lupus erythematosus: a 10-year prospective study on the value of diagnostic tests. Am J Med 1995;99:153–63.
7. ACR Ad Hoc Committee on Neuropsychiatric Lupus Nomenclature. The American College of Rheumatology nomenclature and case definitions for neuropsychiatric lupus syndromes. Arthritis Rheum 1999;42:599–608.
8. Arnett FC, Reveille JD, Moutsopoulos HM, Georgescu L, Elkon KB. Ribosomal P autoantibodies in systemic lupus erythematosus: frequencies in different ethnic groups and clinical and immunogenetic associations. Arthritis Rheum 1996;39:1833–9.
9. Bonfa E, Golombek SJ, Kaufman LD, Skelly S, Weissbach H, Brot N, et al. Association between lupus psychosis and anti-ribosomal P protein antibodies. N Engl J Med 1987;317:265–71.
10. Chan EY, Ko OK, Lawton JW, Lau CS. The use of anti-ribosomal P antibodies in the diagnosis of cerebral lupus: superiority of western blotting over enzyme-linked immunosorbent assay. Hong Kong Med J 1998;4:145–50.
11. Conti F, Alessandri C, Bompane D, Bombardieri M, Spinelli FR, Rusconi AC, et al. Autoantibody profile in systemic lupus erythematosus with psychiatric manifestations: a role for anti-endothelial-cell antibodies. Arthritis Res Ther 2004;6:R366–72.
12. Corres Gonzalez JM, Rodriguez Hernandez C, Zea Mendoza AC, Sequi Navarro J, Sanchidrian Fernandez I. Anti-ribosomal antibodies in systemic lupus erythematosus. Rev Clin Esp 1995;195: 16–21.
13. Gerli R, Caponi L, Tincani A, Scorza R, Sabbadini MG, Danieli MG, et al. Clinical and serological associations of ribosomal P autoantibodies in systemic lupus erythematosus: prospective evaluation in a large cohort of Italian patients. Rheumatology (Oxford) 2002;41:1357–66.
14. Hoffman IE, Peene I, Meheus L, Huizinga TW, Cebecauer L, Isenberg D, et al. Specific antinuclear antibodies are associated with clinical features in systemic lupus erythematosus. Ann Rheum Dis 2004;63:1155–8.
15. Isenberg DA, Garton M, Reichlin MW, Reichlin M. Long-term follow-up of autoantibody profiles in black female lupus patients and clinical comparison with Caucasian and Asian patients. Br J Rheumatol 1997;36:229–33.
16. Isshi K, Hirohata S. Association of anti-ribosomal P protein antibodies with neuropsychiatric systemic lupus erythematosus. Arthritis Rheum 1996;39:1483–90.
17. Johanet C, Andre C, Sibilia J, Baquey A, Oksman F, San Marco M, et al, and the Study Group on Autoimmunity (GEAI). Clinical significance of antiribosomal antibodies. Rev Med Interne 2000; 21:510–6.
18. Jonsen A, Bengtsson AA, Nived O, Ryberg B, Truedsson L, Ronnblom L, et al. The heterogeneity of neuropsychiatric systemic lupus erythematosus is reflected in lack of association with cerebrospinal fluid cytokine profiles. Lupus 2003;12:846–50.
19. Kao CH, Ho YJ, Lan JL, Changlai SP, Liao KK, Chieng PU. Discrepancy between regional cerebral blood flow and glucose metabolism of the brain in systemic lupus erythematosus patients with normal brain magnetic resonance imaging findings. Arthritis Rheum 1999;42:61–8.
20. Mahler M, Kessenbrock K, Raats J, Williams R, Fritzler MJ, Bluthner M. Characterization of the human autoimmune response to the major C-terminal epitope of the ribosomal P proteins. J Mol Med 2003;81:194–204.
21. Massardo L, Burgos P, Martinez ME, Perez R, Calvo M, Barros J, et al. Antiribosomal P protein antibodies in Chilean SLE patients: no association with renal disease. Lupus 2002;11:379–83.
22. Nojima Y, Minota S, Yamada A, Takaku F, Aotsuka S, Yokohari R. Correlation of antibodies to ribosomal P protein with psychosis in patients with systemic lupus erythematosus. Ann Rheum Dis 1992;51:1053–5.
23. Press J, Palayew K, Laxer RM, Elkon K, Eddy A, Rakoff D, et al. Antiribosomal P antibodies in pediatric patients with systemic lupus erythematosus and psychosis. Arthritis Rheum 1996;39: 671–6.
24. Sanna G, Piga M, Terryberry JW, Peltz MT, Giagheddu S, Satta L, et al. Central nervous system involvement in systemic lupus erythematosus: cerebral imaging and serological profile in patients with and without overt neuropsychiatric manifestations. Lupus 2000;9:573–83.
25. Sato T, Uchiumi T, Ozawa T, Kikuchi M, Nakano M, Kominami R, et al. Autoantibodies against ribosomal proteins found with high frequency in patients with systemic lupus erythematosus with active disease. J Rheumatol 1991;18:1681–4.
26. Schneebaum AB, Singleton JD, West SG, Blodgett JK, Allen LG, Cheronis JC, et al. Association of psychiatric manifestations with antibodies to ribosomal P proteins in systemic lupus erythematosus. Am J Med 1991;90:54–62.
27. Teh LS, Bedwell AE, Isenberg DA, Gordon C, Emery P, Charles PJ, et al. Antibodies to protein P in systemic lupus erythematosus. Ann Rheum Dis 1992;51:489–94.
28. Teh LS, Hay EM, Amos N, Black D, Huddy A, Creed F, et al. Anti-P antibodies are associated with psychiatric and focal cerebral disorders in patients with systemic lupus erythematosus. Br J Rheumatol 1993;32:287–90.
29. Teh LS, Lee MK, Wang F, Manivasagar M, Charles PJ, Nicholson GD, et al. Antiribosomal P protein antibodies in different populations of patients with systemic lupus erythematosus. Br J Rheumatol 1993;32:663–5.
30. Tzioufas AG, Tzortzakis NG, Panou-Pomonis E, Boki KA, Sakarellos-Daitsiotis M, Sakarellos C, et al. The clinical relevance of antibodies to ribosomal-P common epitope in two targeted systemic lupus erythematosus populations: a large cohort of consecutive patients and patients with active central nervous system disease. Ann Rheum Dis 2000;59:99–104.
31. Van Dam A, Nossent H, de Jong J, Meilof J, ter Borg EJ, Swaak T, et al. Diagnostic value of antibodies against ribosomal phosphoproteins: a cross sectional and longitudinal study. J Rheumatol 1991;18:1026–34.
32. Weiner SM, Otte A, Schumacher M, Klein R, Gutfleisch J, Brink I, et al. Diagnosis and monitoring of central nervous system involvement in systemic lupus erythematosus: value of F-18 fluorodeoxyglucose PET. Ann Rheum Dis 2000;59:377–85.
33. Williams RC Jr, Sugiura K, Tan EM. Antibodies to microtubule-associated protein 2 in patients with neuropsychiatric systemic lupus erythematosus. Arthritis Rheum 2004;50:1239–47.
34. Yalaoui S, Gorgi Y, Hajri R, Goucha R, Chaabouni L, Kooli C, et al. Autoantibodies to ribosomal P proteins in systemic lupus erythematosus. Joint Bone Spine 2002;69:173–6.
35. Yoshio T, Masuyama J, Ikeda M, Tamai K, Hachiya T, Emori T, et al. Quantification of antiribosomal P0 protein antibodies by ELISA with recombinant P0 fusion protein and their association with central nervous system disease in systemic lupus erythematosus. J Rheumatol 1995;22:1681–7.
36. Elkon K, Skelly S, Parnassa A, Moller W, Danho W, Weissbach H, et al. Identification and chemical synthesis of a ribosomal protein antigenic determinant in systemic lupus erythematosus. Proc Natl Acad Sci U S A 1986;83:7419–23.
37. Ghirardello A, Caponi L, Franceschini F, Zampieri S, Quinzanini M, Bendo R, et al. Diagnostic tests for antiribosomal P protein antibodies: a comparative evaluation of immunoblotting and ELISA assays. J Autoimmun 2002;19:71–7.
38. Mahler M, Kessenbrock K, Raats J, Fritzler MJ. Technical and clinical evaluation of anti-ribosomal P protein immunoassays. J Clin Lab Anal 2004;18:215–23.
39. Rayno K, Reichlin M. Evaluation of assays for the detection of autoantibodies to the ribosomal P proteins. Clin Immunol 2000; 95:99–103.

40. Tan EM, Cohen AS, Fries JF, Masi AT, McShane DJ, Rothfield NF, et al. The 1982 revised criteria for the classification of systemic lupus erythematosus. Arthritis Rheum 1982;25:1271–7.
41. Owens DK, Holodniy M, Garber AM, Scott J, Sonnad S, Moses L, et al. Polymerase chain reaction for the diagnosis of HIV infection in adults: a meta-analysis with recommendations for clinical practice and study design. Ann Intern Med 1996;124:803–15.
42. Ioannidis JP. The value of meta-analysis in rheumatology research. Autoimmun Rev 2004;3 Suppl 1:S57–9.
43. Fleiss JL. The statistical basis of meta-analysis. Stat Methods Med Res 1993;2:121–45.
44. Mantel N, Haenszel W. Statistical aspects of the analysis of data from retrospective studies of disease. J Natl Cancer Inst 1959;22: 719–48.
45. Littenberg B, Moses LE. Estimating diagnostic accuracy from multiple conflicting reports: a new meta-analytic method. Med Decis Making 1993;13:313–21.
46. Moses LE, Shapiro D, Littenberg B. Combining independent studies of a diagnostic test into a summary ROC curve: data-analytic approaches and some additional considerations. Stat Med 1993;12:1293–316.
47. Irwig L, Macaskill P, Glasziou P, Fahey M. Meta-analytic methods for diagnostic test accuracy. J Clin Epidemiol 1995;48:119–30.
48. Lijmer JG, Mol BW, Heisterkamp S, Bonsel GJ, Prins MH, van der Meulen JH, et al. Empirical evidence of design-related bias in studies of diagnostic tests. JAMA 1999;282:1061–6.
49. Ainiala H, Hietaharju A, Loukkola J, Peltola J, Korpela M, Metsanoja R, et al. Validity of the new American College of Rheumatology criteria for neuropsychiatric lupus syndromes: a population-based evaluation. Arthritis Rheum 2001;45:419–23.
50. Hanly JG. ACR classification criteria for systemic lupus erythematosus: limitations and revisions to neuropsychiatric variables. Lupus 2004;13:861–4.
51. Afeltra A, Garzia P, Mitterhofer AP, Vadacca M, Galluzzo S, del Porto F, et al. Neuropsychiatric lupus syndromes: relationship with antiphospholipid antibodies. Neurology 2003;61:108–10.
52. Kovacs JA, Urowitz MB, Gladman DD. Dilemmas in neuropsychiatric lupus. Rheum Dis Clin North Am 1993;19:795–814.
53. Chindalore V, Neas B, Reichlin M. The association between anti-ribosomal P antibodies and active nephritis in systemic lupus erythematosus. Clin Immunol Immunopathol 1998;87:292–6.
54. Hulsey M, Goldstein R, Scully L, Surbeck W, Reichlin M. Anti-ribosomal P antibodies in systemic lupus erythematosus: a case-control study correlating hepatic and renal disease. Clin Immunol Immunopathol 1995;74:252–6.
55. Reichlin M, Broyles TF, Hubscher O, James J, Lehman TA, Palermo R, et al. Prevalence of autoantibodies to ribosomal P proteins in juvenile-onset systemic lupus erythematosus compared with the adult disease. Arthritis Rheum 1999;42:69–75.
56. Arnett FC, Reichlin M. Lupus hepatitis: an under-recognized disease feature associated with autoantibodies to ribosomal P. Am J Med 1995;99:465–72.
57. Kavanaugh AF, Solomon DH, and the American College of Rheumatology Ad Hoc Committee on Immunologic Testing Guidelines. Guidelines for immunologic laboratory testing in the rheumatic diseases: anti-DNA antibody tests. Arthritis Rheum 2002;47:546–55.
58. Solomon DH, Kavanaugh AJ, Schur PH, and the American College of Rheumatology Ad Hoc Committee on Immunologic Testing Guidelines. Evidence-based guidelines for the use of immunologic tests: antinuclear antibody testing. Arthritis Rheum 2002;47:434–44.
59. Benito-Garcia E, Schur PH, Lahita R, and the American College of Rheumatology Ad Hoc Committee on Immunologic Testing Guidelines. Guidelines for immunologic laboratory testing in the rheumatic diseases: anti-Sm and anti-RNP antibody tests. Arthritis Rheum 2004;51:1030–44.

Role of Bone Marrow in the Pathogenesis of Rheumatoid Arthritis

Shunsei Hirohata*

Department of Internal Medicine, Teikyo University School of Medicine, Tokyo 173-8605, Japan

Abstract: Rheumatoid arthritis (RA) is characterized by hyperplasia of synovial lining cells, consisting of macrophage-like type A synoviocytes and fibroblast-like type B synoviocytes. Type A synoviocytes, also called intimal macrophages, have been found to be derived from monocyte precursors in the bone marrow. Accordingly, the spontaneous generation of CD14+ cells from bone marrow CD14- progenitor cells is accelerated in RA, resulting in the facilitated entry of such CD14+ cells into the synovium. Whereas type B synoviocytes, also called fibroblast-like synoviocytes, are thought to arise from the sublining tissue or other support structures of the joint, they might be also derived from bone marrow progenitor cells. Thus, RA bone marrow CD34+ cells show abnormal responses to TNF-α, resulting in their accelerated differentiation into fibroblast-like cells producing MMP-1. On the other hand, persistent neovascularization is crucial for continuous synovial proliferation through delivery of nutrients and recruitment of inflammatory cells. In this regard, RA bone marrow CD34+ cells differentiate into endothelial cells much more effectively than control subjects, suggesting that bone marrow CD34+ cells might play a role in the synovial hyperplasia in RA through mobilization of endothelial progenitor cells that contribute to vasculogenesis. Taken together, these results support the hypothesis that the bone marrow, rather than the synovium, might be the primary-lesion site of RA.

Keywords: Bone marrow, synovium, CD34, CD14, TNF-α, VEGF.

