Hypoxia-induced Abrogation of Contact-dependent Inhibition of Rheumatoid Arthritis Synovial Fibroblast Proliferation

YOSHINORI NONOMURA, FUMITAKA MIZOGUCHI, AKIKO SUZUKI, TOSHIHIRO NANKI, HIROYUKI KATO, NOBUYUKI MIYASAKA, and HITOSHI KOHSAKA

ABSTRACT. Objective. Uncontrolled proliferation of synovial fibroblasts is characteristic of the pathology of rheumatoid arthritis (RA). Since synovial tissues in the rheumatoid joints are hypoxic, we investigated how hypoxia affects RA synovial fibroblast (RASF) proliferation.

Methods. RASF were cultured at 2000 cells (low density culture) or at 5000 cells (high density, growth-inhibitory confluent culture) per microtiter well under hypoxic (10%, 3%, or 1% O2) or normoxic (21% O2) conditions. Some RASF were treated with recombinant human interleukin 1 receptor antagonist (IL-1ra), anti-tumor necrosis factor-α (TNF-α)-neutralizing antibodies, anti-N-cadherin-blocking antibodies, or MG132. 3H-labeled thymidine incorporation was quantified to assess their proliferation. Total RNA and cell lysates were prepared for real-time polymerase chain reaction and Western blot analyses.

Results. Hypoxia exerted no effect on proliferation of RASF cultured at low density. At high density, it abrogated contact-dependent growth inhibition of RASF, but not of human dermal fibroblasts. Addition of anti-TNF-α antibodies or IL-1ra did not affect the results. Upregulated expression of cyclin-dependent kinase inhibitor p27Kip1 was observed in the cells cultured at high density under normoxic conditions, but not under hypoxic conditions. Hypoxia decreased N-cadherin expression on RASF. Addition of anti-N-cadherin-blocking antibodies mimicked the effects of hypoxic culture; it promoted proliferation of RASF cultured at high density under normoxic conditions. This antibody treatment also downmodulated p27Kip1 expression.

Conclusion. Hypoxia downregulates N-cadherin expression on RASF, and thus prevents p27Kip1 upregulation for their contact inhibition. It is likely that hypoxia in rheumatoid synovial tissues contributes to rheumatoid pathology by augmenting proliferation of synovial fibroblasts. (First Release Feb 15 2009; J Rheumatol 2009;36:698–705; doi:10.3899/jrheum.080188)

Key Indexing Terms: HYPOXIA RHEUMATOID ARTHRITIS SYNOVIAL FIBROBLASTS CYCLIN-DEPENDENT KINASE INHIBITOR CADHERIN

Rheumatoid arthritis (RA) is a common autoimmune disease characterized by synovitis and subsequent destruction of cartilage and bone. In affected joints, inflammatory cells including lymphocytes and macrophages are recruited and activated to produce many cytokines at high concentrations. Among them, tumor necrosis factor-α (TNF-α) and interleukin 1 (IL-1) are established targets in treatment of RA, and stimulate RA synovial fibroblasts (RASF) to proliferate and to produce inflammatory mediators. In the normal joint, the synovial lining layer is at most a few cells thick. In the RA inflamed joint, RASF proliferate to form stratified hyperplastic synovial tissues called pannus. When isolated and transferred to in vitro culture, they still proliferate as if they were transformed cells, which show no contact-dependent proliferative inhibition (contact inhibition). Because of the uncontrolled synovial hyperplasia, capillary density becomes insufficient for oxygen demand by synovial cells in the rheumatoid joints. Moreover, synovial fluid retention in affected joints increases the intraarticular pressure, leading to further reduction of blood perfusion.
Thus, rheumatoid synovial tissues support vigorous proliferation of synovial fibroblasts, and are paradoxically hypoxic. Lund-Olesen reported that oxygen levels of synovial fluid in patients with RA are reduced to less than half of those in healthy controls; mean PO2 is 63 mm Hg in the normal joints and 27 mm Hg in the rheumatoid joints.

