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% O_2) or normoxic (21% O_2) 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. ^{3}H-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 $p27^{Kip1}$ 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 $p27^{Kip1}$ expression.

Conclusion. Hypoxia downregulates N-cadherin expression on RASF, and thus prevents $p27^{Kip1}$ upregulation for their contact inhibition. It is likely that hypoxia in rheumatoid synovial tissues contributes to rheumatoid pathology by augmenting proliferation of synovial fibroblasts. (J Rheumatol First Release Feb 15 2009; doi:10.3899/jrheum.080188)

Key Indexing Terms:
HYPOXIA RHEUMATOID ARTHRITIS SYNOVIAL FIBROBLASTS
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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[1]. 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 joints[2]. Moreover, synovial fluid retention in affected joints increases the intraarticular pressure, leading to further reduction of blood perfusion[3].

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Thus, rheumatoid synovial tissues support vigorous proliferation of synovial fibroblasts, and are paradoxically hypoxic[3,4]. 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 pO_2 is 63 mm Hg in the normal joints and 27 mm Hg in the rheumatoid joints[3].

Hypoxia regulates gene expression of various inflammatory mediators and proteinases involved in bone and cartilage destruction[2,5,6], 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 cells[7] and fibroblasts[8,9]. It downregulated cyclin-dependent kinase inhibitor (CDKI) $p21^{Cip1}$ protein expression[9]. It also extended the lifespan of vascular smooth-muscle cells by activating telomerase[10]. In contrast, it halted cell-cycle progression by upregulation of CDKI, $p16^{INK4a}$ in the CV-1P monkey kidney cell line[11], and $p21^{Cip1}$ and $p27^{Kip1}$ in murine embryonic fibroblasts[12,13]. 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 complexes[14]. 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 RA[15,16]. 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 Shimoshizu Hospital, or Shinshu University Hospital. All patients fulfilled the American College of Rheumatology criteria for classification of RA[17]. 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 described[15]. 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% O_2). Hypoxic conditions (10%, 3%, or 1% O_2) were generated in a hypoxic chamber filled with CO_2 (5%) and N_2 (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 3.5×10^5 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 ($CoCl_2$; Sigma). During the last 24-h culture, 0.3 μCi of ^{3}H-labeled thymidine was present for quantification of the incorporated radioactivities.

Real-time polymerase chain reaction (PCR). Total RNA was isolated with an RNeasy 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[18] or $p27^{Kip1}$ cDNA (sense: GCT CTA GAT TTT TTG AGA GTG CGA GAG AG; antisense: GGG GTA GCC GCT TTT AGA GGC AGA TCA TT). Data were standardized with human 28S ribosomal RNA (sense: TTG AAA ATC CGG GGG AGA G; antisense: ACA TTG TTC CAA CAT GCC AG), and were analyzed with the cycle threshold method[19].

Western blot analyses. RASF were cultured for 24–72 h. Some RASF were treated with dimethyl sulfoxide (DMSO); Sigma), or 2.5 μM carbobenzoxy-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[15]. 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 67; 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-1ß, 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 ^{3}H-thymidine incorporation. RASF were first cultured at low density in a microtiter plate for logarithmic cell growth. They grew equally under normoxic (21% O_2) and hypoxic (1% O_2) 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 ^{3}H-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% O_2) and hypoxic conditions (1% O_2), 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-