1. INTRODUCTION

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by continuous synovial proliferation. Thus, joint in RA consists of massive proliferating synovium, forming an invading tissue termed pannus, which results in the destruction of cartilage and bone. The histologic features of synovium in RA can be summarized as follows:

1. Stratification of synovial lining cells.
2. Infiltration of lymphocytes, plasma cells, macrophages, polymorphonuclear neutrophils, dendritic cells, NK cells and mast cells in sublining layers.
3. Formation of pseudo-germinal center.
4. Angiogenesis.

In the lining layer, both type A and type B synoviocytes, also called intimal macrophages and fibroblast-like synoviocytes, respectively, are found to proliferate [1]. In the sublining layer, there is infiltration of a variety of cells, as mentioned above. Of note, lymphoid cluster in RA synovium sometimes results in the formation of pseudo-germinal center, consisting of CD20+ B cells in the center surrounded by CD4+ T cells [1, 2]. In the synovium of RA, neovascularization is usually accompanied by lining cell proliferation and inflammatory cell infiltration [3]. In fact, it has been shown that lining cells and inflammatory cells produce angiogenic growth factors [4]. Therefore, it has been believed that angiogenesis in the synovium of RA might be secondary to inflammation. However, the synovium of patients with RA also showed the neovascularization in the area without either lining cell proliferation or inflammatory cell infiltration [5]. The data therefore strongly suggest that the neovascularization might be the primary abnormal feature that is most proximal to the etiology of RA [5]. On the other hand, increasing attention has been paid to the role of mast cells in the pathogenesis of RA [6]. Accumulating within inflamed synovium in response to stem cell factors produced by fibroblast-like synoviocytes, mast cells produce cytokines and other mediators that may contribute to ongoing synovial inflammation [6].

A number of studies have focused on the abnormalities within the synovial tissues. Thus, the abnormal activation of normal joint constituents, such as synovial lining cells, has been implicated in the pathogenesis of RA [8]. However, increasing attention has been paid to the role of bone marrow in the pathogenesis of RA. The present article overviews an update on the role of bone marrow in the pathogenesis of RA.

2. BONE MARROW ABNORMALITIES IN ANIMAL MODELS OF ARTHRITIS

Bone marrow abnormalities were first noted in an atypical murine polyarthritis model [9]. In this model, polyarthritis is induced in C3H/He mice by intraperitoneal injection of allogenic thymocytes from young BALB/c mice. Such polyarthritis has been shown to be induced by host-versus graft (HvG) reaction. Serial histological examination of joint tissues disclosed the occurrence of synovial proliferation confined to the small canals communicating the bone marrow and joint space at day 5 [10]. Fibroblastic cells apparently migrated from bone marrow into the joint space forming synovial proliferation accompanying vascular invasion [10]. At 3 weeks, remarkable synovitis similar to RA was found to develop.

In other experimental models such as collagen-induced arthritis (CIA) in rats, histopathological evidence of articular

*Address correspondence to this author at the Department of Internal Medicine, Teikyo University School of Medicine, 2-11-1 Kaga, Itabashi-ku, Tokyo 173-8605, Japan; Tel: +81-3-3964-1211 Ext. 1568; Fax: +81-3-5375-1308; E-mail: shunsei@med.teikyo-u.ac.jp

1573-3971/06 $50.00+.00

synovitis appeared on the 10th day after treatment, followed by the appearance of macroscopic foot swelling on day 14 [11]. In this model of arthritis, IL-6 and IL-1 began to increase in bone marrow sera on the 4th and 7th day, respectively, prior to the onset of arthritis [11]. Almost the same phenomena were observed in rats with adjutant arthritis [11]. These data indicate that bone marrow was an important site for the development of arthritis. When fibroblastic stromal cells obtained from bone marrow of untreated Lewis rats were injected into splenectomized Lewis rats and then CIA was induced in these rats, migration of such fibroblastic cells into the joint was demonstrated [12]. Thus, bone marrow derived stromal cells were found to migrate into the joint cavities through canals between the articular margin and the synovial insertion (the bare zone) and therein formed synovial cells, suggesting that the origin of at least some synovial fibroblastic cells in CIA might be bone marrow fibroblastic cells, although there is some evidence that there may be other sources as well [12].

To investigate the role of bone marrow cells (BMC) in the induction of antigen induced arthritis (AIA), Saeki and his colleagues examined whether AIA induced by bovine serum albumin could be transferred by bone marrow transplantation (BMT) [13]. BMT experiments were performed using 2 different mouse genotypes, wild type (IL-6+/+) and IL-6 deficient (IL-6-/-) mice, as a donor. All irradiated IL-6+/+ mice developed typical AIA by transplantation of BMC from immunized IL-6+/+ mice, whereas almost no irradiated IL-6+/+ mice transplanted with BMC from the immunized IL-6-/- mice developed definite arthritis [13]. BMC depleted of T cells and/or B cells still induced a definite arthritis although the histological scores were slightly lower compared with the case of T/B cell undepleted (whole) BMC, suggesting that BMC other than pre-activated mature T and B cells play a unique role in the initiation of AIA. These results indicate that BMC play a critical role and IL-6 is a key cytokine for the induction and progression of AIA.

3. BONE MARROW AND TYPE A SYNOVIOCYTES (INTIMAL MACROPHAGES) IN RA

a. Abnormalities in Peripheral Blood Monocytes in RA

Studies with cell lineage specific monoclonal antibodies have confirmed the presence of cells of the mononuclear phagocyte system both in the subintimal area and the lining of the synovial membrane [14, 15], as well as in subcutaneous nodules [16, 17]. It is thus presumed that the macrophages in the synovial tissue as well as in subcutaneous nodules have been recruited from the systemic circulation. In addition, it has been also described that peripheral blood monocytes in patients with RA have enhanced capacity to bind heat aggregated immunoglobulin G (IgG) and monomeric IgG [18, 19], suggesting that the expression of Fc receptors of peripheral blood monocytes might be increased in RA.

Previous studies demonstrated that bacterial products such as LPS activate monocytes and increase CD14 expression [20]. Conversely, expression and release of CD14 by activated monocytes are suppressed by glucocorticoid *in vivo* and *in vitro* [21]. In this regard, we showed that peripheral blood monocytes in patients with active RA are already activated to express higher densities of CD14 [22]. It is also suggested that peripheral blood monocytes in patients with RA may have intrinsic abnormalities as evidenced by the enhanced expression of FcγR, which is repeatedly observed regardless of the disease activity of RA [22]. Our data are consistent with the previous observation that peripheral blood monocytes in patients with RA have enhanced capacity to bind heat aggregated IgG and monomeric IgG [18, 19] and increased antibody dependent cell mediated cytotoxicity [23]. It has been demonstrated that CD14, FcγRI and FcγRII are involved in the regulation of various functions of monocytes, including the production of cytokines [24] and the expression of adhesion molecules [25]. Therefore, the observed abnormalities in our studies suggest that the recruitment of RA peripheral blood monocytes may result in further activation and adhesion of these cells in local synovial tissues, thus contributing to extending the rheumatoid disease process. More importantly, it is further suggested that abnormalities in bone marrow progenitor cells might play a role in abnormal phenotypes of peripheral blood monocytes in RA.

b. Accelerated Generation of CD14+ Monocyte-Lineage Cells from the Bone Marrow

Although previous studies have suggested a role of dysregulated proliferation of synoviocytes in synovial hyperplasia [26], it was found that rheumatoid synovium rarely evidence of mitosis, and that only 4% of rheumatoid synovial cells showed uptake of thymidine [27]. Thus, there has been no evidence for accelerated or dysregulated proliferation of synoviocytes in rheumatoid synovium.

Previous studies have suggested that abnormal myelopoiesis in the bone marrow might play an important role in the development of RA [28]. Thus, it has been shown that 30% of patients with RA have peripheral blood monocytosis [29] and that the mononuclear cell population in RA patients contains higher numbers of monocytes than that in healthy controls [30]. In this regard, we have shown that the spontaneous generation of CD14+ cells from bone marrow CD14- progenitor cells was accelerated in RA patients compared with control patients [31]. Moreover, the expression of HLA-DR on the bone marrow-derived CD14+ cells was also accelerated in RA patients compared with controls, confirming the accelerated maturation of macrophages in RA bone marrow. Consistently, recent studies have revealed that CD14+ CD16+ blood monocytes with high expression of chemokine receptors and CD54 are increased in active RA [32]. It should be also pointed out that the expression of a variety of chemokines and adhesion molecules is enhanced in vascular endothelium and fibroblast-like synoviocytes in the RA synovium [33-35], possibly facilitating the entry of such CD14+ CD16+ blood monocytes into the synovium. These observations strongly support the hypothesis that the accelerated generation of CD14+ cells from bone marrow progenitor cells and the accelerated maturation of such CD14+ cells into tissue-infiltrative CD16+ monocytes before entry into the joint might play an important role in the pathogenesis of RA.

Recent studies have shown that decreased apoptosis is involved in the synovial hyperplasia in RA [36]. Thus, it has been shown that the resistance of RA synovial fibroblasts to

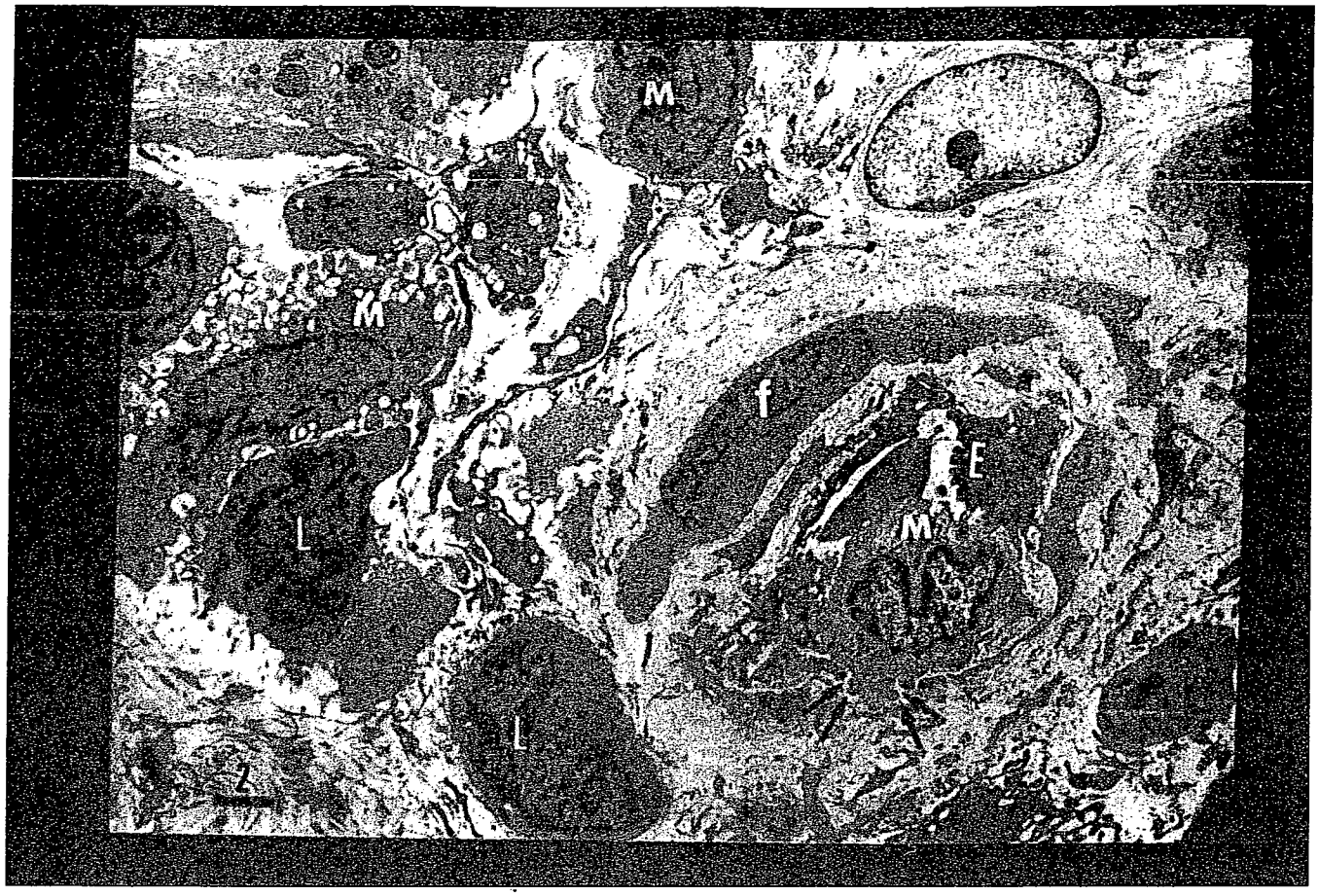


Fig. (1). Transendothelial migration of monocyte into RA synovium (arrows). M: monocyte-like cells, f: fibroblast-like cells, L: lymphocytes.

(Electron microscopy, the scale bar indicates 2 μm).

tumor necrosis factor (TNF-α)-induced apoptosis is mediated by the NF-κB-regulated expression of FLICE-inhibitory protein (FLIP) [37]. In addition, recent studies have also revealed that anti-TNF-α therapy induced apoptosis in synovial monocytes/macrophages [38]. These results indicate that in addition to increased cellular infiltration, reduced apoptosis plays a significant role in the increased cellularity in the synovium.

Of note, accelerated angiogenesis have been demonstrated in RA synovium [27], which might facilitate the recruitment of bone marrow-derived monocytes as well as lymphocytes into the synovium. In fact, the transendothelial migration of monocytes in RA synovium can be observed under by electron microscopy (Fig. 1). Of interest, the formation of synovium-like tissue was also observed at the site of non-union in an RA patient (Fig. 2). Since the formation of the synovium-like tissue took place in the place without original synovial tissues, it is suggested that all the constituents of the newly formed tissue might be recruited from the systemic circulation. These observations strongly support the hypothesis that the accelerated generation and continuous recruitment of bone marrow-derived CD14+ cells might play a critical role in the synovial hyperplasia in RA, thus accounting for the discrepancy between the marked synovial hyperplasia and the lack of evidence for accelerated proliferation of synoviocytes.

c. Abnormal Functions of Bone Marrow Derived CD14+ Cells in RA - Its Capacity to Stimulate RF-Producing B Cells

One of the characteristic serologic abnormalities in RA is the expression of rheumatoid factors (RF), autoantibodies directed against the Fc portion of IgG [39]. A number of *in vitro* experiments have revealed that RF are produced during the course of polyclonal B cell activation in normal human subjects [40, 41]. Thus, RF can be detected in the sera of normal individuals in the setting of infection [42]. In RA, however, RF production has been noted to be disproportionately high compared with total Ig production [39].