Hypoxia regulates gene expression of various inflammatory mediators and proteinases involved in bone and cartilage destruction, and which are suggested to contribute to rheumatoid inflammation. Effects of hypoxia on cellular proliferation depend on the cell types. Hypoxia promoted proliferation of endothelial cells and fibroblasts. It down-regulated cyclin-dependent kinase inhibitor (CDKI) p21 Cip1 protein expression. It also extended the lifespan of vascular smooth-muscle cells by activating telomerase. In contrast, it halted cell-cycle progression by upregulation of CDKI, p16 INK4a in the CV-1 monkey kidney cell line, and p21 Cip1 and p27 Kip1 in murine embryonic fibroblasts. Thus, our objective was to determine how hypoxia affects the cell cycle of RASF.

Cell-cycle progression is largely regulated by kinase activity of cyclin/cyclin-dependent kinase complexes. CDKI are intracellular molecules that halt cell-cycle progression via inhibition of cyclin/CDK kinase activities. We reported previously that gene transfer of CDKI inhibited cell proliferation of endothelial cells and fibroblasts. It down-regulated cyclin-dependent kinase inhibitor (CDKI) p21 Cip1 protein expression. It also extended the lifespan of vascular smooth-muscle cells by activating telomerase. In contrast, it halted cell-cycle progression by upregulation of CDKI, p16 INK4a in the CV-1 monkey kidney cell line, and p21 Cip1 and p27 Kip1 in murine embryonic fibroblasts. Thus, our objective was to determine how hypoxia affects the cell cycle of RASF.

**MATERIALS AND METHODS**

**Cell culture.** Synovial tissues were derived from 10 patients with RA who had responded poorly to antirheumatic drugs and underwent joint replacement or synovectomy at Tokyo Medical and Dental University Hospital, Tokyo Metropolitan Bokuto Hospital, National Shimoshizu Hospital, or Shinhu University Hospital. All patients fulfilled the American College of Rheumatology criteria for classification of RA. All gave their consent to procedures in our studies, which were approved by the ethics committees of Tokyo Medical and Dental University and REN Research Center for Allergy and Immunology.

RASF were isolated and cultured as described. Adult normal human dermal fibroblasts (HDF) were purchased from Cambrex, East Rutherford, NJ, USA. They were used at early passages (passages 5 to 9). These fibroblasts were cultured under normoxic conditions (21% O2). Hypoxic conditions (10%, 3%, or 1% O2) were generated in a hypoxic chamber filled with N2 or under normoxic conditions. Some RASF were treated with 100 ng/ml recombinant human IL-1 receptor antagonist (IL-1ra; Prospect, Rehovot, Israel), 2 µg/ml anti-TNF-α-neutralizing monoclonal antibody (mAb; J2D10, Lab Vision, Fremont, CA, USA), 80 µg/ml anti-N-cadherin-blocking mAb (GC-4; Sigma, St. Louis, MO, USA), control mouse IgG1 mAb (MOPC-31; BD Biosciences, San Diego, CA, USA), and up to 200 µM cobalt chloride (CoCl2; Sigma). During the last 24-h culture, 0.3 µCi of 3H-labeled thymidine was present for quantification of the incorporated radioactivities.

**Cell proliferation assay.** RASF were incubated for 24–72 h in a hypoxic chamber or under normoxic conditions. Some RASF were treated with 100 ng/ml recombinant human IL-1 receptor antagonist (IL-1ra; Prospect, Rehovot, Israel), 2 µg/ml anti-TNF-α-neutralizing monoclonal antibody (mAb; J2D10, Lab Vision, Fremont, CA, USA), 80 µg/ml anti-N-cadherin-blocking mAb (GC-4; Sigma, St. Louis, MO, USA), control mouse IgG1 mAb (MOPC-31; BD Biosciences, San Diego, CA, USA), and up to 200 µM cobalt chloride (CoCl2; Sigma). During the last 24-h culture, 0.3 µCi of 3H-labeled thymidine was present for quantification of the incorporated radioactivities.

**Real-time polymerase chain reaction (PCR).** Total RNA was isolated with an RNasey kit (Qiagen, Valencia, CA, USA) and converted to cDNA with Superscript II (Invitrogen, Carlsbad, CA, USA) reverse transcriptase. Real-time PCR was carried out with iQ Syber Green supermix (Bio-Rad, Hercules, CA, USA) and a set of primers specific to N-cadherin or p27 Kip1 cDNA (sense: GCC GTA GCC GCT TTT AGA GGA AG; antisense: GGG GTA GCC GCT TTT AGA GGA AGA TCA TT). Data were standardized with human 28S ribosomal RNA (sense: TTC AAT CGG GGG AGA G; antisense: ACA TTG TCT CAA CTA GCC AG), and were analyzed with the cycle threshold method.

