Introduction
Rheumatoid arthritis (RA) is a chronic disease characterized by infiltration of inflammatory cells into the joints, leading to synovial inflammation and progressive destruction of cartilage. Although the basic mechanisms of RA are widely accepted, the pathogenesis of the disease is not fully understood.

To identify genes that are useful in the diagnosis and treatment of RA, we compared the patterns of gene expression in cartilage from RA patients with that from osteoarthritic (OA) patients and normal joints using DNA microarray analysis. It was found that monocyte chemoattractant protein-4/CCL13 (MCP-4) was highly expressed in RA cartilage (relative intensity in normal, OA, and RA was 24.9, 55.1, and 408, respectively). MCP-4 is considered to be a major chemoattractant for monocytes, eosinophils and lymphocytes in chronic inflammatory diseases such as asthma and atopic dermatitis. We examined the hypothesis that the expression of MCP-4 is higher among patients with RA than controls and MCP-4 is associated with the pathogenesis of RA.

Materials and Methods
Human articular cartilage specimen were obtained from OA and RA patients (n = 5 in each group) who were undergoing total knee replacement at Tokyo Women’s Medical University, Tokyo, Japan. As controls, normal human articular cartilage was obtained from three patients without history of joint disease who were undergoing joint surgery after femoral neck fracture. OA was diagnosed by physical examination along with radiographic findings, and RA patients had disease that met the 1987 criteria of the American College of Rheumatology (formerly, the American Rheumatism Association). Serum samples were obtained from RA patients (n = 23), OA patients (n = 8) and patients without history of joint disease whose joints were considered to be normal (n = 10). Synovial fluid was obtained from OA and RA patients (n = 24 in each groups). All samples were obtained with informed consent from these patients.

Real-time PCR:
Cartilage was dissected, and total RNA was directly isolated from cartilage with Isogen (Nippon Gene, Tokyo, Japan) and an RNasey Mini Kit (Qiagen, Chatsworth, CA). Complementary DNA was synthesized from 1µg of total RNA, using Superscript II and random hexamers (Invitrogen, San Diego, CA), and real-time PCR was performed using the ABI Prism 7900HT sequence detection system and SYBR Green PCR Master Mix in accordance with the protocol suggested by the manufacturer (PE Applied Biosystems, Foster City, CA). PCR conditions were as follows: 10 minutes at 95°C, followed by 40 cycles each consisting of 15 seconds at 95°C and 1 minute at 59°C. Values were calculated based on standard curves generated for each gene. Normalization of samples was determined by dividing the number of copies of MCP-4/CCL13 by the number of copies of GAPDH.

Enzyme-linked immunosorbent assay (ELISA):
Serum and synovial fluid samples were stored at -20°C until use. MCP-4/CCL13 concentrations in these samples were evaluated by ELISA with the Quantikine human MCP-4 immunoassay according to the instruction of the manufacturer (R&D Systems, Minneapolis, MN).

MTT assay:
Cultured fibroblast-like synoviocytes (FLS) were treated with various concentrations of recombinant MCP-4 protein for 48 hours and cell proliferation was evaluated by measuring the number of viable cells using the MTT assay. As a positive control, FLS were incubated with IL-1β (5ng/ml). Experiments were performed six times and the average was obtained.

Results
The gene expression of MCP-4 was significantly higher in cartilage from RA patients than in that from OA patients (p<0.009) or in normal cartilage (p=0.025). There was no significant difference between OA patients and controls. We next analyzed the concentrations of MCP-4 protein in serum obtained from the three groups, and in synovial fluid obtained from RA patients and OA patients by using ELISA. The concentrations of MCP-4 protein in serum from RA patients (mean 94.7 pg/ml) were significantly higher than that from OA patients (mean 49.2 pg/ml, p=0.0051) and controls (mean 32.6 pg/ml, p=0.0001). The concentration of MCP-4 protein in synovial fluid from RA patients (mean 247.2 pg/ml) was also significantly higher than OA patients (mean 29.6 pg/ml, p=0.000019). Moreover, we studied the biological effects of MCP-4 on the proliferation of FLS by using the MTT assay. MCP-4 significantly enhanced the proliferation of FLS in a dose-dependent manner (Figure 1).

Discussion
Monocyte chemoattractant proteins constitute a subfamily of CC chemokines that share structural and functional features, and they are considered to be major chemoattractants for monocytes, eosinophils, basophils and lymphocytes in chronic inflammatory diseases. Among them, MCP-4/CCL13 is the most recently identified chemokine from human heart cDNA library. There are some reports showing that MCP-4/CCL13 is up-regulated at sites of inflammation and MCP-4/CCL13 may play a major role in the pathophysiologic mechanisms of allergic disorders such as asthma and atopic dermatitis which are considered to be Th2 dominant diseases. To our knowledge, there has been no report showing the relationship between MCP-4/CCL13 and RA, therefore this is the first report that describe the expression of MCP-4/CCL13 in RA patients.

Conclusions
MCP-4 is highly expressed in the RA joints not only at the mRNA level but also at the protein level. Our biological study of MCP-4 shows the role of MCP-4 in the pathogenesis of RA in which MCP-4 secreted from synoviocytes activate the proliferation of rheumatoid synovial cells leading to the joint destruction in RA.

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Figure 1. Enhanced synovial cell proliferation by MCP-4/CCL13. The data are represented as mean ± standard deviation. *P < 0.01