Staphylococcus aureus Cowan I (SAC) was found to enhance the frequencies of IgM-RF-producing precursor cells, but not those of IgM-producing cells, in anti-CD3-stimulated cultures [43]. Previous studies also showed that staphylococcal enterotoxin D (SED) facilitates the selective production of RF [44]. Taken together, these findings suggest that normal subjects and RA patients share a pool of B cells which secrete RF on activation by T helper cells in

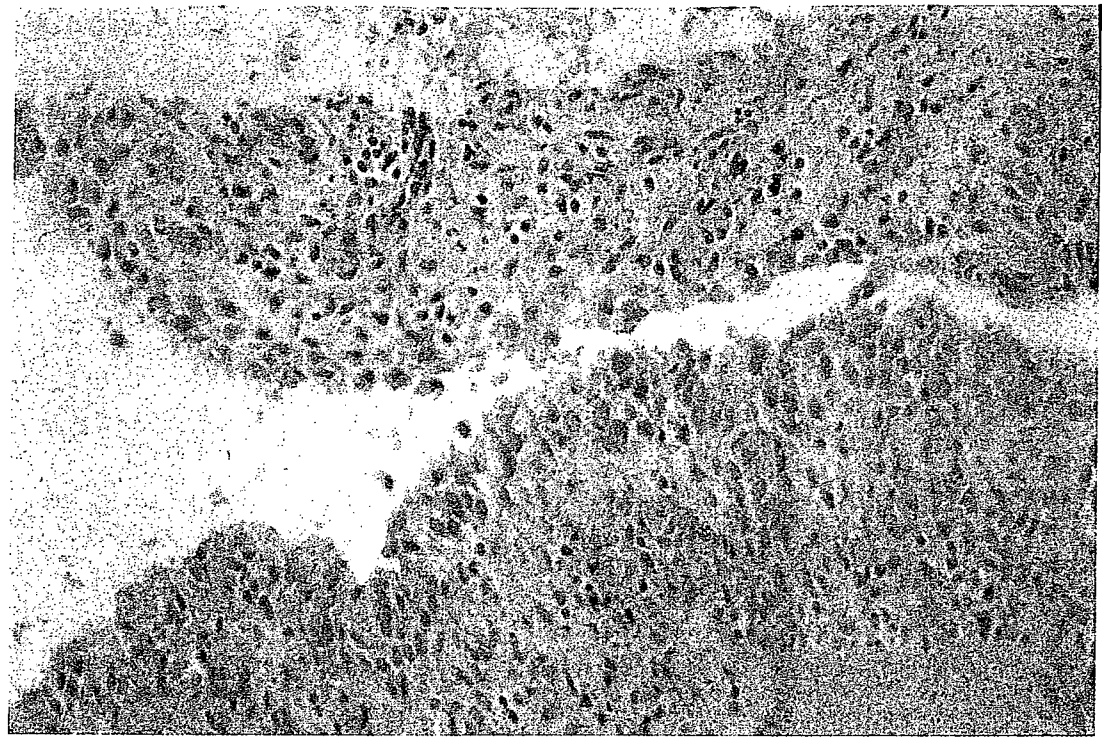


Fig. (2). Synovium-like tissue at non-union lesion in an RA patient. (Hematoxylin and eosin, original magnification x25).

the presence of SED or SAC [44]. These B cells are frequent and obviously anergic in normal individuals, but are ready to be activated by T helper cells alone in RA patients [44]. It is therefore possible that B cells in RA patients might be exposed to stimuli with SED-like effects or SAC-like effects.

Previous studies have also revealed enhanced RF production by B cells in bone marrow [45] as well as in synovial tissue of patients with RA [39]. It should be noted that direct interaction between B cells and monocytes has been shown to play a role in regulating the responses of B cells [46]. Since intimal macrophages as well as sublining macrophages have been considered to be recruited from bone marrow-derived monocytes [15, 31], it is possible that accessory cells of the monocyte lineage generated from RA bone marrow precursors might be able to stimulate RF production in a manner similar to SED or SAC, in the presence of signals delivered by activated T helper cells. In this regard, our study demonstrated that CD14+ cells induced by granulocyte-macrophage colony-stimulating factor (GM-CSF) stimulation of RA bone marrow CD14- precursors significantly shift the antibody repertoire, with selective production of IgM-RF, compared with CD14+ cells induced from osteoarthritis (OA) patient bone marrow CD14- precursors [47]. These data emphasize the role of monocyte-lineage CD14+ cells in the disproportionately high expression of RF in RA. It is thus likely that sublining macorophages might also facilitate the activation of RF-producing B cells in the RA synovium.

4. BONE MARROW AND TYPE B SYNOVIOCYTE IN RA

Type B synoviocytes (fibroblast-like synoviocytes) have the morphologic appearance of fibroblasts as well as the capacity to produce and secrete a variety of factors, including proteoglycans, cytokines, arachidonic acid metabolites, and matrix metalloproteinases (MMPs), that lead to the destruction of joints [48]. Unlike intimal macrophages, the precise origin of type B synoviocytes remains unclear, although they are through to arise from the sublining tissue or other support structures of a joint [48]. On the other hand, a number of studies have shown that peripheral blood dendritic cells accumulate in the synovium, where they undergo phenotypic and functional differentiation in situ [49, 50]. Previous studies have also shown that synovial dendritic cells gradually lose their distinct morphologic appearance and become indistinguishable from fibroblasts *in vitro* [51]. Moreover, Kyogoku *et al.* identified the presence of dendritic cell-like cells that strongly express major histocompatibility complex (MHC) class II antigens and interact with T lymphocytes, in the sublining layers of the RA synovium [52]. They also showed that the sublining dendritic cell-like cells proliferate and differentiate into type A as well as type B synoviocytes to replace the lining layers [52].

Since it was shown that dendritic cells are derived from bone marrow CD34+ cells, it was also likely that type B synoviocytes might also be induced from bone marrow progenitors. In this regard, we have disclosed that CD34+ cells from the bone marrow of RA patients differentiated into cells with fibroblast-like morphology, which expressed prolyl 4-hydroxylase, in the presence of stem cell factor (SCF), GM-CSF, and TNF-α, much more effectively than CD34+ cells from bone marrow of control subjects [53]. The generation of fibroblast-like cells was not at all observed in cultures with SCF, GM-CSF, and interleukin 4 (IL-4) with or without TNF-α. In the absence of TNF-α and IL-4, most cells induced from bone marrow CD34+ cells from RA patients as well as from control subjects expressed CD14 and

HLA-DR (Fig. 3) [53]. The expression of CD14 was higher and the expression of HLA-DR was lower in the cultured bone marrow cells from RA patients compared with those from control subjects, which is consistent with the previous study [22]. It is noteworthy that the addition of TNF-α dramatically reduced the expression of CD14 and HLA-DR in cultured bone marrow cells from RA patients compared with those from OA patients, corresponding with the generation of fibroblast-like cells (Fig. 3). These features are consistent with those of fibroblast-like synoviocytes in patients with RA, as described in previous studies [52, 54, 55].

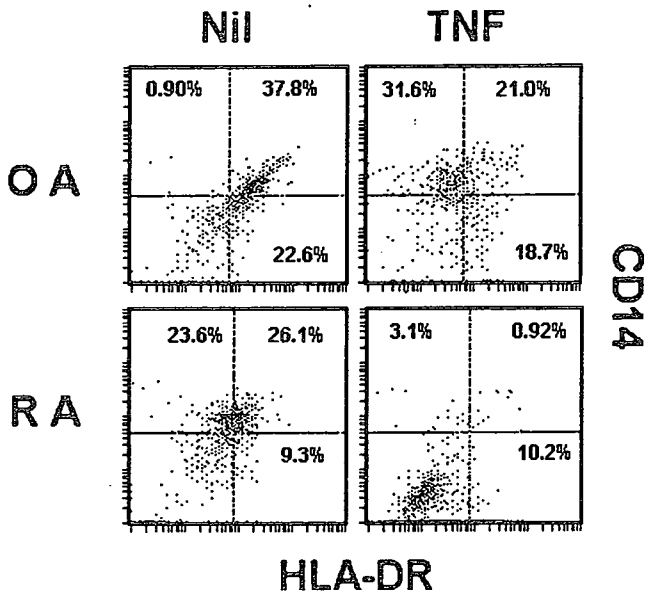


Fig. (3). Representative two-color flowcytometric analysis of the phenotypes of CD34+ cells cultured in the presence of SCF and GM-CSF with or without TNF-α for 4 weeks (modified from ref. 53).

On the other hand, it should be pointed out that generation of fibroblast-like cells from RA bone marrow CD34+ cells by stimulation with SCF, GM-CSF and TNF-α was correlated with MMP-1 levels in culture supernatants [53]. These results indicate that RA bone marrow CD34+ cells have abnormal capacities to respond to TNF-α and to differentiate into fibroblast-like cells producing MMP-1. It is therefore suggested that bone marrow CD34+ progenitor cells might generate type B synoviocytes and thus could play an important role in the pathogenesis of RA. In other words, the presence of abnormal precursors within the bone marrow progenitor cells might play an important role in the pathogenesis of RA by providing a repopulating reservoir of type B synoviocytes, as has been also suggested in other recent studies [56].

5. THE ROLE OF ANTIGEN-PRESENTATION IN BONE MARROW IN THE DEVELOPMENT OF SYNOVITIS: LESSONS FROM EXPERIMENTAL ARTHRITIS MODELS

A number of studies have demonstrated that RA is strongly associated with HLA-DR4 or DR1 [57], which are involved in the presentation of antigens to T cells. Although there has been no evidence for the interactions between antigen presenting cells and T cells in bone marrow in human RA, the role of such interactions in the development of arthritis in animal models has been appreciated. Thus, in the HvG-induced arthritis model, thymocytes of BALB/c mice, which had been injected intraperitoneally, were shown to accumulate in bone marrow and spleen, but not joint tissues, prior to the onset of arthritis in the C3H/He mice [9, 10]. However, when the thymocytes from BALB/c mice were administrated into the C3H/He mice after the development of arthritis, they were shown to accumulate in the synovial tissues as well as in the bone marrow [9, 10]. These results suggest that the antigen presentation involving HLA-DR4 or the shared epitope might take place in bone marrow and spleen and thus play a critical role in the development of synovitis in human RA. Moreover, it is also suggested that the antigen presentation in the bone marrow as well as in the inflamed synovium might be important for the persistence of synovitis in RA. Although the interactions between dendritic cells and T cells, possibly through MHC class II antigens, have been disclosed in the sublining layers of the RA synovium [52], further studies are required to explore the presence of such interactions in the RA bone marrow.

6. BONE MARROW AND ANGIOGENESIS IN RA

A number of studies indicated that neovascularization is crucial to the synovial hyperplasia in RA [4, 5]. Postnatal neovascularization has been attributed to so-called angiogenesis, a process characterized by the sprouting of new capillaries from preexisting blood vessels [58]. Thus, it has been shown that the expression of angiogenic factors, such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor in synovial lining cells and stromal cells, is increased in RA synovium and plays a pivotal role in the angiogenesis [59-61]. It is noteworthy that recent studies have demonstrated that endothelial progenitor cells of bone marrow origin play a significant role in the *de novo* formation of capillaries without preexisting blood vessels, so-called vasculogenesis [62-65]. Moreover, bone marrow-derived endothelial precursor cells have been shown to home to neovascularized hind limb ischemic sites in animal models [62].

We have recently shown that the generation of von Willebrand factor (vWF)-positive cells and CD31+/vWF+ cells from RA bone marrow-derived CD34+ cells was significantly higher than that from control subjects [66]. The generation of vWF+ cells from bone marrow CD34+ cells correlated significantly with the microvessel densities in the synovial tissues. Finally, RA bone marrow CD34+ cells expressed VEGFR2/KDR mRNA at higher levels than OA bone marrow CD34+ cells [66]. These results indicate that RA bone marrow CD34+ cells have enhanced capacities to differentiate into endothelial cells in relation to synovial vascularization. The data therefore suggest that bone marrow CD34+ cells might contribute to synovial neovascularization by supplying endothelial precursor cells and, thus, play an important role in the pathogenesis of RA.

Neovascularization of the synovium is not unique to RA. It has also been observed in OA synovium and has been shown to play an important role in the development of new cartilage and mineralization [67-69]. Of note, recent studies have revealed that levels of expression of the angiogenic

factors VEGF and platelet-derived endothelial cell growth factor are increased in RA as well as in OA, relative to normal subjects, whereas the presence of an activated synovial vasculature was high only in RA [67]. Moreover, the vascular activation by VEGF/KDR was significantly lower in OA than in RA patients, although the activation of the hypoxia inducing factor α (HIFα) pathway was comparable in OA and RA patients [67]. These observations suggest the presence of intrinsic abnormalities in synovial endothelial cells in RA patients. Of note, we have disclosed that the expression of VEGFR-2/KDR mRNA in RA bone marrow CD34+ cells was significantly higher than that in OA bone marrow CD34+ cells [66]. It is therefore likely that the differences in VEGF/KDR vascular activation in bone marrow CD34+ cells might result in differences in their capacity to generate vWF+ cells between RA and OA patients, since signaling through the KDR plays a crucial role in the generation of endothelial cells [59, 70].

Beyond its role in angiogenesis, it is also noteworthy that the abnormality in RA bone marrow CD34+ cells at the mRNA level has been demonstrated for the first time [66]. Further studies to delineate the whole characteristics of bone marrow CD34+ cells would be helpful for a complete understanding not only of the differences in synovial neovascularization in RA and OA, but also of the pathogenesis of RA.