**Western blot analyses.** RASF were cultured for 24–72 h. Some RASF were treated with dimethyl sulfoxide (DMSO; Sigma), or 2.5 μM carbobenzoxyl-L-leucyl-L-leucyl-L-leucinal (MG132; EMD Chemicals, Darmstadt, Germany) during the last 24-h culture. Total protein extraction and sodium dodecyl sulfate-polyacrylamide gel electrophoresis were performed as described. Nuclear extracts were prepared using a Nuclear Extract Kit (Active Motif, Carlsbad, CA, USA). Rabbit anti-human p16 INK4a, p21 Cip1, CDK4 (sc-468, sc-397, and sc-260; Santa Cruz Biotech, Santa Cruz, CA, USA), mouse anti-E-cadherin, N-cadherin, p27 Kip1 (HECD-1, clone 32, and clone 57; BD-Biosciences), cadherin-11 (clone 283416; R&D Systems, Minneapolis, MN, USA), and hypoxia inducible factor-1α (Gene Tex, San Antonio, TX, USA) antibodies were used as primary antibodies. Horseradish peroxidase-conjugated anti-rabbit (NA-934; GE Healthcare Biosciences, Piscataway, NJ, USA) or anti-mouse IgG antibodies (6175-05; Southern Biotech, Birmingham, AL, USA) were used as secondary antibodies. Bound antibodies were visualized with ECL (GE Healthcare Biosciences). Signal intensities were quantified with ImageJ software (US National Institutes of Health, Bethesda, MD, USA).

**ELISA.** ELISA kits for IL-1α, IL-1β, IL-6, and TNF-α (Biosource International, Camarillo, CA, USA) were employed to quantify protein levels in the culture supernatants.

**Statistics.** Student’s paired t test was used for statistical comparisons.

**RESULTS**

**RASF proliferation accelerated by hypoxia in high density culture.** The effect of hypoxia on RASF growth was studied with 3H-thymidine incorporation. RASF were first cultured at low density in a microtiter plate for logarithmic cell growth. They grew equally under normoxic (21% O2) and hypoxic (1% O2) conditions. When cultured at high density under normoxic conditions, they stopped growing and incorporated less thymidine. Although more cells were present in the high density culture under normoxic conditions, these cells incorporated an amount of 3H-thymidine comparable to those in the low density culture. However, under hypoxic conditions, they incorporated more thymidine than those under normoxic conditions (Figure 1A). Thus, hypoxia attenuated growth suppression that was induced by high density culture.

**Under normoxic (21% O2) and hypoxic conditions (1% O2), the dissolved oxygen concentrations in the culture supernatants of RASF cultured at low density were 8.01 ± 0.06 and 2.15 ± 0.14 mg/dl, and those of RASF cultured at high density were 8.00 ± 0.03 and 2.14 ± 0.07 mg/dl, respec-
Figure 1. Hypoxia-induced augmentation of fibroblast proliferation. A. RA synovial fibroblasts (RASF) were cultured in microtiter plates at high or low density for 72 h under normoxic (21% O2) or hypoxic (1% O2) conditions. RASF proliferation was assessed using [3H]-thymidine incorporation in 5 wells per RASF sample, and the mean values were calculated. Columns and bars represent mean and SD values of 5 RASF samples. *p < 0.05. B. Effect of hypoxia on proliferation of human dermal fibroblasts was assessed in the same manner. Representative results of 1 of 2 experiments are shown. Columns and bars represent mean and standard deviations of 5 wells. C. The effects of hypoxia on the proliferation of RASF were assessed as in panel A. RASF were cultured in microtiter plates at high density for 72 hours under normoxic (21% O2) or hypoxic (10% and 3% O2) conditions. Columns and bars represent mean and SD of 3 RASF samples. D. RASF were cultured at high density for 24, 48, or 72 hours under normoxic (21% O2) or hypoxic (1% O2) conditions as described above. The effect of hypoxia on the proliferation of RASF was assessed in the same manner. Columns and bars represent mean and SD of 3 RASF samples. The relative [3H]-thymidine incorporations by RASF cultured in hypoxic conditions standardized with cultures in normoxic conditions at the same timepoints are shown at the bottom. *p < 0.05. E. The effect of hypoxia on the proliferation of RASF cultured at high density was assessed as in A. RASF were treated with culture medium only (control), 100 ng/ml human recombinant IL-1ra, 2 µg/ml isotype IgG1 control antibody, or 2 µg/ml anti-TNF-α-neutralizing mAb. Representative results of 1 of 2 experiments are shown. Columns and bars represent mean and SD of 5 wells. *p < 0.05. cpm: counts per minute; ns: not significant.
respectively (mean ± SD of 3 samples). In these experiments, the cell density was found not to affect dissolved oxygen concentrations.

