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

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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 mediators1. 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)1.

Because of the uncontrolled synovial hyperplasia, capillary density becomes insufficient for oxygen demand by synovial cells in the rheumatoid joints2. Moreover, synovial fluid retention in affected joints increases the intraarticular pressure, leading to further reduction of blood perfusion3.
Thus, rheumatoid synovial tissues support vigorous proliferation of synovial fibroblasts, and are paradoxically hypoxic3-4. 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 joints3.

Hypoxia regulates gene expression of various inflammatory mediators and proteinases involved in bone and cartilage destruction3-5,6, 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 cells7 and fibroblasts8,9. It down-regulated cyclin-dependent kinase inhibitor (CDKI) p21Cip1 protein expression7. It also extended the lifespan of vascular smooth-muscle cells by activating telomerase10. In contrast, it halted cell-cycle progression by upregulation of CDKI, p16INK4a in the CV-1P monkey kidney cell line11, and p21Cip1 and p27Kip1 in murine embryonic fibroblasts12,13. 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 complexes14. 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 RASF proliferation in vitro, and that intraarticular CDKI gene therapy ameliorated animal models of RA15,16. Thus, RASF proliferation is crucial in the pathology of RA, and could be a target of treatment for RA. We investigated the effect of hypoxia on RASF proliferation in the context of CDKI expression.

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 Shimoshi University Hospital, or Shinshu University Hospital. All patients fulfilled the American College of Rheumatology criteria for classification of RA17. All gave their consent to procedures in our studies, which were approved by the ethics committees of Tokyo Medical and Dental University and RIEN Research Center for Allergy and Immunology.

RASF were isolated and cultured as described15. 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 CO₂ (5%) and N₂ (85%, 92%, or 94%) gas mixture. Oxygen concentration was monitored with an oxygen electrode (Cosmo or Jiko, Tokyo, Japan). Dissolved oxygen levels in culture supernatants were measured with a dissolved oxygen monitor (Central Kagaku, Tokyo, Japan). RASF and HDF were cultured in a microtiter-plate at 5000 cells per well (high density culture) or 2000 cells per well (low density culture). RASF for RNA, total protein, and nuclear extraction were cultured at 3.5 × 10⁵ cells per 60 mm dish (high density culture).

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; Prospec, 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-cadherin18 or p27Kip1 cDNA (sense: GCT GTA GAT TTT TTT TTG CGA GAG AG; antisense: GGG GTA GCC GCT TTT TTG AGA GCA TCA TT). Data were standardized with human 28S ribosomal RNA (sense: TTG AAA ATC CGG GGG AGA G; antisense: ACA TTG TTC CAA CAT GGC AG), and were analyzed with the cycle threshold method19.

Western blot analyses. RASF were cultured for 24–72 h. Some RASF were treated with dimethyl sulfoxide (DMSO); Sigma), or 2.5 µM carbonoxyl-1-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 described15. Nuclear extracts were prepared using a Nuclear Extract Extract Kit (Active Motif, Carlsbad, CA, USA). Rabbit anti-human p16INK4a, p21Cip1, CDK4 (sc-468, sc-397, and sc-260; Santa Cruz Biotech, Santa Cruz, CA, USA), mouse anti-E-cadherin, N-cadherin, p27Kip1 (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 only 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. cpms: 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 joints. 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 timepoints 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-1α 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 suppressed the ³H-thymidine incorporation and matrix metalloproteinase-3 production by RASF stimulated with 10 pg/ml IL-1α and 10 pg/ml TNF-α (data not shown).

It has been reported that IL-6 suppresses RASF proliferation. 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 P27Kyle 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 p16INK4a, p21Cip1, and/or p27Kyle, depending on the cell types. Our previous studies revealed that RASF in confluent culture upregulated expression of these CDKI when they were incubated for more than 4 days. We examined the effect of hypoxia in CDKI expression. When RASF were cultured at high density for 3 days, the protein level of p27Kyle was upregulated. p16INK4a and p21Cip1 protein were not upregulated at this timepoint. However, the high density culture did not induce p27Kyle upregulation under hypoxic conditions (Figure 2A, 2B). The protein level of p27Kyle was not upregulated when RASF were cultured at low density for 3 days (data not shown). Quantitative PCR of p27Kyle mRNA transcripts showed that the difference did not depend on alteration of the p27Kyle mRNA expression (Figure 2C). It has been reported that the p27Kyle protein is degraded via the ubiquitin-proteasome pathway. To elucidate whether this pathway is involved in the attenuation of p27Kyle protein expression by hypoxia, a proteasome inhibitor, MG132, was added to the culture medium. MG132 upregulates p27Kyle protein expression through inhibition of protein degradation.

**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 CDKIs 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. p.c.: 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. n.s: 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 p27^Kip1 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^2-5. 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 p27^Kip1 expression. The same
changes were not observed in HDF. Thus, hypoxia-induced augmentation of proliferation via N-cadherin and p27Kip1 might be one specific feature of RASF. RASF were found to be prone to express a few types of CDKI in longterm in vitro cultures14. Although the short-term hypoxic culture in our experiments disclosed only its effect on p27Kip1, 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 Hg3. 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 hypoxia30. 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 was increased gradually with time, and peaked after 72 hours of culture. Further, hypoxia downregulated the expression of p27Kip1 and N-cadherin to a statistically significant level. However, the absolute level of 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 p27Kip1, but the latter effect was not significant. Thus, some mechanism other than the attenuation of p27Kip1 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)31. Its expression was upregulated in the rheumatoid synovial tissues, reflecting the hypoxic environment of the affected joints2. In a separate experiment, HIF-1 in cultured RASF was upregulated artificially with CoCl22. However, this treatment did not affect the RASF proliferation or the protein level of p27Kip1 (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 p21Cip1 and p27Kip1, that inhibit cell-cycle progression. E-cadherin interaction mediates contact-dependent proliferative inhibition by peak.
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 RASF and contributes to organization of the lining-like structure of synovial tissues. Thus, there is another link between hypoxia and cadherins.

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REFERENCES