7. BONE MARROW TRANSPLANTATION IN RA

Since 1996, autologous hematopoietic stem cell transplantation (HSCT) has been used to treat severe RA in limited case reports [71, 72]. However, a study with large numbers of patients has disclosed that recurrence of RA is frequent in patients who received autologous HSCT [73, 74]. Frequent recurrence after autologous HSCT for RA suggest that abnormalities in bone marrow stem cells persist after the treatment. It has been recently reported that bone marrow CD34+ progenitor cell reserve and function are defective in RA probably due, at least in part, to a TNF-α mediated effect [75, 76]. Accordingly, significant restoration of the disturbed hematopoiesis was obtained following anti-TNF-α treatment [75, 76]. It should be noted that blockade of TNF-α is not curative for RA, although it ameliorate disease activities of RA [77]. Thus, recurrence of RA is noted after discontinuation of blockade of TNF-α or even during anti-TNF-α therapy [77]. It is therefore likely that intrinsic abnormalities in bone marrow progenitor cells rather that TNF-α itself might cause such defects that lead to recurrence of RA. In this regard, bone marrow CD34+ cells have been shown to display aberrant responses to TNF-α and to differentiate into fibroblast-like cells producing MMP-1 [53]. Therefore, further studies to identify the nature of intrinsic abnormalities in bone marrow would be important for a complete understanding of the pathogenesis and etiology of RA as well as for the establishment of the curative treatment of RA.

8. FUTURE PROSPECTS

In the past decade, the importance of TNF-α in the pathogenesis of RA has come to be increasingly appreciated [78]. Thus, anti-TNF-α mAbs and soluble TNF receptor-Ig fusion proteins have been demonstrated to have beneficial effects in the treatment of RA [77]. We have revealed that CD34+ cells from bone marrow of RA patients have abnormal responsiveness to TNF-α, resulting in their accelerated differentiation into fibroblast-like cells producing MMP-1 [53]. However, the precise sequelae of abnormal responses of CD34+ cells from bone marrow of RA patients to TNF-α remain unclear. It is likely that mRNA abnormalities of bone marrow CD34+ cells might be involved in the abnormal responsiveness to TNF-α [66]. Further studies that explore in detail such abnormalities in CD34+ cells form bone marrow of RA patients would be helpful in gaining a complete understanding of the etiology and pathogenesis of RA.

REFERENCES

[1] Tak PP. Examination of the synovium and synovial fluid. In "Rheumatoid arthritis: Frontiers on pathogenesis and treatment". Firestein GS, Panayi GS, Wollheim RA, eds. Oxford University Press, New York. 2000, pp.55-68.

[2] Hirohata S. Involvement of B cells in the pathogenesis of rheumatoid arthritis. Igakunoayumi 2004; 209: 796-800.

[3] Firestein GS. Starving the synovium: angiogenesis and inflammation in rheumatoid arthritis. J Clin Invest 1999; 103: 3-4.

[4] Koch AE. Angiogenesis: implications for rheumatoid arthritis. Arthritis Rheum 1998; 41: 951-62.

[5] Hirohata S, Sakakibara J. Angioneogenesis as a possible elusive triggering factor in rheumatoid arthritis. Lancet 1999; 353: 1331.

[6] Nigrovic PA, Lee DM. Mast cells in inflammatory arthritis. Arthritis Res Ther 2005; 7: 1-11.

[7] Kiener HP, Hofbauer R, Tohidast-Akrad M, *et al.* Tumor necrosis factor alpha promotes the expression of stem cell factor in synovial fibroblasts and their capacity to induce mast cell chemotaxis. Arthritis Rheum 2000; 43: 164-74.

[8] Shiozawa S, Tokuhisa T. Contribution of synovial mesenchymal cells to the pathogenesis of rheumatoid arthritis. Semin Arthritis Rheum 1992; 21: 267-73.

[9] Tanabe S, Ochi T, Ono K. Chronic polyarthritis in inbred C3H/He mice after systemic administration of allogeneic thymocytes. J Rheumatol 1981; 8: 895-901.

[10] Ochi T. Disease subsets of rheumatoid arthritis and the role of bone marrow in the pathogenesis (in Japanese). Rymachi 2002; 42: 205.

[11] Hayashida K, Ochi T, Fujimoto M, *et al.* Bone marrow changes in adjuvant-induced and collagen-induced arthritis. Interleukin-1 and interleukin-6 activity and abnormal myelopoiesis. Arthritis Rheum 1992; 35: 241-5.

[12] Nakagawa S, Toritsuka Y, Wakitani S, *et al.* Bone marrow stromal cells contribute to synovial cell proliferation in rats with collagen induced arthritis. J Rheumatol 1996; 23: 2098-103.

[13] Kobayashi H, Ohshima S, Nishioka K, *et al.* Antigen induced arthritis (AIA) can be transferred by bone marrow transplantation: evidence that interleukin 6 is essential for induction of AIA. J Rheumatol 2002; 29: 1176-82.

[14] Burmester GR, Locher P, Koch B, *et al.* The tissue architecture of synovial membranes in inflammatory and non-inflammatory joint diseases. I. The localization of the major synovial cell populations as detected by monoclonal reagents directed towards Ia and monocyte-macrophage antigens. Rheumatol Int 1983; 3: 173-81.

[15] Hogg N, Palmer DG, Revell PA. Mononuclear phagocytes of normal and rheumatoid synovial membrane identified by monoclonal antibodies. Immunology 1985; 56: 673-81.

[16] Hedfors E, Klareskog L, Lindblad S, Forsum U, Lindahl G. Phenotypic characterization of cells within subcutaneous rheumatoid nodules. Arthritis Rheum 1983; 26: 1333-9.

[17] Palmer DG, Hogg N, Highton J, Hessian PA, Denholm I. Macrophage migration and maturation within rheumatoid nodules. Arthritis Rheum 1987; 30: 728-36.

[18] Katayama S, Chia D, Nasu H, Knutson DW. Increased Fc receptor activity in monocytes from patients with rheumatoid arthritis: a study of monocyte binding and catabolism of soluble aggregates of IgG *in vitro*. J Immunol 1981; 127: 643-7.

[19] MacKinnon SK, Starkebaum G. Monocyte Fc receptor function in rheumatoid arthritis. Enhanced cell-binding of IgG induced by rheumatoid factors. Arthritis Rheum 1987; 30: 498-506.

[20] Heidenreich S, Schmidt M, August C, Cullen P, Rademaekers A, Pauels HS. Regulation of human monocyte apoptosis by the CD14 molecule. J Immunol 1997; 159: 3178-88.

[21] Nockher WA, Scherberich JE. Expression and release of the monocyte lipopolysaccharide receptor antigen CD14 are suppressed by glucocorticoids *in vivo* and *in vitro*. J Immunol 1997; 158: 1345-52.

[22] Shinohara S, Hirohata S, Inoue T, Ito K. Phenotypic analysis of peripheral blood monocytes isolated from patients with rheumatoid arthritis. J Rheumatol 1992; 19: 211-5.

[23] Waytz PH, Douglas SD. Increased antibody-dependent cell-mediated cytotoxicity by monocytes from patients with rheumatoid arthritis. Arthritis Rheum 1979; 22: 490-4.

[24] Krutmann J, Kirnbauer R, Kock A, *et al.* Cross-linking Fc receptors on monocytes triggers IL-6 production. Role in anti-CD3-induced T cell activation. J Immunol 1990; 145: 1337-42.

[25] Lauener RP, Geha RS, Vercelli D. Engagement of the monocyte surface antigen CD14 induces lymphocyte function-associated antigen-1/intercellular adhesion molecule-1-dependent homotypic adhesion. J Immunol 1990; 145: 1390-4.

[26] Lafyatis R, Remmers EF, Roberts AB, Yocum DE, Sporn MB, Wilder RL. Anchorage-independent growth of synoviocytes from arthritic and normal joints. Stimulation by exogenous platelet-derived growth factor and inhibition by transforming growth factor-beta and retinoids. J Clin Invest 1989; 83: 1267-76.

[27] Harris ED Jr. Etiology and pathogenesis of rheumatoid arthritis. In: Kelley WN, Harris ED Jr, Ruddy S, Sledge CB, Editors. Textbook of Rheumatology. Forth ed. Philadelphia, WB Saunders, 1993.

[28] Hamilton JA. Rheumatoid arthritis: opposing actions of haemopoietic growth factors and slow-acting anti-rheumatic drugs. Lancet 1993; 342: 536-9.

[29] Buchan GS, Palmer DG, Gibbins BL. The response of human peripheral blood mononuclear phagocytes to rheumatoid arthritis. J Leukoc Biol 1985; 37: 221-30.

[30] Seitz M, Deimann W, Gram N, Hunstein W, Gemsa D. Characterization of blood mononuclear cells of rheumatoid arthritis patients. I. Depressed lymphocyte proliferation and enhanced prostanoid release from monocytes. Clin Immunol Immunopathol 1982; 25: 405-16.

[31] Hirohata S, Yanagida T, Itoh K, *et al.* Accelerated generation of CD14+ monocyte-lineage cells from the bone marrow of rheumatoid arthritis patients. Arthritis Rheum 1996; 39: 836-43.

[32] Kawanaka N, Yamamura M, Aita T, *et al.* CD14+,CD16+ blood monocytes and joint inflammation in rheumatoid arthritis. Arthritis Rheum 2002; 46: 2578-86.

[33] Oppenheimer-Marks N, Lipsky PE. Adhesion molecules in rheumatoid arthritis. Springer Semin Immunopathol 1998; 20: 95-114.

[34] Patel DD, Zachariah JP, Whichard LP. CXCR3 and CCR5 ligands in rheumatoid arthritis synovium. Clin Immunol 2001; 98: 39-45.

[35] Kanbe K, Takagishi K, Chen Q. Stimulation of matrix metalloprotease 3 release from human chondrocytes by the interaction of stromal cell-derived factor 1 and CXC chemokine receptor 4. Arthritis Rheum 2002; 46: 130-7.

[36] Pope RM. Apoptosis as a therapeutic tool in rheumatoid arthritis. Nat Rev Immunol 2002; 2: 527-35.

[37] Bai S, Liu H, Chen KH, *et al.* NF-kappaB-regulated expression of cellular FLIP protects rheumatoid arthritis synovial fibroblasts from tumor necrosis factor alpha-mediated apoptosis. Arthritis Rheum 2004; 50: 3844-55.

[38] Catrina AI, Trollmo C, af Klint E, *et al.* Evidence that anti-tumor necrosis factor therapy with both etanercept and infliximab induces apoptosis in macrophages, but not lymphocytes, in rheumatoid arthritis joints: extended report. Arthritis Rheum 2005; 52: 61-72.

[39] Wernick RM, Lipsky PE, Marban-Arcos E, Maliakkal JJ, Edelbaum D, Ziff M. IgG and IgM rheumatoid factor synthesis in rheumatoid synovial membrane cell cultures. Arthritis Rheum 1985; 28: 742-52.

[40] Koopman WJ, Schrohenloher RE. *In vitro* synthesis of IgM rheumatoid factor by lymphocytes from healthy adults. J Immunol 1980; 125: 934-9.

[41] Levinson AI, Tar L, Carafa C, Haidar M. *Staphylococcus aureus* Cowan I. Potent stimulus of immunoglobulin M rheumatoid factor production. J Clin Invest 1986; 78: 612-7.

[42] Williams RC, Kunkel HG: Rheumatoid factor, complement, and conglutinin aberrations in patients with subacute bacterial endocarditis. J Clin Invest 1962; 41: 666-75.

[43] Hirohata S, Inoue T, Miyamoto T. Frequency analysis of human peripheral blood B cells producing IgM-rheumatoid factor. Differential effects of stimulation with monoclonal antibodies to CD3 and *Staphylococcus aureus*. J Immunol 1990; 145: 1681-6.

[44] He X, Goronzy JJ, Weyand CM. The repertoire of rheumatoid factor-producing B cells in normal subjects and patients with rheumatoid arthritis. Arthritis Rheum 1993; 36: 1061-9.

[45] Panush RS, Bittner AK, Sullivan M, Katz P, Longley S. IgM rheumatoid factor elaboration by blood, bone marrow, and synovial mononuclear cells in patients with rheumatoid arthritis. Clin Immunol Immunopathol 1985; 34: 387-91.

[46] Boswell HS, Sharrow SO, Singer A. Role of accessory cells in B cell activation. I. Macrophage presentation of TNP-Ficoll: evidence for macrophage-B cell interaction. J Immunol 1980; 124: 989-96.

[47] Hirohata S, Yanagida T, Koda M, Koiwa M, Yoshino S, Ochi T. Selective induction of IgM rheumatoid factors by CD14+ monocyte-lineage cells generated from bone marrow of patients with rheumatoid arthritis. Arthritis Rheum 1995; 38: 384-8.

[48] Firestein GS. Invasive fibroblast-like synoviocytes in rheumatoid arthritis. Passive responders or transformed aggressors? Arthritis Rheum 1996; 39: 1781-90.

[49] Zvaifler NJ, Steinman RM, Kaplan G, Lau LL, Rivelis M. Identification of immunostimulatory dendritic cells in the synovial effusions of patients with rheumatoid arthritis. J Clin Invest 1985; 76: 789-800.

[50] Thomas R, Davis LS, Lipsky PE. Rheumatoid synovium is enriched in mature antigen-presenting dendritic cells. J Immunol 1994;152: 2613-23.

[51] Hendler PL, Lavoie PE, Werb Z, Chan J, Seaman WE. Human synovial dendritic cells. Direct observation of transition to fibroblasts. J Rheumatol 1985; 12: 660-4.

[52] Kyogoku M, Sawai T, Murakami K, Ito J. Histopathological characteristics of rheumatoid arthritis -as a clue to elucidate its pathogenesis-. Nippon Rinsho 1992; 50: 483-89.

[53] Hirohata S, Yanagida T, Nagai T, *et al.* Induction of fibroblast-like cells from CD34(+) progenitor cells of the bone marrow in rheumatoid arthritis. J Leukoc Biol 2001; 70: 413-21.

[54] Dechanet J, Briolay J, Rissoan MC, *et al.* IL-4 inhibits growth factor-stimulated rheumatoid synoviocyte proliferation by blocking the early phases of the cell cycle. J Immunol 1993; 151: 4908-17.

[55] Goto M, Sasano M, Yamanaka H, *et al.* Spontaneous production of an interleukin 1-like factor by cloned rheumatoid synovial cells in long-term culture. J Clin Invest 1987; 80: 786-96.

[56] Sen M, Lauterbach K, El-Gabalawy H, Firestein GS, Corr M, Carson DA. Expression and function of wingless and frizzled homologs in rheumatoid arthritis. Proc Natl Acad Sci USA 2000; 97: 2791-6.

[57] Nepom GT. The role of the DR4 shared epitope in selection and commitment of autoreactive T cells in rheumatoid arthritis. Rheum Dis Clin North Am 2001; 27: 305-15.

[58] Folkman J, Shing Y. Angiogenesis. J Biol Chem 1992; 267: 10931-4.