To determine if this was a general feature of fibroblasts, HDF were cultured in the same conditions. The results showed that hypoxia (1% O₂) did not promote proliferation of HDF in high density culture (Figure 1B).

The mean pO₂ is reported to be 63 mm Hg in normal joints and 27 mm Hg in rheumatoid joints³. To simulate oxygen supplies in normal and rheumatoid joints, we examined the effects of 10% O₂ (mean O₂ level in normal joints) or 3% O₂ (mean O₂ level in rheumatoid joints) on proliferation of RASF cultured at high density. RASF cultured under 3% O₂ incorporated slightly more ³H-thymidine than fibroblasts under 10% O₂, but the differences were not statistically significant (Figure 1C).

To elucidate the time course of accelerated RASF proliferation by hypoxia, we examined the ³H-thymidine incorporation by RASF cultured at high density under normoxic (21% O₂) or hypoxic (1% O₂) conditions at 24 hours, 48 hours and 72 hours. The relative ³H-thymidine incorporation by RASF under hypoxic conditions standardized with those under normoxic conditions at the same time points increased gradually with time, and it reached 2.64 ± 0.51 after 72 hours of culture (Figure 1D).

**Effects of IL-1 and TNF-α on hypoxia-induced proliferation.** When IL-1α, IL-1β, and TNF-α in the supernatants of RASF culture were quantified with specific ELISA, they were all below detection limits (IL-1α and IL-1β < 3.9 pg/ml, TNF-α < 1.7 pg/ml). This suggested that hypoxia does not stimulate RASF to produce these cytokines. Even when IL-1ra or anti-TNF-α-blocking mAb were included in the medium, RASF in the high density culture grew more under hypoxic conditions (Figure 1E). In separate experiments, the same concentrations of these blocking reagents under hypoxic conditions, they proliferated more than those treated with the blocking mAb (data not shown).

It has been reported that IL-6 suppresses RASF proliferation²⁰. The supernatants of RASF cultured at high density under normoxic conditions and those cultured under hypoxic conditions contained comparable levels of IL-6: 4.6 ± 4.9 ng/ml and 6.4 ± 6.5 ng/ml (mean ± SD of 3 samples), respectively. Thus, IL-6 was not responsible for the overgrowth of RASF under the hypoxic conditions.

**Downregulation of CDKI expression by hypoxia.** In general, logarithmic cell growth of nontransformed cells can be inhibited in high density culture. This contact inhibition involves upregulation of the CDKI p16⁺NK4a, p21⁺Cip1, and/or p27⁺Kip1, depending on the cell types²¹-²³. Our previous studies revealed that RASF in confluent culture upregulated expression of these CDKI when they were incubated for more than 4 days¹⁵. We examined the effect of hypoxia in CDKI expression. When RASF were cultured at high density for 3 days, the protein level of p27⁺Kip1 was upregulated. p16⁺NK4a and p21⁺Cip1 protein were not upregulated at this timepoint. However, the high density culture did not induce p27⁺Kip1 upregulation under hypoxic conditions (Figure 2A, 2B). The protein level of p27⁺Kip1 was not upregulated when RASF were cultured at low density for 3 days (data not shown). Quantitative PCR of p27⁺Kip1 mRNA transcripts showed that the difference did not depend on alteration of the p27⁺Kip1 mRNA expression (Figure 2C). It has been reported that the p27⁺Kip1 protein is degraded via the ubiquitin-proteasome pathway. To elucidate whether this pathway is involved in the attenuation of p27⁺Kip1 protein expression by hypoxia, a proteasome inhibitor, MG132, was added to the culture medium. MG132 upregulates p27⁺Kip1 protein expression through inhibition of protein degradation²⁴. MG132 2.5 µM upregulated p27⁺Kip1 protein concentrations in RASF cultured at high density under hypoxia (1% O₂; Figure 2D). This suggests that attenuation of p27⁺Kip1 by hypoxia is regulated via the ubiquitin-proteasome pathway. These findings were in agreement with the fact that the protein level of p27⁺Kip1 was primarily controlled at the posttranscriptional level, via the ubiquitin-proteasome pathway²⁵. In contrast, when HDF were cultured at high density, the p27⁺Kip1 protein was upregulated even in hypoxic conditions (Figure 2E).

