Id-2 induced by hypoxia promotes synovial fibroblast-dependent osteoclastogenesis

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Abstract

Objective: The aim of our study was to map hypoxic areas in the arthritic synovium and to establish the relevance of low oxygen levels to the phenotype of synovial fibroblasts with special focus on bone degradation.

Methods: To analyze the distribution of hypoxia in arthritic joints, the hypoxia marker EF-5 was administered to mice with collagen-induced arthritis (CIA). To evaluate the effect of hypoxia on rheumatoid arthritis synovial fibroblasts (RASF), reverse subtractive hybridisation and cDNA array were used. Real-time PCR, Western blot, and immunohistochemistry were used to evaluate the expression of inhibitor of differentiation-2 (Id-2). To look at the function of Id-2 in RASF, cells were either transfected with Id-2 vector or Id-2 specific siRNA.

Results: EF-5 staining showed the presence of hypoxia in arthritic joints, particularly at sites of synovial invasion into bone. Differential expression analysis revealed that Id-2 is strongly induced by hypoxia in RASF. Immunohistochemistry on CIA and human RA synovium showed a strong expression of Id-2 by RASF at sites of synovial invasion into bone. Overexpression of Id-2 in RASF significantly induced the expression of several factors promoting osteoclastogenesis. The biological relevance of the potent osteoclastogenesis-promoting effects was shown by co-culture assays of Id-2 overexpressing RASF with bone marrow cells leading to an increased differentiation of osteoclasts from bone marrow precursors.

Conclusion: The data show that hypoxic conditions are present at sites of inflammation and synovial invasion into bone in arthritic synovium. Hypoxia induced Id-2 may contribute to joint destruction in RA patients by promoting synovial fibroblast-dependent osteoclastogenesis.

Introduction

A number of studies have suggested that the microenvironment of the rheumatoid synovium is ischemic and hypoxic (1). This hypothesis is supported by the analysis of the oxygen tension in the synovial fluid, which demonstrated that the mean oxygen levels in patients with rheumatoid arthritis (RA) are reduced compared to healthy controls. In addition, indirect markers of hypoxia have been found in the RA synovium including an increased level of lactate together with acidosis (2).

In general, the expression of hypoxia-regulated genes is mediated by a highly conserved transcription factor called hypoxia inducible factor (HIF), which consists of one of the hypoxia stabilized alpha subunits (HIF-1α, HIF-2α , HIF-3α) and an ubiquitously expressed beta subunit (HIF-1β) (1). HIF-1α is expressed in RA synovial biopsies, but not in synovial biopsies from patients with osteoarthritis (OA) (3). Moreover, data obtained by Cramer et al. provide a strong link between hypoxia signaling and synovial inflammation (4). However, the relevance of low oxygen levels to joint destruction (one of the key features of RA) has not been addressed.

The inhibitor of differentiation (Id-2) is a member of the helix-loop-helix (HLH) protein family (Id 1-4) forming high-affinity heterodimers with basic HLH transcription factors (bHLH). Id proteins lack a basic amino acid domain and are therefore unable to bind to DNA. Binding of Id proteins to bHLH inhibits dimerization of bHLH with other transcription factors, thereby leading to the inhibition of the transcription of specific genes driven by bHLH (5). The interaction of Id-2 with bHLH transcription factors plays a critical role in determining cell lineage and cell phenotype (6-10).

In this study, we used the hypoxia marker EF-5, which is well established for the detection of hypoxia in tumors (11), to analyze the presence and distribution of hypoxic sites in

the collagen-induced arthritis model of RA. We showed that hypoxic cells can be found in inflammatory infiltrates and at sites of synovial invasion into bone. We identified the inhibitor of differentiation (Id-2) as a downstream molecule of hypoxia in synovial fibroblasts. Consistent with the distribution of hypoxia in synovium, Id-2 was strongly expressed by synovial fibroblasts at sites of invasion into bone in synovial biopsies from patients with RA. Functional co-culture experiments with Id-2 transfected cells showed that overexpression of Id-2 in synovial fibroblasts significantly promotes the differentiation of osteoclasts from bone marrow precursors. Taken together, these data suggest that Id-2 induced by hypoxia at sites of synovial invasion into bone may trigger bone destruction by fibroblast-dependent osteoclastogenesis in RA.

Material and Methods

Patients

Synovial tissue specimens and bone marrow cells were obtained during synovectomy and arthroplastic surgery from consented patients with RA, OA, and from trauma patients (Clinic of Orthopedic Surgery, Schulthess Hospital, Zurich). The study protocol was approved by the local ethics committees.