[59] Koch AE, Harlow LA, Haines GK, *et al.* Vascular endothelial growth factor. A cytokine modulating endothelial function in rheumatoid arthritis. J Immunol 1994; 152: 4149-56.

[60] Fava RA, Olsen NJ, Spencer-Green G, *et al.* Vascular permeability factor/endothelial growth factor (VPF/VEGF): accumulation and expression in human synovial fluids and rheumatoid synovial tissue. J Exp Med 1994; 180: 341-6.

[61] Fajardo LF, Kwan HH, Kowalski J, Prionas SD, Allison AC. Dual role of tumor necrosis factor-alpha in angiogenesis. Am J Pathol 1992; 140: 539-44.

[62] Asahara T, Murohara T, Sullivan A, *et al.* Isolation of putative progenitor endothelial cells for angiogenesis. Science 1997; 275: 964-7.

[63] Gehling UM, Ergun S, Schumacher U, *et al. In vitro* differentiation of endothelial cells from AC133-positive progenitor cells. Blood 2000; 95: 3106-12.

[64] Bhattacharya V, McSweeney PA, Shi Q, *et al.* Enhanced endothelialization and microvessel formation in polyester grafts seeded with CD34(+) bone marrow cells. Blood 2000; 95: 581-5.

[65] Lin Y, Weisdorf DJ, Solovey A, Hebbel RP. Origins of circulating endothelial cells and endothelial outgrowth from blood. J Clin Invest 2000; 105: 71-7.

[66] Hirohata S, Yanagida T, Nampei A, *et al.* Enhanced generation of endothelial cells from CD34+ cells of the bone marrow in rheumatoid arthritis: possible role in synovial neovascularization. Arthritis Rheum 2004; 50: 3888-96.

[67] Giatromanolaki A, Sivridis E, Maltezos E, *et al.* Upregulated hypoxia inducible factor-1alpha and -2alpha pathway in rheumatoid arthritis and osteoarthritis. Arthritis Res Ther 2003; 5: R193-201.

[68] Brown RA, Weiss JB, Tomlinson IW, Phillips P, Kumar S. Angiogenic factor from synovial fluid resembling that from tumours. Lancet 1980; 1: 682-5.

[69] Haywood L, McWilliams DF, Pearson CI, *et al.* Inflammation and angiogenesis in osteoarthritis. Arthritis Rheum 2003; 48: 2173-7.

[70] Giatromanolaki A, Sivridis E, Athanassou N, *et al.* The angiogenic pathway "vascular endothelial growth factor/flk-1(KDR)-receptor" in rheumatoid arthritis and osteoarthritis. J Pathol 2001; 194: 101-8.

[71] Joske DJ. Autologous bone-marrow transplantation for rheumatoid arthritis. Lancet 1997; 350: 337-8.

[72] Durez P, Toungouz M, Schandene L, Lambermont M, Goldman M Remission and immune reconstitution after T-cell-depleted stem-cell transplantation for rheumatoid arthritis. Lancet 1998; 352: 881.

[73] Snowden JA, Passweg J, Moore JJ, *et al.* Autologous hemopoietic stem cell transplantation in severe rheumatoid arthritis: a report from the EBMT and ABMTR. J Rheumatol 2004; 31: 482-8.

[74] Bingham SJ, Moore JJ. Stem cell transplantation for autoimmune disorders. Rheumatoid arthritis. Best Pract Res Clin Haematol 2004; 17: 263-76.

[75] Papadaki HA, Kritikos HD, Gemetzi C, *et al.* Bone marrow progenitor cell reserve and function and stromal cell function are defective in rheumatoid arthritis: evidence for a tumor necrosis factor alpha-mediated effect. Blood 2002; 99: 1610-19.

[76] Porta C, Caporali R, Epis O, *et al.* Impaired bone marrow hematopoietic progenitor cell function in rheumatoid arthritis patients candidated to autologous hematopoietic stem cell transplantation. Bone Marrow Transplant 2004; 33: 721-8.

[77] Feldmann M, Maini RN. Anti-TNF alpha therapy of rheumatoid arthritis: what have we learned? Annu Rev Immunol 2001; 19: 163-96.

[78] Feldmann M, Brennan FM, Maini RN. Role of cytokines in rheumatoid arthritis. Annu Rev Immunol 1996; 14: 397-440.

Received: February 7, 2005 Revised: May 5, 2005 Accepted: June 8, 2005

Research article

Enhanced expression of mRNA for nuclear factor κB1 (p50) in CD34+ cells of the bone marrow in rheumatoid arthritis

Shunsei Hirohata[1], Yasushi Miura[2], Tetsuya Tomita[3], Hideki Yoshikawa[3], Takahiro Ochi[4] and Nicholas Chiorazzi[5]

[1]Department of Internal Medicine, Teikyo University School of Medicine, Tokyo 173-8605, Japan
[2]Department of Rheumatology, Kobe University FHS School of Medicine, Kobe 654-0142, Japan
[3]Department of Orthopedic Surgery, Osaka University Medical School, Osaka 565-0871, Japan
[4]Sagamihara National Hospital, Kanagawa 228-8522, Japan
[5]Experimental Immunology and Rheumatology, North Shore-LIJ Research Institute, Manhasset, NY 11030, USA

Corresponding author: Shunsei Hirohata, shunsei@med.teikyo-u.ac.jp

Received: 1 Oct 2005 Revisions requested: 8 Nov 2005 Revisions received: 27 Jan 2005 Accepted: 9 Feb 2006 Published: 6 Mar 2006

Arthritis Research & Therapy 2006, **8**:R54 (doi:10.1186/ar1915)
This article is online at: http://arthritis-research.com/content/8/2/R54

Abstract

Bone marrow CD34+ cells from rheumatoid arthritis (RA) patients have abnormal capacities to respond to tumor necrosis factor (TNF)-α and to differentiate into fibroblast-like cells producing matrix metalloproteinase (MMP)-1. We explored the expression of mRNA for nuclear factor (NF)κB in RA bone marrow CD34+ cells to delineate the mechanism for their abnormal responses to TNF-α. CD34+ cells were purified from bone marrow samples obtained from 49 RA patients and 31 osteoarthritis (OA) patients during joint operations via aspiration from the iliac crest. The mRNAs for NFκB1 (p50), NFκB2 (p52) and RelA (p65) were examined by quantitative RT-PCR. The expression of NFκB1 mRNA in bone marrow CD34+ cells was significantly higher in RA than in OA, whereas there was no significant difference in the expression of mRNA for NFκB2 and RelA. The expression of NFκB1 mRNA was not correlated with serum C-reactive protein or with the treatment with methotrexate or oral steroid. Silencing of NFκB1 by small interfering RNA abrogated the capacity of RA bone marrow CD34+ cells to differentiate into fibroblast-like cells and to produce MMP-1 and vascular endothelial growth factor upon stimulation with stem cell factor, granulocyte-macrophage colony stimulating factor and TNF-α without influencing their viability and capacity to produce β2-microglobulin. These results indicate that the enhanced expression of NFκB1 mRNA in bone marrow CD34+ cells plays a pivotal role in their abnormal responses to TNF-α and, thus, in the pathogenesis of RA.

Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by hyperplasia of synovial lining cells, consisting of macrophage-like type A synoviocytes and fibroblast-like type B synoviocytes [1]. It has been appreciated that type A synoviocytes, which are also called intimal macrophages, are derived from monocyte precursors in the bone marrow [1]. On the other hand, type B synoviocytes, which are also called fibroblast-like synoviocytes, have the morphological appearance of fibroblasts as well as the capacity to produce and secrete a variety of factors, including proteoglycans, cytokines, arachidonic acid metabolites, and matrix metalloproteinases (MMPs), that lead to the destruction of joints [1]. Apart from type A synoviocytes, the origin of type B synoviocytes has been unclear [1]. Of note, we have recently demonstrated that bone marrow CD34+ cells from RA patients have abnormal capacities to respond to tumor necrosis factor (TNF)-α and to differentiate into fibroblast-like cells producing MMP-1, suggesting that bone marrow CD34+ progenitor cells might generate type B synoviocytes and thus could play an important role in the pathogenesis of RA [2].

TNF-α is one of the first triggers to be found effective for the activation of nuclear factor (NF)κB in RA synovium [3]. This

β_2MG = β_2-microglobulin; ELISA = enzyme-kinked immunosorbent assay; GM-CSF = granulocyte-macrophage colony stimulating factor; HSCT = hematopoietic stem cell transplantation; MFI = mean fluorescence intensity; MMP = matrix metalloproteinase; MTX = methotrexate; NFκB = nuclear factor kappa B; OA = osteoarthritis; PCR = polymerase chain reaction; PE = phychoerythrin; RA = rheumatoid arthritis; SCF = stem cell factor; siRNA = small interfering RNA; TNF-α = tumor necrosis factor-alpha; VEGF = vascular endothelial growth factor.

mechanism of activation was followed by up-regulation of several inflammatory genes usually found in active RA. Accordingly, a number of studies have shown that TNF-α blockade has beneficial effects in the treatment of RA [4]. Moreover, inhibition of NFκB by the antioxidant N-acetylcysteine significantly reduced TNF-α- and NFκB-dependent gene expression and synovial proliferation [3]. We thus hypothesized that abnormal responses of RA bone marrow CD34+ cells to TNF-α might result from abnormal expression of NFκB genes. The current studies were undertaken, therefore, to explore the expression of mRNA for various components of NFκB in bone marrow CD34+ cells in RA.

Materials and methods

Patients and samples

Bone marrow samples were obtained from 49 patients with RA (8 males and 41 females: mean age, 58.6 years; age range, 35 to 78 years) who satisfied the American College of Rheumatology 1987 revised criteria for RA [5] and gave informed consent in accordance with the World Medical Association Declaration of Helsinki Ethical Principles for Medical Research Involving Human Subjects. The samples were taken during joint operations via aspiration from the iliac crest under anesthesia. As a control, bone marrow samples were similarly obtained from 31 patients with osteoarthritis (OA; 3 males and 28 females; mean age, 71.2 years; age range, 49 to 81 years) who gave informed consent. Most patients with RA and OA were taking non-steroidal anti-inflammatory drugs. Of the 45 patients with RA, 23 were treated with low dose methotrexate (MTX) and 33 were taking oral steroids when bone marrow samples were obtained. No OA patients were taking MTX or oral steroid.

Preparation of bone marrow CD34+ cells

Mononuclear cells were isolated by centrifugation of heparinized bone marrow aspirates over sodium diatrizoate-Ficoll gradients. CD34+ cells were purified from the mononuclear cells by positive selection with magnetic beads (CD34 progenitor cell selection system; Dynal, Oslo, Norway). The cells thus prepared were >95% CD34+ cells and <0.5% CD19+ B cells, as previously described [2]. In addition, CD34+ cells derived from bone marrow aspirates from the iliac crests of healthy individuals (purity >95%) were purchased from BioWhittaker (Walkersville, MD, USA).

RNA isolation and real-time quantitative PCR

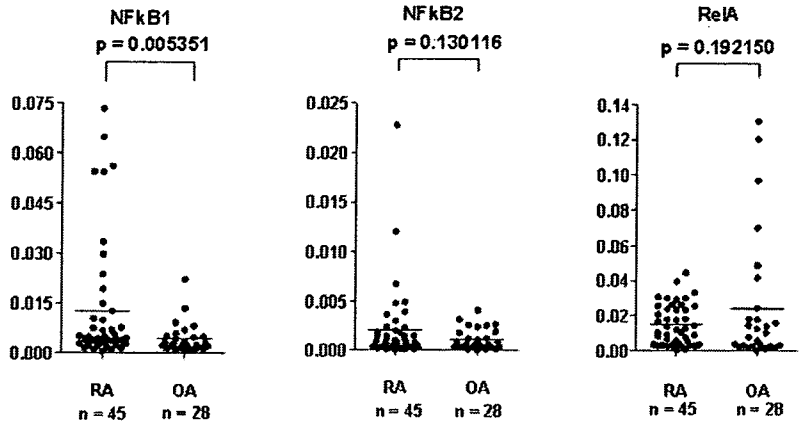
Total RNA was isolated from purified bone marrow CD34+ cells using the Trizol reagent (Life Technologies, Grand Island, NY, USA) according to the manufacturer's instructions. cDNA samples were prepared from 1 μg of total RNA using the SuperScript reverse transcriptase preamplification system (Life Technologies) with oligo (dT) primer and subjected to PCR. Real-time quantitative PCR was performed using the LightCycler rapid thermal cycler system (Roche Diagnostics, Lewes, UK) with primer sets for NFκB1, NFκB2, RelA or β-actin and Light Cycler-Fast Start DNA master SYBR Green I (Roche Diagnostics). The primers were designed using Oligo Primer Analysis Software version 5.0 (Takara Bio Inc., Ohtsu, Japan). The detail of primer sequences is shown in Table 1. Quantitative analysis was performed using LightCycler Software v.3.5. PCR reaction conditions composed of denaturing at 95°C for 10 minutes for 1 cycle, followed by 40 cycles of denaturing (10 seconds at 95°C), annealing (10 seconds at 60°C (NFκB2, RelA) or 62°C (NFκB1, β-actin)), and extension (5 seconds (NFκB1), 6 seconds (NFκB2, RelA), or 10 seconds (β-actin) at 72°C).

Table 1

Primer sequences used in real-time quantitative PCR for analysis of mRNA for various nuclear factor κB components

Gene product (GenBank accession no.)	Primer sequences	Nucleotides
NFκB1 [M58603]	Forward: 5'-TCC ACA AGG CAG CAA ATA GA-3'	3,125–3,144
	Reverse: 5'-GGG GCA TTT TGT TGA GAG TT-3'	3,244–3,263
NFκB2 [NM_002502]	Forward: 5'-TTC TGA AGG CTG GTG CTG AC-3'	2,220–2,239
	Reverse: 5'-AGT GAG GTC AAG AGG CGT GT-3'	2,352–2,371
RelA [NM_021925]	Forward: 5'-GAA GAA GAG TCC TTT CAG CG-3'	1,011–1,030
	Reverse: 5'-GGG AGG ACG TAA AGG GAT AG-3'	1,116–1,135
β-actin [X00351]	Forward: 5'-GCA AAG ACC TGT ACG CCA AC-3'	910–929
	Reverse: 5'-CTA GAA GCA TTT GCG GTG GA-3'	1,150–1,169

Figure 1

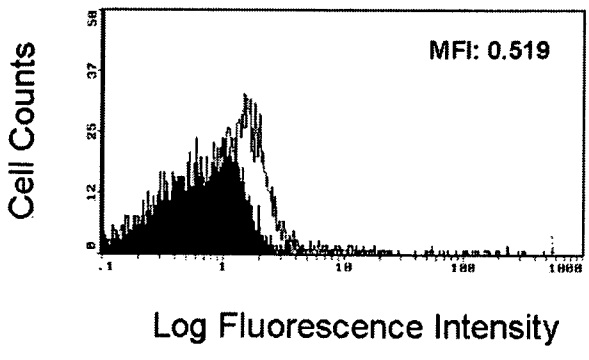


The expression of mRNAs for nuclear factor (NF)κB1 (p50), NFκB2 (p52) and RelA (p65) in bone marrow CD34+ cells. Total RNA was isolated from purified bone marrow CD34+ cells. The expression of mRNAs for NFκB1, NFκB2, RelA and β-actin was evaluated by real-time quantitative PCR. The data are expressed as the ratio of the mRNA copy numbers to those of β-actin. Horizontal lines indicate the mean values. Statistical significance was evaluated by Welch's *t* test. OA, osteoarthritis; RA, rheumatoid arthritis.