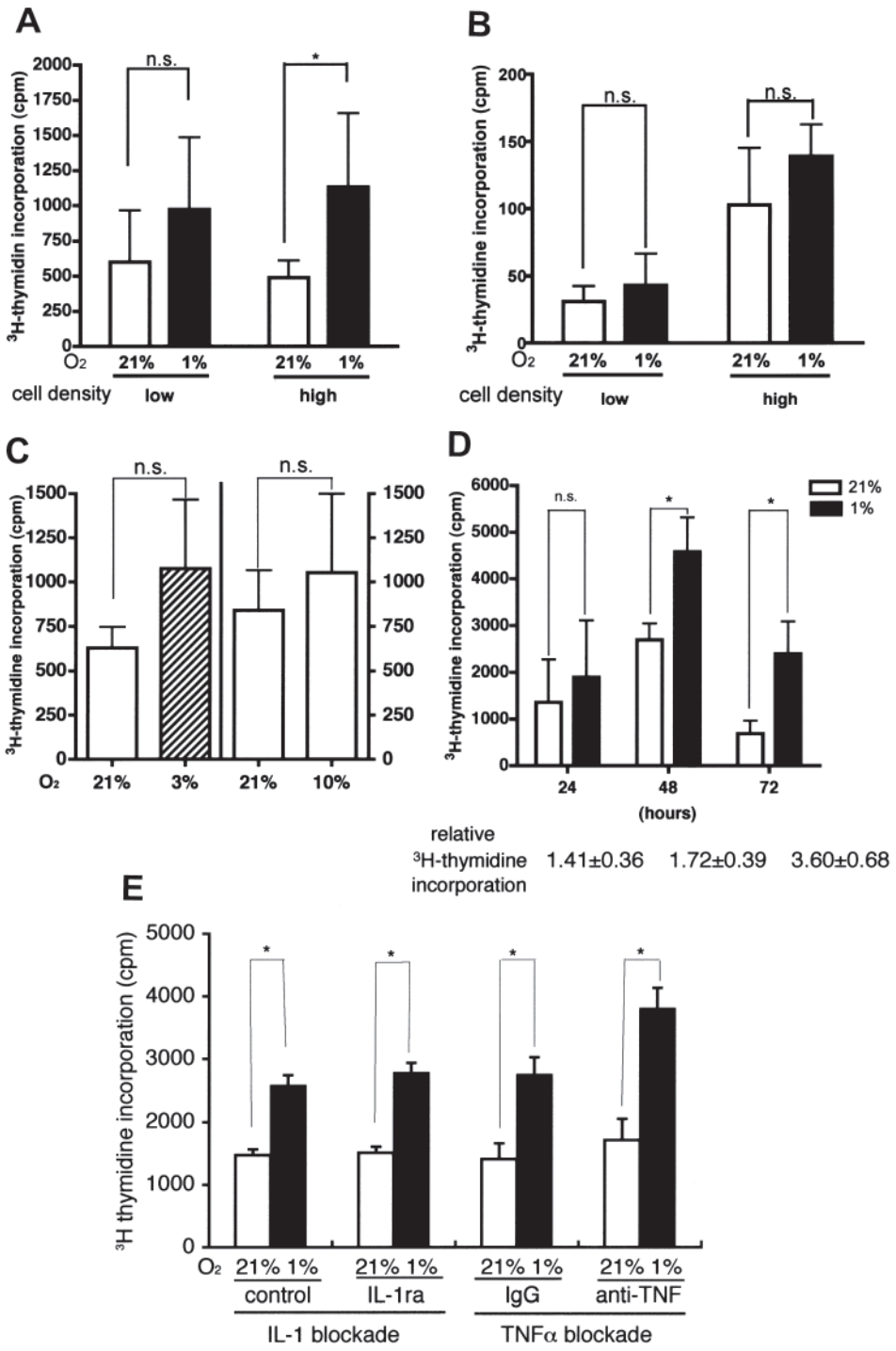

Figure 1. Hypoxia-induced augmentation of fibroblast proliferation. A. RA synovial fibroblasts (RASF) were cultured in microtiter plates at high or low density for 72 h under normoxic (21% O_2) or hypoxic (1% O_2) conditions. RASF proliferation was assessed using ^{3}H-thymidine incorporation in 5 wells 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% O_2) or hypoxic (10% and 3% O_2) 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% O_2) or hypoxic (1% O_2) 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 ^{3}H-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.

tively (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% O_2) did not promote proliferation of HDF in high density culture (Figure 1B).

The mean pO_2 is reported to be 63 mm Hg in normal joints and 27 mm Hg in rheumatoid joints[3]. To simulate oxygen supplies in normal and rheumatoid joints, we examined the effects of 10% O_2 (mean O_2 level in normal joints) or 3% O_2 (mean O_2 level in rheumatoid joints) on proliferation of RASF cultured at high density. RASF cultured under 3% O_2 incorporated slightly more ^{3}H-thymidine than fibroblasts under 10% O_2, but the differences were not statistically significant (Figure 1C).