**Hypoxia-induced N-cadherin downregulation responsible for accelerated RASF proliferation.** Cadherins are a group of cell-surface molecules that recognize direct cell-cell contact and mediate signal pathways into the cells. Homophilic interaction of surface N-cadherin and E-cadherin promotes p27⁺Kip1 protein accumulation for contact inhibition²⁶,²⁷. Recently, it was reported that cadherin-11 is expressed by RASF and contributes to organization of the lining-like structure of RA synovial tissues²⁸,²⁹. RASF grown under normoxic conditions expressed N-cadherin and cadherin-11, but not E-cadherin. When the same cells were cultured under hypoxic conditions, their expression of N-cadherin, but not cadherin-11, was reduced (Figure 3A, 3B). Quantitative PCR of p27⁺Kip1 mRNA transcripts showed that the difference did not depend on alteration of the p27⁺Kip1 mRNA expression (Figure 2C). It has been reported that hypoxia suppressed N-cadherin expression, resulting in augmentation of RASF proliferation at high density. In support of this, hypoxia did not alter the N-cadherin protein expression in HDF (Figure 3C).

Next, anti-N-cadherin blocking mAb was used to interfere with homophilic interaction of N-cadherins. When RASF were cultured with this mAb at high density under normoxic conditions, they proliferated more than those treated with control IgG (Figure 3D). In analogy to hypoxia, treatment with the blocking mAb downmodulated expression of p27⁺Kip1 protein in the cultured RASF (Figure 3E).

**DISCUSSION** Hypoxia promoted proliferation of RASF by attenuating...
Figure 2. Downregulation of p27Kip1 expression by hypoxia. A. RASF were cultured at high density under normoxic (21% O2) or hypoxic (1% O2) conditions, and whole-cell lysates were collected after indicated number of days. Expression of CDK1 p16INK4a, p21Cip1, and p27Kip1 by RASF was immunodetected by Western blot. CDK4 was stained as loading control. Expression levels of p27Kip1 protein standardized with those of the CDK4 protein in the same blot are shown at the bottom. pc: positive control. B. p27Kip1 levels cultured under normoxic (21% O2) and hypoxic (1% O2) conditions for 3 days were standardized with levels of CDK4. Columns and bars represent mean and SD of 3 samples. *p < 0.05. C. RASF were cultured for 3 days at high density under normoxic (21% O2) or hypoxic (1% O2) conditions before extraction of total RNA. p27Kip1 mRNA expression was quantified by real-time PCR using 28S ribosomal RNA as an endogenous control. Columns and bars represent mean and SD of 3 samples. ns: not significant. D. RASF were cultured at high density under normoxic (21% O2) or hypoxic (1% O2) conditions, and whole-cell lysates were collected after 3 days. RASF were treated with culture medium alone (med), 0.1% DMSO, or 2.5 µM MG132 during the last 24 hours of culture. p27Kip1 expression by RASF was immunodetected by Western blot. Representative results of 2 samples are shown. Expression levels of p27Kip1 protein standardized with those of the CDK4 protein in the same blot are shown at the bottom. E. Human dermal fibroblasts were cultured for 3 days at high density under normoxic (21% O2) or hypoxic (1% O2) conditions before extraction of whole-cell lysates. Expression of p27Kip1 and CDK4 was assessed as in A. Representative blots of 2 independent experiments are shown.
their contact inhibition. The accelerated proliferation was mediated by suppressed expression of N-cadherin, which should otherwise upregulate p27Kip1 expression on their homophilic interaction. It has been reported that hypoxia stimulates RASF to produce several inflammatory mediators and proteinases that are involved in the pathology of RA. We observed that hypoxia may contribute to RA pathology by promoting proliferation of RASF as well (Figure 4).

