Objective: Rheumatoid arthritis (RA) is a systemic inflammatory disease that mainly affects multiple joints. The inflammatory tissues of RA patients are metabolically active, leading to increased oxygen consumption, while the oxygen supply is deficient because the intra-articular pressure exceeds the capillary perfusion pressure in the chronically inflamed joints during movement. Therefore, hypoxia has been implicated in the development of RA. Indeed, oxygen tension in the synovial fluid or tissues is lower in patients with RA than in those with other pathological conditions. Hypoxia-inducible factor 1α (HIF-1α), a hypoxia-inducible transcriptional factor, is strongly expressed in the RA synovial tissue in response to the hypoxic condition, whereas none or little of it is expressed in the tissue from healthy individuals. These findings suggest stress proteins under the control of HIF-1α are involved in the pathogenesis of RA. We focused on heme oxygenase-1 (HO-1), the expression of which is regulated by HIF-1α. We have demonstrated last year on this convention that HO-1 is abundantly expressed in the joint tissues from patients with RA. Although those from OA also expressed HO-1 proteins, the expression level was much less than RA. In contrast, very little HO-1 was detected in non-inflammatory synovial tissues. HO-1 plays cytoprotective roles in various pathological conditions including inflammatory disorders. HO-1 is an inducible isofrom of HO, which degrades heme to biliverdin, Fe2+, and carbon monoxide (CO). Biliverdin is subsequently converted to bilirubin by biliverdin reductase. Fe2+ stimulates synthesis of ferritin. Both bilirubin and ferritin act as antioxidants, whereas CO suppresses apoptosis and the synthesis of inflammatory mediators such as proinflammatory cytokines, nitric oxide, and prostaglandins. Thus, multiple biochemical actions of the heme degradation products and their metabolic derivatives contribute to the cytoprotective functions of HO. Here we examined the relationship between HO and RA synovial cell lines. We also assessed which product of HO-1 is involved in the suppression of cytokine synthesis in these cell lines.

Methods: Patients. Synovial tissue samples were obtained during arthroscopy, arthroplasty or synovectomy from 14 patients with RA (all female, mean age; 57.1±16.6 years). This study was approved by the ethics committee of our institute, and all subjects in the study gave their written informed consent.

Isolation of RA synovial cells and establishment of the cell lines. Synovial tissues were minced aseptically, and then digested enzymatically with 1.0 mg/ml collagenase in Dulbecco’s modified Eagle’s medium (DMEM) for 2 hours at 37°C. Single-cell suspensions harvested from the tissues were filtered through a nylon mesh (0.22 μm). The cells were then plated in culture dishes containing DMEM supplemented with 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 10% fetal calf serum and allowed to adhere to the dishes at 37°C. Adherent synovial cells were removed by trypsin-EDTA. The collected synovial cells were used at the second to sixth passages for subsequent experiments.

Transfection with siRNA duplexes in vitro. RA synovial cell lines (4×10^4 cells/well) were seeded into 24-well plates for 24 hours prior to transfection with siRNA oligonucleotide. After the cells had been transfected for 24 hours with 0.2 μg/well siRNA by using the RNAiFect transfection reagent, they were exposed to 0.1 μg/ml auranofin or 10 ng/ml LPS for 6 hours or 24 hours. At the end of the cultures, the cells lyse, mRNAs, and culture supernatants were collected.