Collagen induced arthritis

CIA was induced by immunising male DBA/1 mice (8-10 weeks old) by intradermal injection of 150 µg of chicken collagen type II (Sigma-Aldrich, St. Louis, MO) emulsified in complete Freund's adjuvant (CFA, Sigma). Mice were then challenged with 150 µg of collagen II in CFA on day 21 (12). Control group received an injection of PBS. DBA/1 mice were bred and housed at the animal facility of the Institute of Rheumatology, Warsaw, Poland.

Administration of EF-5

The hypoxia marker, EF-5 (Radiation Oncology Imaging Service Center, University of Pennsylvania, PE, USA) was administered intraperitoneally to CIA mice and control mice 14 days after the second immunization (10 µl of 10 mM EF-5 in 0.9% saline / g body weight) (13). In viable, hypoxic cells but not in necrotic cells, EF-5 is biochemically reduced to a product that binds covalently to thiols of proteins (11). Specific monoclonal antibodies (ELK3-51, University of Pennsylvania) recognizing adducts of EF-5 in cells allow its detection (11). Four hours after the injection, all mice were sacrificed and paws were snap-frozen and store at -80 ^{0}C for further analysis. All animal experiments in this study were carried out in accordance with the Polish Home Office guidelines.

Cell cultures

Human synovial fibroblasts, skin fibroblasts, wild type mouse embryonic fibroblasts (MEF) and MEF lacking HIF-1α (kindly provided by R. Johnson (14)) were cultured under standard normoxic conditions (5% CO_2, 74% N_2, 21% O_2) as described (15). For hypoxic conditions, cells were incubated in a hypoxic workstation ($InVivo_2$-400, Ruskinn Technology, Leeds, UK) with continuous flow of a gas mixture (either of 1% O_2, 5% CO_2, 94% N_2 or 0.3% O_2, 5% CO_2, 94.7% N_2). Cells were incubated under hypoxic or normoxic conditions for 48 hours or 4 weeks. In some experiments, after 48h of hypoxia, cells were exposed to 4h normoxia.

Gene screening methods

Two RA synovial fibroblasts (RASF) lines were cultured for 48h under normoxic (20% oxygen) and hypoxic (1% oxygen) conditions followed by RNA isolation. Subtractive hybridization was performed as described previously (16) with normoxic cDNA as a tester. The Atlas human 1.2 gene array No. 7850-1 (BD Clontech, Palo Alto, CA, USA) was applied to the cDNA array experiment. Data were evaluated by using AtlasImage 2.0 software (Clontech). (17)

SYBR Green and TaqMan Real-time PCR

The expression of genes were quantified by SYBR Green Real-time PCR or TaqMan Real-time PCR as described (15, 18). Sequences of primers and probes are shown in Supplementary Table 1 and 2.

Western blotting

Nuclear proteins (50 µg/well) isolated from RASF were separated on 15% SDS-PAGE and transferred onto PVDF (Bio-Rad, Hercules, CA, USA). Membranes were incubated with rabbit anti-Id-2 antibodies (dilution 1/200, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Bands were detected by further incubation with peroxidase-conjugated swine anti-rabbit IgGs (dilution 1/3000, Dako, Glostrup, Denmark) followed by visualization using the ECL system (Amersham, Uppsala, Sweden). Control membranes were incubated with anti-nucleoporin p62 antibodies (1/500, BD Pharmingen, San Diego, CA, USA) or mouse anti-α-tubulin antibodies (1/1000, Sigma).

Immunohistochemistry on paraffin embedded sections

Human and mouse tissues were prepared as described (19) and stained with (I) rabbit anti-human/murine Id-2 antibodies (1 µg/ml, Santa Cruz) or (II) double stained as described previously (15) with rabbit anti-human/murine Id-2 antibodies and mouse anti-human vimentin Abs (10 µg/ml, Dako) or (III) control IgGs, followed by incubation with alkaline phosphatase conjugated goat anti-mouse-IgG antibodies (dilution 1/40, Dako), and peroxidase conjugated goat anti-rabbit IgGs (dilution 1/1000, Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Id-2 positive cells were counted in the lining and sublining layer in four randomly selected fields of each section at x 20 magnification.

Immunohistochemistry on frozen sections

Sections from mouse paws were prepared and mounted onto slides which were incubated with biotinylated ELK3-51 antibodies (25 µg/ml) or with mouse IgG1 (Dako) for control. Next,

sections were incubated with Avidin DH and a biotinylated horseradish peroxidase macromolecular complex (ABC system, Vector Laboratories, Burlingame, CA, USA).

