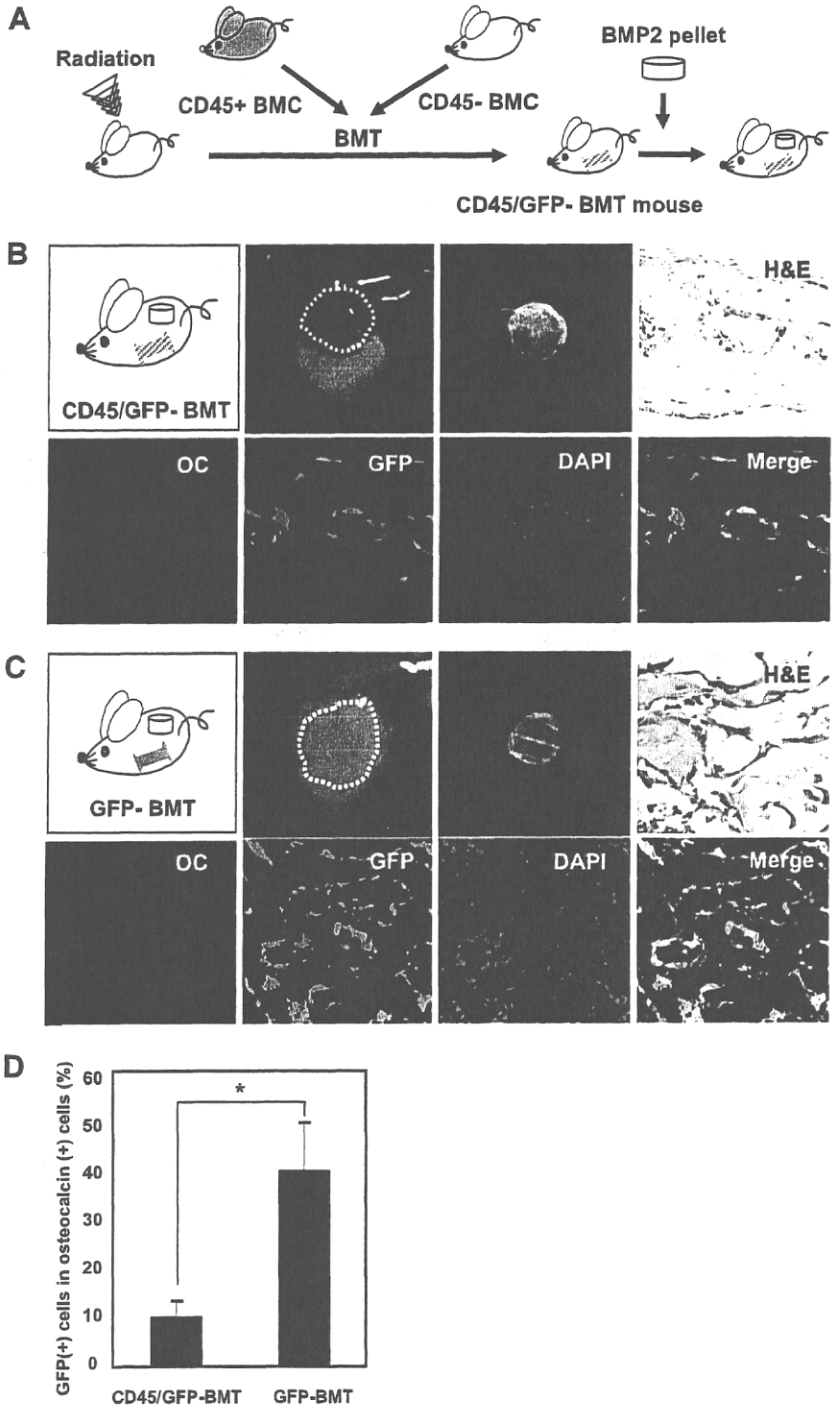

Figure 2. CD45-negative fraction of bone marrow cells predominantly participated in the BMP-2-induced ectopic bone formation. **(A):** CD45-negative bone marrow cells from wild-type mice and CD45-positive bone marrow cells from GFP-transgenic mice were transplanted into a lethal-dose-irradiated wild-type mouse (CD45/GFP-BMT) before the BMP-2-pellet implantation. **(B):** The ectopic bone (circled with a dotted line) in the CD45/GFP-BMT mouse showed weak GFP fluorescence. A soft x-ray photo and H&E-stained histologic section showed successful ectopic bone formation in the CD45/GFP-BMT mice. Immunofluorescence staining revealed fewer GFP-positive cells in the ectopic bone of the CD45/GFP-BMT mice than in the ectopic bone of the GFP-BMT mice. Magnification, ×400. **(C):** Total bone marrow cells from GFP transgenic mice were transplanted to lethally irradiated wild-type mice (GFP-BMT mouse). A BMP-2 pellet in the GFP-BMT mouse showed stronger GFP fluorescence (circled with a dotted line). A soft x-ray photo and histologic H&E-stained section showed bone formation in the BMP-2 pellet in the GFP-BMT mice as well. Immunofluorescence staining revealed that more GFP-positive cells expressed OC in the newly formed bone. Magnification, ×200. **(D):** Quantitative analysis showed that the percentage of GFP-positive/osteocalcin-positive osteoblasts in the osteocalcin-positive osteoblasts lining the trabecular bone significantly decreased in the CD45/GFP-BMT mice compared with the GFP-BMT mice. *, $p = .00127$. Abbreviations: BMC, bone marrow cell; BMP, bone morphogenetic protein; BMT, bone marrow transplantation; DAPI, 4′,6-diamidino-2-phenylindole; GFP, green fluorescent protein; OC, osteocalcin.

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