Immunofluorescence staining and analysis

Purified bone marrow CD34+ cells (obtained from three RA patients and three OA patients) were treated with IntraPrep™ Permeabilization Reagent (Immunotech, Marseille, France), followed by staining with phycoerythrin (PE)-conjugated anti-NFκB p50 (E-10; a mouse IgG1 monoclonal antibody against amino acids 120 to 239 mapping at the amino terminus of human NFκB p50; Santa Cruz Biotech, Santa Cruz, CA, USA) or PE-conjugated normal mouse IgG1 (Santa Cruz). The cells were analyzed using an EPICS XL flow cytometer (Coulter, Hialeah, FL, USA) equipped with an argon-ion laser at 488 nm. A combination of low-angle and 90° light scatter measurements (forward scatter versus side scatter) was used to generate a bit map gating to identify bone marrow cells using Cyto-Trol™ Control Cells (Coulter) and Immuno-Trol™ Cells (Coulter) as standards. Specific mean fluorescence intensity (MFI) for NFκB1 (p50) was calculated by subtracting the non-specific MFI of staining with the isotype-matched control mouse IgG1.

Figure 2



Expression of nuclear factor (NF)κB1 (p50) protein in bone marrow CD34+ cells. Purified bone marrow CD34+ cells from a rheumatoid arthritis patient were permeabilized and then stained with phychoerythrin-conjugated anti-NFκB p50 monoclonal antibody or phychoerythrin-conjugated normal mouse IgG1, followed by analysis with flow cytometry. The level of NFκB1 protein was expressed by mean fluorescence intensity as described in Materials and methods.

Culture medium and cytokines

RPMI 1640 medium (Life Technologies) supplemented with L-glutamine (0.3 mg/ml) and 10% fetal bovine serum (Life Technologies) was used for all cultures. Recombinant human stem cell factor (SCF), granulocyte-macrophage colony stimulating factor (GM-CSF), and TNF-α were purchased from Pepro Tech EC (London, UK).

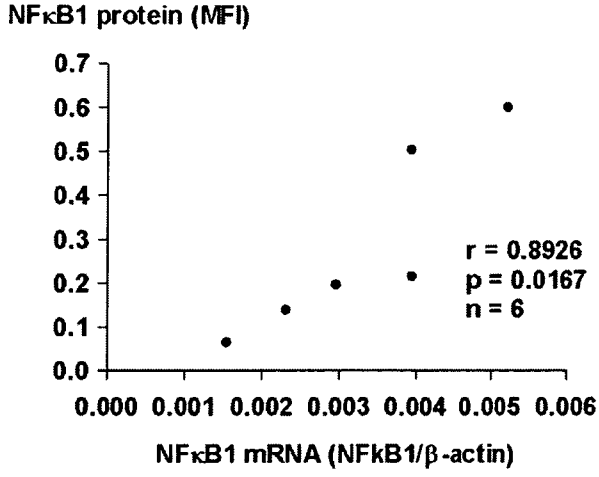
Silencing of NFκB1 in bone marrow CD34+ cells by small interfering RNA

SMARTpool® small interfering RNA (siRNA) for NFκB1 (p50) gene and nonsense scrambled control siRNA were purchased from Dharmacon (Lafayette, CO, USA). Chemical transfection of siRNAs into bone marrow CD34+ cells was performed using siPORT™ Amine Transfection Agent (Ambion, Austin, TX, USA) according to the manufacturer's directions. Briefly, purified bone marrow CD34+ cells were cultured in a 24-well microtiter plate (N0. 3524; Costar, Cambridge, MA, USA) at 2 × 10^5 cells per well in 0.2 ml culture medium in the presence of SCF (10 ng/ml) and GM-CSF (1 ng/ml). After 24 hours of incubation, chemical transfection of siRNAs was performed, and incubated for 4 hours.

Cell cultures and measurement of MMP-1 and vascular endothelial growth factor

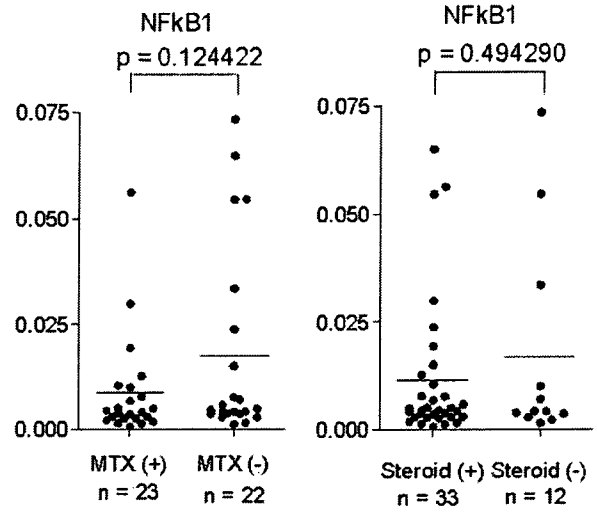
After transfection of siRNAs, the cells were cultured with SCF (10 ng/ml) and GM-CSF (1 ng/ml) in 1.0 ml culture medium for

Figure 3



Comparison of the expression of nuclear factor (NF)κB1 (p50) protein with that of NFκB1 mRNA in bone marrow CD34+ cells. Purified bone marrow CD34+ cells were permeabilized and then stained with phychoerythrin-conjugated anti-NFκB p50 monoclonal antibody or phychoerythrin-conjugated normal mouse IgG1, followed by analysis with flow cytometry. The NFκB1 protein levels as expressed by mean fluorescence intensity were compared with NFκB1 mRNA levels (expressed as the ratio of the mRNA copy numbers to those of β-actin) in bone marrow CD34+ cells from six patients (three rheumatoid arthritis patients and three osteoarthritis patients). Statistical significance was evaluated by linear regression test.

Figure 4



The relevance of treatment with the expression of mRNAs for nuclear factor (NF)κB1 mRNA in bone marrow CD34+ cells. Total RNA was isolated from purified bone marrow CD34+ cells from 45 rheumatoid arthritis patients. The expression of mRNAs for NFκB1 and β-actin was evaluated by real-time quantitative PCR. The data are expressed as the ratio of the mRNA copy numbers to those of β-actin. Effect of treatment with methotrexate (MTX) or oral steroids (Steroid) was evaluated by Welch's *t* test. Horizontal lines indicate the mean values.

48 hours and then harvested for RNA extraction. Alternatively, the cells were cultured in a 24-well microtiter plate at 2×10^5 cells per well in 1.0 ml culture medium for 4 weeks in the presence of SCF (10 ng/ml), GM-CSF (1 ng/ml) and TNF-α (10 ng/ml) without medium change, as previously described [2]. The differentiation of fibroblast-like cells was observed under the phase-contrast light microscopy. The concentrations of MMP-1 and vascular endothelial growth factor (VEGF) in the culture supernatants were measured using the Biotrak human MMP-1 ELISA system (Amarsham Pharmacia Biotech, Buckinghamshire, UK) and human VEGF immunoassay kit (BioSource International, Camarillo, CA, USA), respectively. The concentrations of β_2-microglobulin (β_2MG) were determined by an ELISA as previously described [6].

Statistics

Comparison between RA and OA patients and between RA patients with MTX or steroid and those without MTX or steroid was carried out using Welch's *t* test. Significance of the effects of siRNA transfection on the generation of fibroblast-like cells and on the production of MMP-1 and VEGF was evaluated by Wilcoxon's signed rank test. Correlation between serum C-reactive protein and NFκB1 mRNA in bone marrow CD34+ cells and that between NFκB1 mRNA and protein were evaluated using a liner regression test. Correlation between NFκB1 mRNA in bone marrow CD34+ cells and the generation of fibroblast-like cells was analyzed using a Spearman's rank correlation test.

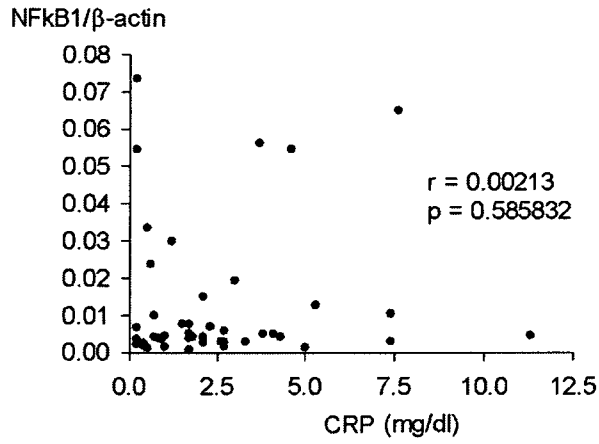
Results

Expression of mRNAs for various components of NFκB in bone narrow CD34+ cells

The expression of mRNA for NFκB1 (p50), NFκB2 (p52), and RelA (p65) in bone marrow CD34+ cells is shown as the ratio of the copy numbers to those of β-actin mRNA in Figure 1. The expression of NFκB1 mRNA was significantly higher in RA bone marrow CD34+ cells than in OA bone marrow CD34+ cells ($p = 0.005351$), whereas there were no significant differences in the expression of NFκB2 mRNA ($p = 0.130116$). Although the expression of RelA mRNA appeared to be lower in RA bone marrow CD34+ cells than in OA bone marrow CD34+ cells, it did not reach statistical significance ($p = 0.192150$). These results indicate that the expression of mRNA for components of NFκB1 is exclusively enhanced in bone marrow CD34+ cells from patients with RA.

Next, experiments were carried out to examine whether the elevation of NFκB1 mRNA expression parallels the elevation of NFκB1 protein expression in bone marrow CD34+ cells. The protein expression of NFκB1 was evaluated by staining of permeabilized bone marrow CD34+ cells from three RA patients and three OA patients with anti-NFκB p50 monoclonal antibody, followed by analysis with flow cytometry. As can be seen

Figure 5



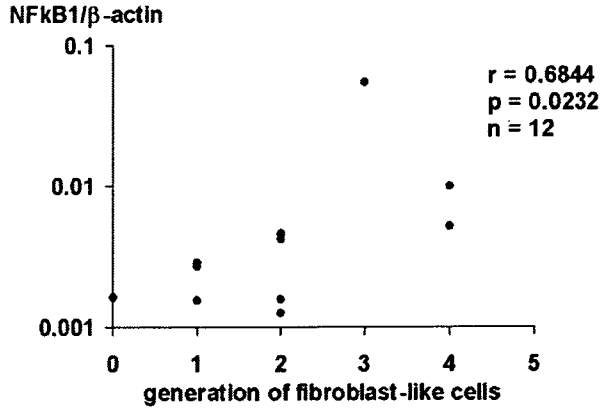
The correlation of the expression of mRNAs for nuclear factor (NF)κB1 mRNA in bone marrow CD34+ cells with serum C-reactive protein (CRP). Total RNA was isolated from purified bone marrow CD34+ cells from 45 rheumatoid arthritis patients. The expression of mRNAs for NFκB1 and β-actin was evaluated by real-time quantitative PCR. The data are expressed as the ratio of the mRNA copy numbers to those of β-actin. Statistical significance was evaluated by linear regression test.

in Figure 2, bone marrow CD34+ cells express NFκB1 (p50) protein, the quantity of which can be expressed as MFI. Moreover, there is significant correlation between MFI for NFκB1 and NFκB1 mRNA in the six bone marrow CD34+ cells (Figure 3). The results indicate that the elevation of NFκB1 mRNA leads to the increase in NFκB1 protein expression.

Relevance of expression of NFκB1 mRNA in bone marrow CD34+ cells from RA patients to treatment and clinical parameters

Of note, 22 and 33 of the 45 RA patients were treated with MTX and oral steroids, respectively, whereas no OA patients were taking either MTX or oral steroids. It is therefore possible that MTX and oral steroids might have affected the expression of NFκB1 mRNA in bone marrow CD34+ cells. As shown in Figure 4, however, there were no significant differences in the expression of NFκB1 mRNA in bone marrow CD34+ cells between RA patients taking MTX or oral steroids and those who were not, although the expression of NFκB1 mRNA appeared to be lower in RA patients taking MTX or oral steroids. It is unlikely, therefore, that the medication the RA patients were taking would have resulted in the upregulation of NFκB1 mRNA expression in bone marrow CD34+ cells. It should be also noted that the expression of NFκB1 mRNA in bone marrow CD34+ cells was not significantly correlated with serum C-reactive protein (CRP) levels in RA patients (Figure 5). The data thus indicate that the upregulation of NFκB1 mRNA in bone marrow CD34+ cells is independent of the activity of the systemic inflammation, as reflected by serum CRP.

Figure 6



Comparison of the expression of nuclear factor (NF)κB1 (p50) mRNA in bone marrow CD34+ cells with their capacity to differentiate into fibroblast-like cells. The expression of NFκB1 mRNA in bone marrow CD34+ cells from 12 rheumatoid arthritis patients was evaluated by real-time quantitative PCR prior to the culture. The bone marrow CD34+ cells were incubated in culture medium with stem cell factor (10 ng/ml), granulocyte-macrophage colony stimulating factor (1 ng/ml) and tumor necrosis factor-α (10 ng/ml) for 4 weeks with no medium changes. Morphological changes were evaluated under light microscopy. The percentages of fibroblast-like cells were calculated from two view fields at ×20 magnifications. The degree of the generation of fibroblast-like cells were scored as follows: 0, fibroblast-like cells <5%; 1, fibroblast-like cells 5% to 25%; 2, fibroblast-like cells 25% to 50%; 3, fibroblast-like cells >50%; 4, formation of a pile or a cluster in at least one view field. Statistical significance was evaluated by Spearman's rank correlation test.