Transfection of RASF with Id-2 plasmid, Id-2 lentivirus, HIF-1α siRNA, HIF-2α siRNA and Id-2 siRNA

Id-2 cDNA (Gen Bank number: D13891) was cloned into pcDNA 3.1+ (Invitrogen, Carlsbad, CA, USA) and into pLenti6/UbC/V5-Dest (K4990-00, Invitrogen) using Hind III and Xho I restriction enzymes (Invitrogen). RASF were transfected with the Id-2 pcDNA or mock plasmid (2µg/5x10^5cells) by nucleofection (Amaxa Biosystem, Cologne, Germany) or by Id-2 lentivirus particles or control lentivirus particles (10^5 dilution of the stock) by using Lipofectamine 2000 (Invitrogen). To obtain stable transfected cells, culture medium was replaced once per week with fresh medium containing geneticin (300µg/ml) for plasmid transfected cells or with blasticidin (1.6 µg/ml) for lentivirus transfected cells. Cells were maintained in culture for at least 2-3 passages (1 month) prior to harvesting. Transfection of RASF with predesigned siRNA targeting HIF-1α (Ambion, Huntingdon, UK) or HIF-2α or Id-2 (20) was performed as previously described. Briefly, transfection of cells was performed by nucleofection. Six hours after transfection, the medium was changed, and the cells were either exposed to hypoxia (1% oxygen) or were cultured under normoxic conditions. After 48 hours, RNA was isolated and analyzed by Real-time PCR as described (15) (18).

Coculture of RASF with bone marrow cells

Bone marrow cells from three donors or Saos-2 cells (1 x 10^6 per well) were seeded in 12 well plates on transwell inserts with polyester membranes with 0.4 µm pore size (Costar, Cambridge,

MA). Bone marrow cells were directly used for the experiments without further manipulation. RASF stably transfected with the Id-2 plasmid or mock plasmid were seeded on the plate at a density of 8 x 10^4 per well. Experiments were performed in α-MEM as described (21). In some experiments, anti-M-CSF (25 μg/ml), anti-BMP-2 (25 μg/ml), anti-PTHrP (25 mg/ml) or isotype controls were added at the beginning of the co-culture. Total RNA from bone marrow cells and Saos-2 was isolated as indicated. TRAP staining was performed on day 7 and 14 (21).

TRAP staining

Membranes from the upper compartment of the transwell system containing bone marrow cells and sections from CIA mice (paraffin embedded tissue) were incubated with TRAP substrates (Sigma). Bone marrow cells cultured in the presence of RANKL and M-CSF (both 20 ng/ml) served as a positive control. As a negative control a membrane without TRAP substrates was used. Quantification of osteoclasts was performed by counting TRAP-positive cells (violet signal) in four randomly selected fields at x 20 magnification.

Statistical analysis

Data are shown as mean ± SEM. Statistical analysis was performed using the Mann-Whitney U test.

Results

The hypoxia marker EF-5 accumulates at sites of synovial invasion into bone in mice with collagen-induced arthritis

The hypoxia marker EF-5 or 0.9% saline was administered to control mice and CIA mice (13). Synovial tissue specimens from control mice and CIA mice, both injected with 0.9% saline, and control mice injected with EF-5 **(Fig. 1A and data not shown)** showed no staining after incubation with antibodies recognizing EF-5 cell protein adducts. In contrast, all CIA mice injected with EF-5 showed strong staining for EF-5 adducts in the synovium **(Figs. 1B-E)**. Notably, the strongest EF-5 staining was detected at sites of inflammation **(Fig. 1C)** and at sites of synovial invasion into bone **(Figs. 1C-E).** CIA tissue specimens incubated with isotype controls showed no staining **(Figs. 1F).** These findings indicate that synovial cells, in particular cells in inflammatory infiltrates and cells invading bone, are exposed to reduced oxygen concentrations.