To elucidate the time course of accelerated RASF proliferation by hypoxia, we examined the ^{3}H-thymidine incorporation by RASF cultured at high density under normoxic (21% O_2) or hypoxic (1% O_2) conditions at 24 hours, 48 hours and 72 hours. The relative ^{3}H-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-1ß, and TNF-α in the supernatants of RASF culture were quantified with specific ELISA, they were all below detection limits (IL-1α and IL-1ß < 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 suppressed the ^{3}H-thymidine incorporation and matrix metalloproteinase-3 production by RASF stimulated with 10 pg/ml IL-1ß and 10 pg/ml TNF-α (data not shown).

It has been reported that IL-6 suppresses RASF proliferation[20]. 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 P27^{Kip1} 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^{INK4a}, p21^{Cip1}, and/or p27^{Kip1}, depending on the cell types[21-23]. Our previous studies revealed that RASF in confluent culture upregulated expression of these CDKI when they were incubated for more than 4 days[15]. 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^{INK4a} 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 degradation[24]. MG132 2.5 μM upregulated p27^{Kip1} protein concentrations in RASF cultured at high density under hypoxia (1% O_2; 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 post-transcriptional level, via the ubiquitin-proteasome pathway[25]. 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 inhibition[26,27]. Recently, it was reported that cadherin-11 is expressed by RASF and contributes to organization of the lining-like structure of RA synovial tissues[28,29]. 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). Quantitation of mRNA with real-time PCR revealed that this reduction was regulated at the mRNA level (Figure 3B). These data suggested 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