Hypoxia did not affect logarithmic-phase proliferation of RASF, but abrogated their contact inhibition by modulating N-cadherin and subsequent p27Kip1 expression. The same
changes were not observed in HDF. Thus, hypoxia-induced augmentation of proliferation via N-cadherin and p27^Kip1 might be one specific feature of RASF. RASF were found to be prone to express a few types of CDKI in longterm *in vitro* cultures. Although the short-term hypoxic culture in our experiments disclosed only its effect on p27^Kip1, we assume that expression of other CDKI might be affected by hypoxia.

Our results showed that RASF proliferation was augmented in the presence of 1% O2, but not in the presence of 3% O2. Treuhaft, *et al.* reported that the knees of 30% of patients with RA exhibited profound hypoxia, with pO2 less than 10 mm Hg, and the lowest pO2 in a rheumatoid joint was 8 mm Hg. Thus, 1% O2 corresponds to the lowest levels in rheumatoid joints. Further, it has been reported that movement of inflamed joints can reduce the blood flow in synovial capillaries, and thus further aggravate synovial hypoxia. Hypoxia may augment RASF proliferation in such severely hypoxic rheumatoid joints, especially during exercise.

The time-course experiments revealed that the relative 3H-thymidine incorporation by RASF peaked after 48 hours, and decreased thereafter, i.e., at 72 hours, even under hypoxic conditions. These data suggest that hypoxia-induced augmentation of RASF proliferation does not completely overcome contact inhibition. After culture for 48 hours, hypoxia augmented RASF proliferation at a statistically significant level, and downregulated the protein expression of p27^Kip1, but the latter effect was not significant. Thus, some mechanism other than the attenuation of p27^Kip1 protein expression may be involved in the hypoxia-induced augmentation of RASF proliferation.

It is known that quite a few cellular responses to hypoxia are mediated by hypoxia inducible factor-1 (HIF-1). Its expression was upregulated in the rheumatoid synovial tissues, reflecting the hypoxic environment of the affected joints. In a separate experiment, HIF-1 in cultured RASF was upregulated artificially with CoCl2. However, this treatment did not affect the RASF proliferation or the protein level of p27^Kip1 (data not shown), suggesting that an HIF-1-independent pathway should operate in the hypoxia-triggered augmentation of RASF proliferation.

Cadherins maintain the integrity of multicellular structures. One of the intracellular outcomes of cadherin activation is induction of CDKI, including p21^Cip1 and p27^Kip1, that inhibit cell-cycle progression. E-cadherin interaction mediates contact-dependent proliferative inhibition by

---

*Figure 4. Abrogation of contact-dependent proliferative inhibition of RASF by hypoxia. Hypoxia downregulates N-cadherin and subsequently p27^Kip1 expression to augment RASF proliferation. It also upregulates inflammatory mediators and proteinases involved in RA. These results suggest that hypoxia in rheumatoid synovial tissues should contribute to joint destruction through multiple pathways. MMP: matrix metalloproteinase; VEGF: vascular endothelial growth factor.*
increasing the level of p27Kip1 expression in thyroid cancer cells, while N-cadherin interaction induces contact inhibition through upregulation of p27Kip1 in CHO cells. These data point to a link between cadherin signaling and CDKI protein expression. In addition, hypoxia suppresses E-cadherin expression on cancer cell lines and N-cadherin expression in cortex proximal renal tubules. Thus, there is another link between hypoxia and cadherins.

RASF expressed N-cadherins, but not E-cadherins. Recently, it was reported that cadherin-11 is expressed by RASF and contributes to organization of the lining-like structure of synovial tissues. A cadherin-11–Fc fusion protein and an anti-cadherin-11–blocking mAb prevented and reduced arthritis in mouse models. Thus, cadherins, which should control proliferative reactions to hypoxia, appear to be crucial molecules for formation and maintenance of the rheumatoid synovial structure.

ACKNOWLEDGMENT

We thank Drs. T. Muneta, K. Goto and K. Taniguchi for providing synovial samples, Dr. I. Morita for monitoring of oxygen concentration, M. Toyomoto for technical assistance, and Dr. H. Tanaka for critical advice.

REFERENCES


Nonomura, et al: Hypoxia and synovial fibroblasts

www.jrheum.org Downloaded on March 14, 2022 from www.jrheum.org