Genes regulated by hypoxia in rheumatoid arthritis synovial fibroblasts (RASF)

To evaluate the molecular effects of the hypoxic environment on RASF, two established gene expression-screening methods were used: cDNA array and reverse suppression subtractive hybridization. Eleven genes with an induction/suppression ratio >1.4 (after global normalization) and with a signal threshold of 200% above background (17) **(supplementary Table 3**) were obtained. Reverse suppression subtractive hybridization method was used to screen for genes that had not been fully characterized and were not included in the array. Three additional genes were identified as downregulated under hypoxic conditions. DNA sequencing revealed that one

of those genes was unknown gene (clone3, cDNA nr FLJ14901 fis), the second one encoded for the signal recognition particle 54 (SRP 54) and the third one encoded for ATP synthase. To verify the results obtained from the screening, the differential expression of the identified genes was evaluated by quantitative Real-time PCR in additional RASF cultures. Six out of 11 genes (54%) obtained in cDNA array analysis and 2 out of 3 genes obtained from subtractive hybridization were confirmed to be regulated by hypoxia in all tested RASF samples. RASF treated with hypoxia (1% oxygen) for 48h expressed significantly higher levels of vascular endothelial growth factor (VEGF), Id-2, neurotrophin-3 (NT-3) and insulin like growth factor binding protein-3 (IGF-BP3) compared to cells cultured in normoxic conditions **(Supplement Fig. 1A)**. In parallel, after 48h of hypoxia the expression of proliferating cell nuclear antigen (PCNA), growth-related c-myc-responsive gene (RLC), and FLJ14901 fis cDNA (clone3) was significantly decreased in these cells **(Supplement Fig. 1B)**.

It is likely that synovial fibroblasts in RA joints are constantly exposed to low oxygen levels and that 48h of hypoxic condition may not adequately resemble the situation in vivo. Therefore, in the next set of experiments, RASF were cultured in hypoxic conditions (1% oxygen) for 4 weeks. After this time the expression of VEGF, Id-2, NT-3 and IGF-BP3 was increased. Moreover, the expression of Id-2 was significantly higher after 4 weeks of hypoxia than after 48h (4.6 ± 1.1 fold versus 2.4 ± 0.2 fold) **(Supplement Fig. 1A)**. Interestingly, exposure to long-term hypoxia decreased the expression of SRP 54 and FLJ14901 fis cDNA (clone3) compared to 48h exposure (21.9 ± 6.3 fold versus 2.0 ± 0.1 fold) **(Supplement Fig. 1B)**.

Hypoxia is a potent regulator of Id-2 expression

Among several genes that were found to be affected by hypoxia in our screening, Id-2 gene was particularly interesting as it belongs to the family of transcriptional regulators. To analyze the effects of hypoxia on all four members of the Id family, SYBR Green Real-time PCR, with specific primers, was performed. Hypoxia up-regulated Id-2 expression in both RASF (3.24 ± 0.7 fold), and to a lower extend, in OASF (n=5, 2.32 ± 1.1 fold, data not shown). Hypoxia did not induce Id-2 expression in primary skin fibroblasts (n=5, 1.21 ± 0.2 fold, data not shown). The induction of Id-2 mRNA expression in RASF was dose-dependent **(Fig. 2A)**. On the contrary, the expression of other members of the Id family was not affected (Id-1) or only slightly modulated by severe hypoxia (Id-3) in RASF **(Fig. 2A)**. Expression of Id-4 was not detectable. The induction of Id-2 by hypoxia in RASF was confirmed by immunoblotting, which showed a consistent induction of Id-2 protein at 1% oxygen compared to RASF cultured in normoxic conditions **(Fig. 2B).** To explore the stability of Id-2 expression triggered by hypoxia, RASF that had been exposed to hypoxia for 48h were incubated in normoxic conditions for an additional 4h. Re-oxygenation decreased the expression of Id-2 by $54\pm12\%$ compared to the levels obtained after 48 h of hypoxia (data not shown).

Because HIF-1 is the major transcription factor mediating the intracellular effects of hypoxia, we next evaluated the role of HIF-1α in the expression of Id-2. Mouse embryonic fibroblasts (MEF) deficient for the HIF-1α gene (HIF-1α -/-) as well as wild-type fibroblasts (HIF-1α +/+) were cultured under hypoxic and normoxic conditions. As shown in **Fig. 2C,** hypoxia induced the expression of Id-2 in both HIF-1α +/+ as well as in HIF-1α -/- fibroblasts. To confirm these data, RASF were transfected with siRNA against HIF-1α and exposed to hypoxia. HIF-1α protein was inhibited by HIF-1α specific siRNA but not control siRNA (data

not shown). Again, the induction of Id-2 by hypoxia was not affected by inhibition of HIF-1 α signaling **(Fig. 2D)**. Altogether, these data suggest that HIF-1α does not play an essential role for the hypoxia induced expression of Id-2. Next, we looked at effects of HIF-2α inhibition by siRNA. In contrast to HIF-1α, inhibition of HIF-2α decreased the hypoxia-induced induction of Id-2 by 54 ± 11% as compared to mock-transfected controls, suggesting that HIF-2α might at least partially mediate the hypoxia-induced expression of Id-2 **(Fig. 2E)**