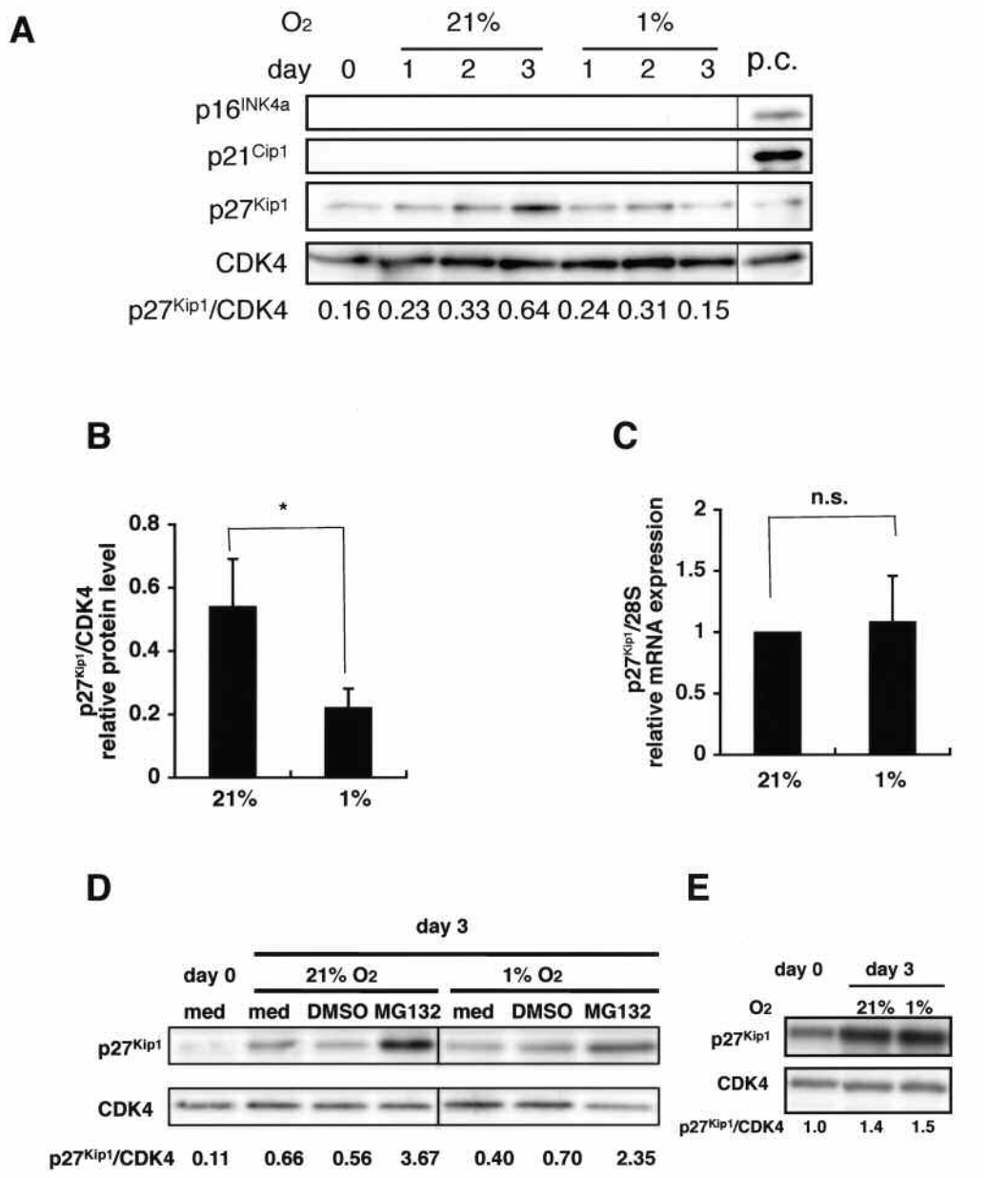

Figure 2. Downregulation of $p27^{Kip1}$ expression by hypoxia. A. RASF were cultured at high density under normoxic (21% O_2) or hypoxic (1% O_2) conditions, and whole-cell lysates were collected after indicated number of days. Expression of CDKI $p16^{INK4a}$, $p21^{Cip1}$, and $p27^{Kip1}$ by RASF was immunodetected by Western blot. CDK4 was stained as loading control. Expression levels of $p27^{Kip1}$ protein standardized with those of the CDK4 protein in the same blot are shown at the bottom. pc: positive control. B. $p27^{Kip1}$ levels cultured under normoxic (21% O_2) and hypoxic (1% O_2) 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% O_2) or hypoxic (1% O_2) conditions before extraction of total RNA. $p27^{Kip1}$ 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% O_2) or hypoxic (1% O_2) 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. $p27^{Kip1}$ expression by RASF was immunodetected by Western blot. Representative results of 2 samples are shown. Expression levels of $p27^{Kip1}$ 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% O_2) or hypoxic (1% O_2) conditions before extraction of whole-cell lysates. Expression of $p27^{Kip1}$ and CDK4 was assessed as in A. Representative blots of 2 independent experiments are shown.