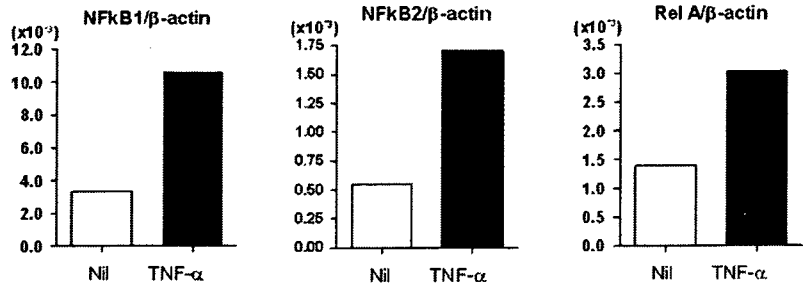
Relevance of the expression of NFκB1 mRNA to the generation of fibroblast-like cells

There was a variation in the expression of NFκB1 mRNA among the RA patients. We next examined the relationship of the initial levels of NFκB1 mRNA in RA bone marrow CD34+ cells with their capacity to differentiate into fibroblast-like cells. As shown in Figure 6, there was a significant correlation between the NFκB1 mRNA expression and the generation of fibroblast-like cells from bone marrow CD34+ cells upon stimulation with SCF, GM-CSF and TNF-α for 4 weeks in 12 RA patients. The data indicate that the enhanced expression of NFκB1 mRNA is important for the enhanced generation of fibroblast-like cells.

Effect of TNF-α on the expression of mRNAs for various components of NFκB in bone marrow CD34+ cells

Previous studies have demonstrated that TNF-α plays a critical role in the pathogenesis of RA [4]. It is possible, therefore, that the up-regulation of NFκB1 mRNA in bone marrow CD34+ cells might be secondary to the increased levels of TNF-α in the born marrow; experiments were carried out to test this possibility. Highly purified bone marrow CD34+ cells from healthy individuals were cultured in the presence of TNF-α (10 ng/ml) for 24 hours, after which the expression of mRNA for various components of NFκB was examined. As shown in Fig-

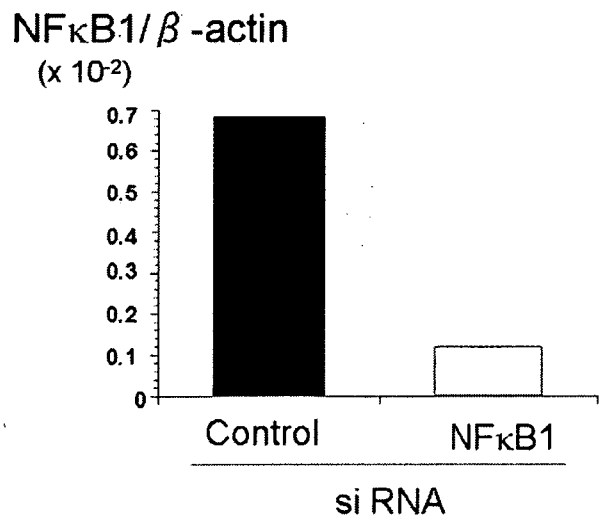
Figure 7



Effect of tumor necrosis factor (TNF)-α on the expression of mRNAs for nuclear factor (NF)κB1 (p50), NFκB2 (p52) and RelA (p65) in bone marrow CD34+ cells. Bone marrow CD34+ cells from healthy individuals were incubated in culture medium with or without TNF-α (10 ng/ml) for 24 hours. After the incubation, total RNA was isolated for evaluation of the expression of mRNAs for NFκB1, NFκB2, RelA and β-actin by real-time quantitative PCR. The data are expressed as the ratio of the mRNA copy numbers to those of β-actin. The data are representative of two different experiments.

ure 7, treatment of bone marrow CD34+ cells with TNF-α upregulated not only the expression of NFκB1 (p50) mRNA, but that of NFκB2 (p52) mRNA and RelA (p65) mRNA. Since only the expression of NFκB1 mRNA, but not that of NFκB2 mRNA and RelA mRNA, was significantly upregulated in RA bone marrow CD34+ cells, the increased expression of NFκB1 mRNA in RA bone marrow CD34+ cells might not be accounted for simply by the increased levels of TNF-α in the bone marrow.

Figure 8

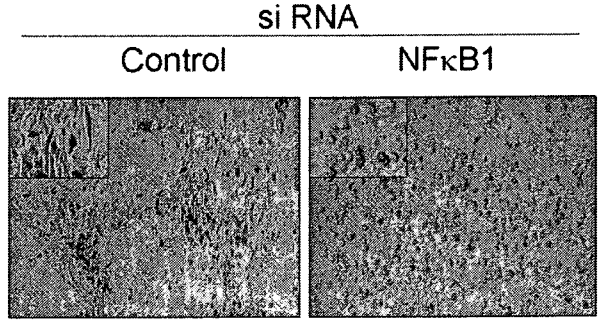


Silencing of nuclear factor (NF)κB1 mRNA in bone marrow CD34+ cells by small interfering RNA (siRNA) for NFκB1. Purified bone marrow CD34+ cells were transfected with siRNA for NFκB1 or a scrambled sequence control siRNA after a 24 hours incubation in culture medium with stem cell factor (10 ng/ml) and granulocyte-macrophage colony stimulating factor (1 ng/ml). After the transfection, the cells were further incubated for 48 hours in culture medium with stem cell factor and granulocyte-macrophage colony stimulating factor, and total RNA was isolated for evaluation of the expression of NFκB1 mRNA and β-actin mRNA by real-time quantitative PCR. The data are expressed as the ratio of the mRNA copy numbers to those of β-actin.

Effect of silencing mRNA for NFκB1 on differentiation of RA bone marrow CD34+ cells into fibroblast-like cells upon stimulation with SCF, GM-SCF and TNF-α

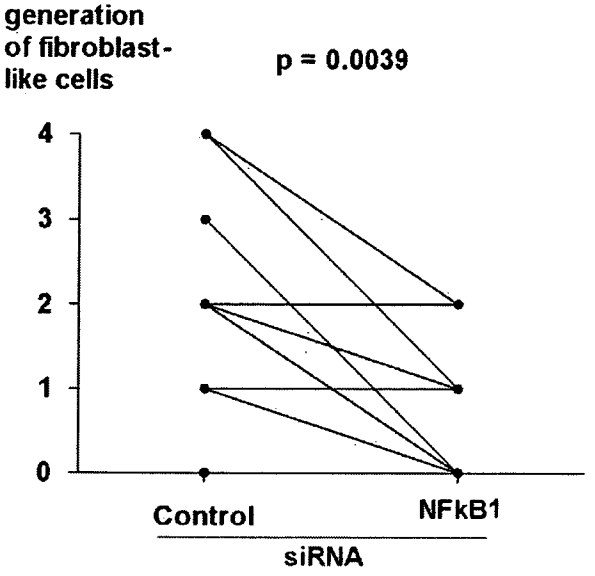
We next examined whether silencing of NFκB1 (p50) mRNA in RA bone marrow CD34+ cells might correct their abnormal responses to TNF-α. As shown in Figure 8, treatment of bone marrow CD34+ cells with siRNA for NFκB1 reduced the expression of NFκB1 mRNA by approximately 80%. More importantly, reduction of NFκB1 mRNA markedly suppressed the generation of fibroblast-like cells from RA bone marrow CD34+ cells upon stimulation with SCF, GM-CSF and TNF-α (Figures 9 and 10). Accordingly, silencing of NFκB1 by siRNA

Figure 9



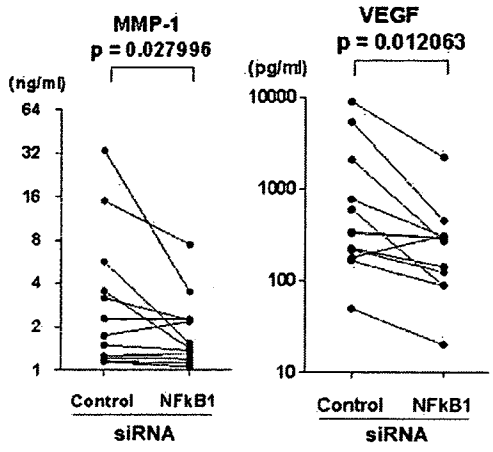
Inhibition of the generation of fibroblast-like cells by silencing nuclear factor (NF)κB1 mRNA in bone marrow CD34+ cells from patients with rheumatoid arthritis. Purified bone marrow CD34+ cells were transfected with small interfering RNA (siRNA) for NFκB1 or a scrambled sequence control, after which the cells were incubated in culture medium with stem cell factor (10 ng/ml), granulocyte-macrophage colony stimulating factor (1 ng/ml) and tumor necrosis factor-α (10 ng/ml) for 4 weeks with no medium changes. Morphological changes were observed under light microscopy (original magnification, ×20; inset, ×50 magnification). The data are representative of 12 different experiments.

Figure 10



Inhibition of the generation of fibroblast-like cells by silencing nuclear factor (NF)κB1 mRNA in bone marrow CD34+ cells from patients with rheumatoid arthritis. Purified bone marrow CD34+ cells were transfected with small interfering RNA (siRNA) for NFκB1 or a scrambled sequence control siRNA, after which the cells were incubated in culture medium with stem cell factor (10 ng/ml), granulocyte-macrophage colony stimulating factor (1 ng/ml) and tumor necrosis factor-α (10 ng/ml) for 4 weeks with no medium changes. Morphological changes were observed under light microscopy. The percentages of fibroblast-like cells were calculated from two view fields at ×20 magnifications. The degree of the generation of fibroblast-like cells were scored as follows: 0, fibroblast-like cells <5%; 1, fibroblast-like cells 5% to 25%; 2, fibroblast-like cells 25% to 50%; 3, fibroblast-like cells >50%; 4, formation of a pile or a cluster in at least one view field. Statistical significance was evaluated by Wilcoxon's signed rank test.

Figure 11



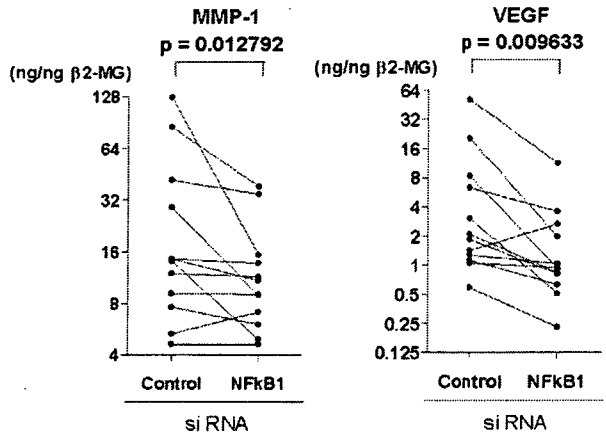
Suppression of the production of matrix metalloproteinase (MMP)-1 and vascular endothelial growth factor (VEGF) by silencing nuclear factor (NF)κB1 mRNA in bone marrow CD34+ cells from patients with rheumatoid arthritis. Purified bone marrow CD34+ cells from 12 patients with rheumatoid arthritis were transfected with small interfering RNA (siRNA) for NFκB1 or a scrambled sequence control siRNA, after which the cells were further incubated in culture medium with stem cell factor (10 ng/ml), granulocyte-macrophage colony stimulating factor (1 ng/ml) and tumor necrosis factor-α (10 ng/ml) for 4 weeks with no medium changes. After the incubation, the supernatants were harvested and assayed for MMP-1 and VEGF by ELISA. Statistical significance was evaluated by Wilcoxon's signed rank test.

significantly decreased the levels of MMP-1 and VEGF in culture supernatants of RA bone marrow CD34+ cells (Figure 11). Since bone marrow CD34+ cells proliferate in response to SCF, GM-CSF and TNF-α, it was possible that differences in MMP-1 and VEGF might be a result of alteration in cell proliferation by NFκB1 siRNA. Previous studies disclosed that β_2MG is produced by a number of cell types, including lymphocytes, myeloid cells, and tumor cells [7-9]. The production of β_2MG generally correlates with cell proliferation [6-9]. In fact, the levels of β_2MG in the culture supernatants paralleled the viable cell counts of bone marrow CD34+ cells stimulated with SCF, GM-CSF and TNF-α. Of note, silencing of NFκB1 also significantly decreased the ratios of MMP-1 and VEGF to β_2MG (MMP-1/β_2MG and VEGF/β_2MG) in culture supernatants of RA bone marrow CD34+ cells (Figure 12). Consistently, whereas siRNA for NFκB1 inhibited the differentiation of RA bone marrow CD34+ cells stimulated with SCF, GM-CSF and TNF-α into fibroblast-like cells (Figure 13), it significantly influenced neither the viable cell numbers nor the levels of β_2MG in the culture supernatants (Figure 14). These results confirm that the enhanced expression of NFκB1 mRNA in RA bone marrow CD34+ cells led to their abnormal capacity to differentiate into fibroblast-like cells producing MMP-1 upon stimulation with SCF, GM-CSF and TNF-α without affecting cell viability or proliferation. The data suggest, therefore, that the enhanced expression of NFκB1 mRNA in bone marrow hematopoietic stem cells might play a pivotal role in the pathogenesis of RA.