Immunoblotting analysis. Cultured cells were treated for 30 minutes on ice with lysis buffer. The supernatants were recovered by centrifugation at 15000 rpm for 30 minutes. Aliquots of the proteins from each sample were boiled and resolved electrophoretically on a 4%-20% gradient of polyacrylamide gel and transferred onto a polyvinylidene difluoride membrane. The membrane was incubated with optimally diluted anti-HO-1 murine mAb, anti-COX-2 rabbit polyclonal IgG for 1 hour at room temperature, and subsequently for 1 hour incubation with HRP-conjugated anti-mouse secondary antibody or rabbit anti-goat IgG HRP conjugate. The signals were developed by using the ECL chemiluminescent detection system. RNA extraction and real-time PCR. Total RNA was isolated from cells by using TRIzol. cDNA was generated from the RNA with reverse transcriptase, SuperScript II. Panels of primers of human HO-1, TNFα, and GAPDH mRNAs were purchased from PE Applied Biosystems. Real-time PCR was performed by using a TaqMan Universal Master Mix, and the data were analyzed by the ABI prism 7700 sequence detection system.

ELISA. Concentrations of IL-6 and IL-8 in the culture supernatants were determined by ELISA using optimal pairs of capture and detecting biotinylated antibodies. Statistical analysis. Comparisons of 2 independent data sets were made by using Mann-Whitney U and chi-square (χ²) tests. P values of less than 0.05 were considered statistically significant.

Results

Effects of HO-1-specific siRNA on proinflammatory cytokine synthesis by synovial cells. We examined the effects of HO-1-specific siRNA on proinflammatory cytokine synthesis by the synovial cell lines. Real-time PCR analysis revealed that the HO-1 mRNA level in the cell lines in the presence of the siRNA. Under this condition, the spontaneous mRNA expression level of TNFα was significantly increased. Furthermore, the cell lines transfected with the siRNA secreted larger amounts of IL-6 and IL-8 in response to LPS than those treated with the control siRNA. Thus, targeting the HO-1 gene augmented proinflammatory cytokine synthesis by synovial cell lines.

Effect of DMOARDs on HO-1 expression in synovial cells. We next examined the influences of auranofin, D-penicillamine, and dexamethasone on HO-1 expression by RA synovial cells. Auranofin strongly induced HO-1 expression at both mRNA and protein levels in a concentration-dependent manner from 10 to 1000 ng/ml. In contrast, neither D-penicillamine nor dexamethasone affected HO-1 expression at their therapeutic concentrations at all.

Influence of HO-1-specific siRNA on TNFα synthesis reduced by auranofin. We found that auranofin significantly suppressed spontaneous TNFα mRNA expression level and COX-2 expression by the cell lines in a dose-dependent manner. Interestingly, the expression levels of TNFα mRNA and COX-2 protein were negatively correlated with the level of HO-1 protein, suggesting that the anti-rheumatic properties of auranofin depended on HO-1 induction. To determine the role of HO-1 in the pharmacological effects of auranofin, we treated the cell lines with HO-1-specific siRNA in the presence of auranofin. As expected, these oligonucleotides almost completely abrogated the HO-1-inducing effects of auranofin. Furthermore, auranofin-dependent suppression of TNFα synthesis was abrogated by the addition of the HO-1-specific siRNA. These data suggest that the pharmacological effects of auranofin depended on HO-1 induction, at least in part.

Effects of heme degradation products on proinflammatory cytokine synthesis. As mentioned earlier, the anti-inflammatory properties of HO-1 are known to be mediated by the heme degradation products, Fe2+, CO, and bilirubin. To determine which product is involved in the suppression of cytokine synthesis, we examined the effects of DFO, which chelates Fe2+, Hb, a CO scavenger, and bilirubin on LPS-induced cytokine synthesis by the cell lines. The concentration of each agent was chosen with reference to the literature. We found that the suppressive effects of auranofin on IL-6 and IL-8 secretion were canceled by the addition of Hb, but not by that of DFO. Bilirubin did not affect the cytokine production. In addition, Hb enhanced LPS-induced IL-6 production even in the absence of auranofin. These results suggest that CO, not Fe2+ or bilirubin is responsible for the suppressive effects of endogenously expressed or chemically induced HO-1 on the cytokine synthesis.

Conclusions: Our data demonstrate that HO-1 and CO plays regulatory roles in the development of inflammation. HO-1 induction and local CO generation may be a novel therapeutic strategy for RA.