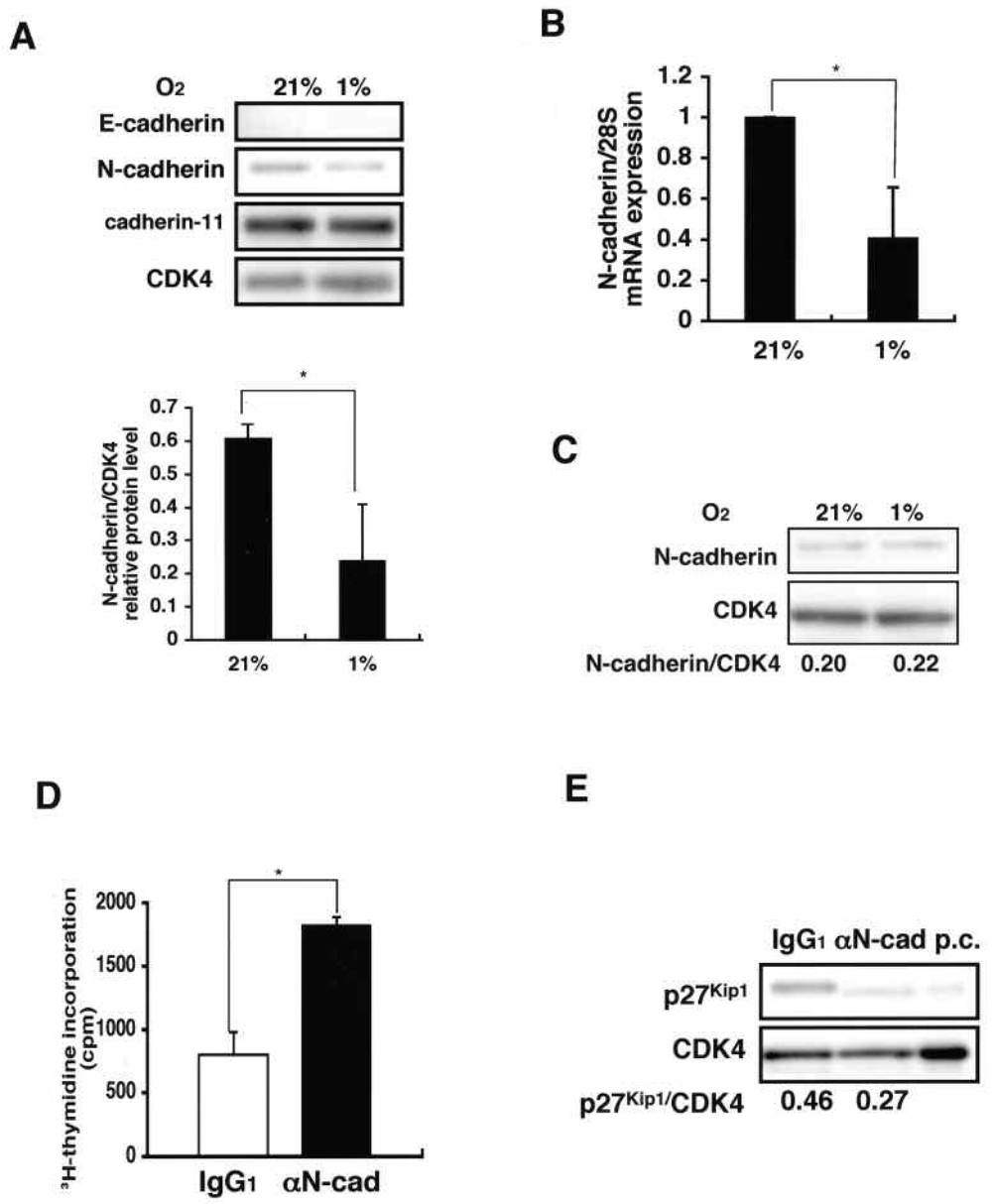

Figure 3. Downregulation of N-cadherin expression by hypoxia. A. RASF were cultured at high density under normoxic (21% O_2) or hypoxic (1% O_2) conditions for 3 days before collection of whole-cell lysates. N-cadherin protein expression was immunodetected by Western blot. Representative blots of 3 samples are shown in the upper panel. Expression levels of N-cadherin protein were standardized with those of CDK4 protein (lower panel). Columns and bars represent mean and SD of the 3 samples. *$p < 0.05$. B. RASF were cultured as in A before extraction of total RNA. N-cadherin mRNA expression was quantified by real-time PCR using 28S as an endogenous control. Columns and bars represent mean and SD of the 3 samples. *$p < 0.05$. C. Expression of N-cadherin and CDK4 in human dermal fibroblasts cultured under normoxic (21% O_2) or hypoxic (1% O_2) conditions was assessed as in A. Representative blots of 2 independent experiments are shown. D. RASF were cultured at high density, and treated with anti-N-cadherin-blocking mAb (αN-cad) or isotype IgG1 control mAb. After 3 days, effect of N-cadherin blockade on RASF proliferation was examined by ^{3}H-thymidine incorporation. Representative results of 3 samples are shown. Columns and bars represent mean and SD of 5 wells. *$p < 0.05$. E. Expression of $p27^{Kip1}$ and CDK4 by RASF treated with αN-cad or IgG1 for 3 days were immunodetected by Western blot. Representative results of 2 independent experiments are shown. pc: positive control