Discussion

The importance of TNF-α in the pathogenesis of RA has been well appreciated. Thus, anti-TNF-α antibodies and soluble TNF receptors have been demonstrated to have beneficial effects in the treatment of RA [4]. On the other hand, increasing attention has been paid to the role of bone marrow abnormalities in the pathogenesis of RA. In this regard, we demonstrated that RA bone marrow CD34+ cells have abnormal capacities to respond to TNF-α and to differentiate into fibroblast-like cells producing MMP-1 [2]. It should be noted that NFκB plays an important role in signal transduction and expression of a variety of genes, including MMP-1, under the influence of TNF-α [3]. The results in the current study have demonstrated that the expression of mRNA for NFκB1 is increased in RA bone marrow CD34+ cells. Of note, the expression of NFκB1 mRNA was significantly correlated with that of NFκB1 protein. Moreover, the initial levels of NFκB1 mRNA in RA bone marrow CD34+ cells were correlated with

Figure 12



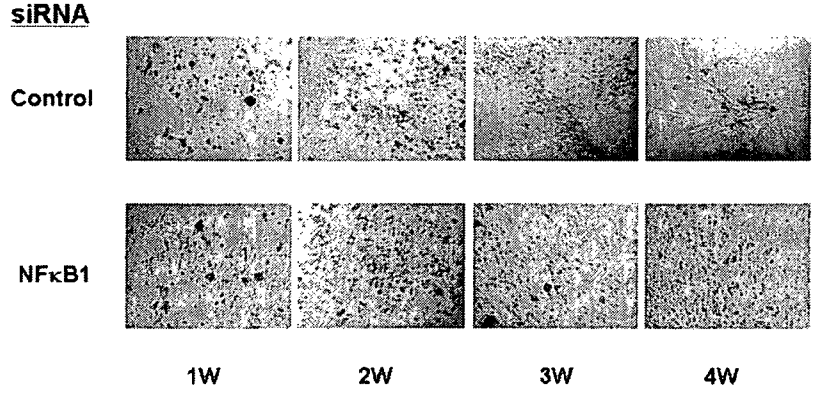
Suppression of the production of matrix metalloproteinase (MMP)-1 and vascular endothelial growth factor (VEGF) by silencing nuclear factor (NF)κB1 mRNA in bone marrow CD34+ cells from patients with rheumatoid arthritis. Purified bone marrow CD34+ cells from 12 patients with rheumatoid arthritis were transfected with small interfering RNA (siRNA) for NFκB1 or a scrambled sequence control siRNA, after which the cells were further incubated in culture medium with stem cell factor (10 ng/ml), granulocyte-macrophage colony stimulating factor (1 ng/ml) and tumor necrosis factor-α (10 ng/ml) for 4 weeks with no medium changes. After the incubation, the supernatants were harvested and assayed for MMP-1, VEGF and β_2-microglobulin (β_2MG) by ELISA. Statistical significance was evaluated by Wilcoxon's signed rank test.

their capacity to differentiate into fibroblast-like cells upon stimulation with TNF-α. The data suggest that the increased expression of NFκB1 mRNA might lead to constitutive overproduction of NFκB p50 molecules and thus result in abnormal responses to TNF-α of RA bone marrow CD34+ cells. Of note, bee venom and its major component melittin have been shown to display anti-arthritic effects through inactivation of NFκB [10]. Since bee venom and melittin delay and reduce nuclear translocation of the p50 subunit of NFκB but not p65 (RelA) [10], the importance of NFκB p50 rather than p65 in the pathogenesis of inflammatory arthritides has been underscored.

In the present study, significant numbers of RA patients were treated with MTX and oral steroids. However, there were no significant differences in the expression of NFκB1 mRNA in bone marrow CD34+ cells between RA patients receiving MTX or oral steroids and those who were not, although the expression of NFκB1 mRNA appeared to be lower in RA patients receiving these drugs. It is suggested, therefore, that administration of MTX and oral steroids might have made the differences in the expression of NFκB1 mRNA in bone marrow CD34+ cells between RA and OA less marked. On the other hand, the expression of NFκB1 mRNA in bone marrow CD34+ cells was not correlated with serum CRP levels in RA patients. The upregulation of NFκB1 mRNA in bone marrow CD34+ cells might not, therefore, be secondary to systemic inflammation, but may be a primary abnormality intrinsic to RA.

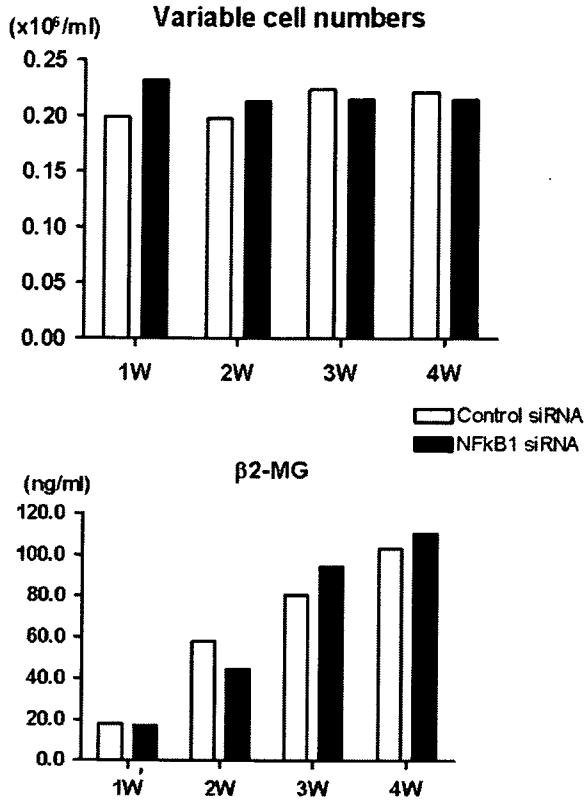
In the present study, the expression of mRNA for RelA (p65) appeared to be decreased in RA bone marrow CD34+ cells compared with that in OA bone marrow CD34+ cells, although this decrease did not reach statistical significance. Of note, a previous study demonstrated that embryonic fibroblasts from RelA-deficient mice are defective in the TNF-α mediated induction of mRNAs for IκBα [11]. Moreover, in RelA deficient fibroblasts, IκBβ protein was absent, presumably due

Figure 13



Time-kinetic effect of silencing nuclear factor (NF)κB1 mRNA in bone marrow CD34+ cells from patients with rheumatoid arthritis on the generation of fibroblast-like cells. Purified bone marrow CD34+ cells from patients with rheumatoid arthritis were transfected with small interfering RNA (siRNA) for NFκB1 or scrambled sequence control siRNA, after which the cells were further incubated in culture medium with stem cell factor (10 ng/ml), granulocyte-macrophage colony stimulating factor (1 ng/ml) and tumor necrosis factor-α (10 ng/ml) up to 4 weeks with no medium changes. After various periods of incubation (W, weeks), the morphological changes of the cells were observed under light microscopy. The data are representative of three different experiments.

Figure 14



Time-kinetic effect of silencing nuclear factor (NF)κB1 mRNA in bone marrow CD34+ cells from patients with rheumatoid arthritis on the viable cell counts and the production of β_2-microblobulin (β_2MG). Purified bone marrow CD34+ cells from patients with rheumatoid arthritis were transfected with small interfering RNA (siRNA) for NFκB1 or scrambled sequence control siRNA, after which the cells were further incubated in culture medium with stem cell factor (10 ng/ml), granulocyte-macrophage colony stimulating factor (1 ng/ml) and tumor necrosis factor-α (10 ng/ml) up to 4 weeks with no medium changes. After various periods of incubation (W, weeks), the cells were counted and the quantities of β_2MG in the culture supernatants were determined by ELISA. This is the same experment as shown in Figure 13. Data are representative of three different experiments.

to the decreased stability of IκBβ mRNA [11]. Since IκB plays an important role in inhibition of translocation of NFκB into the nucleus, the decrease in RelA mRNA might result in enhanced activation of NFκB related genes through upregulation of the translocation of NFκB. It is suggested, therefore, that the decreased expression of RelA mRNA in RA bone marrow CD34+ cells might also contribute to abnormal response to TNF-α.

It is possible that the upregulation of NFκB1 mRNA in bone marrow CD34+ cells might be secondary to the increased levels of TNF-α in the bone marrow. In fact, the treatment of bone marrow CD34+ cells from healthy individuals with TNF-α resulted in the increased expression of NFκB1 mRNA. However, TNF-α also enhanced the expression of mRNAs for NFκB2 and RelA in bone marrow CD34+ cells from healthy individuals. Of note, the expression of RelA mRNA appeared to be rather decreased in RA bone marrow CD34+ cells as mentioned above. Taken together, these data strongly suggest that the enhanced expression of NFκB1 mRNA might not be due simply to the increased levels of TNF-α in the bone marrow. Further studies to explore the mechanism of abnormal expression of NFκB1 mRNA in bone marrow CD34+ cells would be important for delineation of the pathogenesis of RA.

The role of the enhanced expression of NFκB1 mRNA in RA bone marrow CD34+ cells in their abnormal responses to TNF-α was further confirmed by the experiments of selective silencing of NFκB1 mRNA. Reduction of NFκB1 mRNA in RA bone marrow CD34+ cells by transfection of siRNA for NFκB1 markedly suppressed the generation of fibroblast-like cells as well as the production of MMP-1 and VEGF under the influence of TNF-α without affecting the viability or the capacity to produce β_2MG. These results indicate that upregulation of NFκB1 mRNA expression leads to the enhanced responses of RA bone marrow CD34+ cells to TNF-α. Thus, the enhanced NFκB1 mRNA expression might be a critical defect in RA bone marrow CD34+ cells.

Autologous hematopoietic stem cell transplantation (HSCT) has been used to treat severe RA in limited case reports [12,13]. However, a study with large numbers of patients has disclosed that recurrence of RA is frequent in patients who received autologous HSCT [14,15]. Frequent recurrence after autologous HSCT for RA suggests that abnormalities in bone marrow stem cells might persist after the treatment [16,17]. It is possible that the enhanced expression of NFκB1 mRNA might be closely related with such abnormalities in bone marrow stem cells, although further studies are required to confirm this point. It would also be important to explore whether there might be another transcription factor that could be inhibited without suppressing the differentiation of bone marrow CD34+ cells into fibroblast-like cells in order to confirm the importance of NFκB1 mRNA expression in the pathogenesis of RA.

Conclusion

The present study has revealed the enhanced expression of NFκB1 mRNA in RA bone marrow CD34+ cells as possible intrinsic abnormalities in bone marrow, resulting in abnormal responses to TNF-α. Further studies to delineate the mechanisms for the abnormal NFκB1 mRNA expression would be important for a complete understanding of the pathogenesis and etiology of RA.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

SH designed the study, and participated in experimental procedures, collection, analysis, and interpretation of data, and manuscript preparation. YM and NC contributed to analysis and interpretation of data. TT, HY, and TO contributed to collection and analysis of data. All authors read and approved the final text before submission of the manuscript.

Acknowledgements

This work is supported by a grant-in-aidfromthe Health Science Research grant from theMinistry of Health and Welfare of Japan and grants from Aventis Pharma Co., Ltd, Tokyo, and from Eisai Co., Ltd, Tokyo, Japan. The authors wish to thank Tamiko Yanagida, PhD, for her technical assistance.

References

1. Tak PP: **Examination of the synovium and synovial fluid.** In *Rheumatoid arthritis: Frontiers on pathogenesis and treatment* Edited by: Firestein GS, Panayi GS, Wollheim RA. New York: Oxford University Press; 2000:55-68.
2. Hirohata S, Yanagida T, Nagai T, Sawada T, Nakamura H, Yoshino S, Tomita T, Ochi T: **Induction of fibroblast-like cells from CD34(+) progenitor cells of the bone marrow in rheumatoid arthritis.** *J Leukoc Biol* 2001, **70**:413-421.
3. Müller-Ladner U, Gay RE, Gay S: **Role of nuclear factor kappaB in synovial inflammation.** *Curr Rheumatol Rep* 2002, **4**:201-207.
4. Feldmann M, Maini RN: **Anti-TNF alpha therapy of rheumatoid arthritis: what have we learned?** *Annu Rev Immunol* 2001, **19**:163-196.
5. Arnett FC, Edworthy SM, Bloch DA, McShane DJ, Fries JF, Cooper NS, Healey LA, Kaplan SR, Liang MH, Luthra HS, *et al.*: **The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis.** *Arthritis Rheum* 1988, **31**:315-324.
6. Kawai M, Hirohata S: **Cerebrospinal fluid beta(2)-microglobulin in neuro-Behcet's syndrome.** *J Neurol Sci* 2000, **179**:132-139.
7. Evrin PE, Nilsson K: **Beta 2-microglobulin production in vitro by human hematopoietic, mesenchymal, and epithelial cells.** *J Immunol* 1974, **112**:137-144.
8. Child JA, Kushwaha MR: **Serum beta 2-microglobulin in lymphoproliferative and myeloproliferative diseases.** *Hematol Oncol* 1984, **2**:391-401.
9. Bataille R, Grenier J, Commes T: **In vitro production of beta 2 microglobulin by human myeloma cells.** *Cancer Invest* 1988, **6**:271-277.
10. Park HJ, Lee SH, Son DJ, Oh KW, Kim KH, Song HS, Kim GJ, Oh GT, Yoon do Y, Hong JT: **Antiarthritic effect of bee venom: inhibition of inflammation mediator generation by suppression of NF-κB through interaction with the p50 subunit.** *Arthritis Rheum* 2004, **50**:3504-3515.
11. Beg AA, Sha WC, Bronson RT, Ghosh S, Baltimore D: **Embryonic lethality and liver degeneration in mice lacking the RelA component of NF-κB.** *Nature* 1995, **376**:167-170.
12. Joske DJ: **Autologous bone-marrow transplantation for rheumatoid arthritis.** *Lancet* 1997, **350**:337-338.
13. Durez P, Toungouz M, Schandene L, Lambermont M, Goldman M: **Remission and immune reconstitution after T-cell-depleted stem-cell transplantation for rheumatoid arthritis.** *Lancet* 1998, **352**:881.
14. Snowden JA, Passweg J, Moore JJ, Milliken S, Cannell P, Van Laar J, Verburg R, Szer J, Taylor K, Loske D, *et al.*: **Autologous hemopoietic stem cell transplantation in severe rheumatoid arthritis: a report from the EBMT and ABMTR.** *J Rheumatol* 2004, **31**:482-488.
15. Bingham SJ, Moore JJ: **Stem cell transplantation for autoimmune disorders. Rheumatoid arthritis.** *Best Pract Res Clin Haematol* 2004, **17**:263-276.
16. Papadaki HA, Kritikos HD, Gemetzi C, Koutala H, Marsh JCW, Boumpas DT, Eliopoulos GD: **Bone marrow progenitor cell reserve and function and stromal cell function are defective in rheumatoid arthritis: evidence for a tumor necrosis factor alpha-mediated effect.** *Blood* 2002, **99**:1610-1619.
17. Porta C, Caporali R, Epis O, Ramaioli I, Invernizzi R, Rovati B, Comolli G, Danova M, Montecucco C: **Impaired bone marrow hematopoietic progenitor cell function in rheumatoid arthritis patients candidated to autologous hematopoietic stem cell transplantation.** *Bone Marrow Transplant* 2004, **33**:721-728.