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

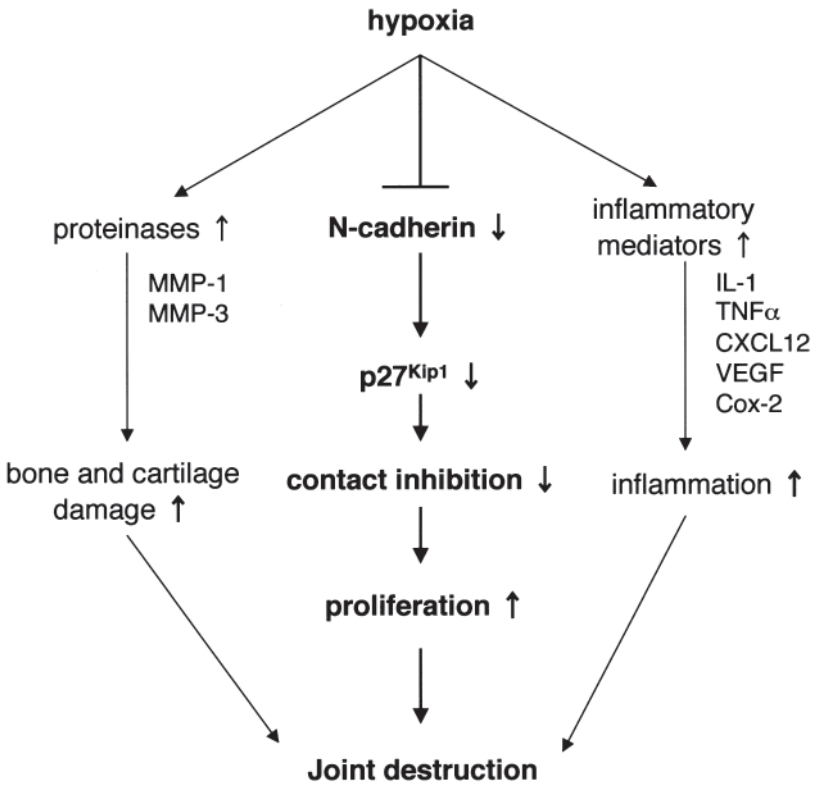

Figure 4. Abrogation of contact-dependent proliferative inhibition of RASF by hypoxia. Hypoxia downregulates N-cadherin and subsequently $p27^{Kip1}$ expression to augment RASF proliferation. It also upregulates inflammatory mediators and proteinases involved in RA. These results suggest that hypoxia in rheumatoid synovial tissues should contribute to joint destruction through multiple pathways. MMP: matrix metalloproteinase; VEGF: vascular endothelial growth factor.

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[14]. 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% O_2, but not in the presence of 3% O_2. Treuhaft, *et al* reported that the knees of 30% of patients with RA exhibited profound hypoxia, with pO_2 less than 10 mm Hg, and the lowest pO_2 in a rheumatoid joint was 8 mm Hg[4]. Thus, 1% O_2 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[30]. Hypoxia may augment RASF proliferation in such severely hypoxic rheumatoid joints, especially during exercise.

The time-course experiments revealed that the relative ^{3}H-thymidine incorporation by RASF was increased gradually with time, and peaked after 72 hours of culture. Further, hypoxia downregulated the expression of $p27^{Kip1}$ and N-cadherin to a statistically significant level. However, the absolute level of ^{3}H-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)[31]. Its expression was upregulated in the rheumatoid synovial tissues, reflecting the hypoxic environment of the affected joints[2]. In a separate experiment, HIF-1 in cultured RASF was upregulated artificially with $CoCl_2$ [2]. 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

increasing the level of $p27^{Kip1}$ expression in thyroid cancer cells[32], while N-cadherin interaction induces contact inhibition through upregulation of $p27^{Kip1}$ in CHO cells[26]. These data point to a link between cadherin signaling and CDKI protein expression. In addition, hypoxia suppresses E-cadherin expression on cancer cell lines[33] and N-cadherin expression in cortex proximal renal tubules[34]. 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[28,29]. A cadherin-11–Fc fusion protein and an anti-cadherin–11-blocking mAb prevented and reduced arthritis in mouse models[35]. Thus, cadherins, which should control proliferative reactions to hypoxia, appear to be crucial molecules for formation and maintenance of the rheumatoid synovial structure.

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