Id-2 is overexpressed in the synovium of patients with rheumatoid arthritis

Immunohistochemistry was performed on synovial specimens from RA, OA and trauma patients. Id-2 protein was expressed at higher levels in the synovium of RA patients than in the synovium from OA and trauma patients (32 ± 13% positive cells in RA synovium, 18 ± 11% in OA synovium and 8 ± 3% in normal synovium, $p < 0.05$) **(Figs. 3A-D)**. Some RA patients showed a strong expression of Id-2 in the sublining layer **(Fig. 3A),** while in others, Id-2 was mainly found in the lining layer **(Fig. 3B)**. In addition, Id-2 was expressed in blood vessels of all synovial specimens as described before (22). Consistent with the EF-5 data, which suggested that hypoxia occurs at sites of inflammation, expression of Id-2 in the sublining layer was most abundant at sites of inflammatory infiltrates.

Id-2 is expressed by synovial fibroblasts at sites of synovial invasion into bone

Based on the EF-5 experiments, which revealed that the lowest oxygen levels were present at sites of synovial invasion into bone, we next investigated the expression of Id-2 in tissue specimens from RA patients containing the bone-synovium interface. A strong and abundant expression of Id-2 protein was found at sites of synovial invasion into bone **(Figs. 4A).**

Similar results were obtained with tissue specimens from CIA mice **(Figs. 4B)**. To characterize the cell types expressing Id-2 protein double immunohistochemistry with antibodies against Id-2 and against vimentin (a marker for fibroblasts) was performed. As shown in **Figs. 4C**, cells expressing Id-2 and invading bone were predominantly fibroblasts. In addition, immunostaining for Id-2 and TRAP on subsequent slides indicated that Id-2 positive fibroblasts were located closely to bone-degrading TRAP positive osteoclasts. Interestingly, multinuclear, TRAP positive osteoclasts also expressed Id-2 **(Fig. 4D-E).**

Id-2 triggers the expression of genes involved in bone turnover in rheumatoid arthritis synovial fibroblasts

RASF invading bone are a source of numerous factors regulating bone turnover (23, 24). To test the hypothesis that Id-2 regulates the expression of these factors at sites of invasion in RA patients, we overexpressed Id-2 in RASF **(Fig. 5A)**. Indeed, a large number of genes involved in bone turnover were expressed at significantly higher levels in Id-2 transfected RASF than in mock-transfected controls **(Fig. 5B).** The majority of these factors are known to support bone degradation and osteoclastogenesis. These include macrophage-colony stimulating factor (M-CSF) (23, 25), parathyroid hormone related protein (PTHrP) (26, 27) as well as factors having a dual role in bone turnover such as bone morphogenic protein-2 (BMP-2) (28, 29) **(Fig. 5B)**. In addition, there was also a small, but significant induction of osteoprotegerin (OPG), which is known to inhibit osteoclastogenesis (30, 31). Other molecules regulating bone-turnover such as cyclooxygenase-2 (COX-2) (32, 33) and metalloproteinase-13 (MMP-13) (34, 35) were not changed in Id-2 transfected RASF. The expression of the receptor activator of NF-κB ligand (RANKL) (25, 36) was below the detection level of the Real-time PCR in both mock and Id-2

transfected RASF. However, the expression of this molecule was easily inducible upon TNFα stimulation in both mock and Id-2 transfected cells (data not shown). We also investigated whether silencing of basal expression of Id-2 in RASF by specific siRNA **(Fig. 5C)** is able to influence the expression of genes involved in bone turnover. Indeed, the most consistent downregulation was found for PTHrP, which was inhibited by 49.6 ± 2.8% ($p < 0.05$) and for BMP-2 **(Fig. 5D).**

Rheumatoid arthritis synovial fibroblasts overexpressing Id-2 promote the induction of osteoclastogenesis

Given that the majority of factors induced by Id-2 in RASF promote osteoclastogenesis, we hypothesized that RASF overexpressing Id-2 are able to favor the development of osteoclasts from their bone marrow precursors. RASF transfected either with Id-2 or mock vector were cocultured with bone marrow cells in a transwell system and the number of TRAP positive osteoclasts in the bone marrow cell compartment was determined after 7 and 14 days. Indeed, after 7 days of culture TRAP positive, single nuclei, immature osteoclasts appeared in both cocultures **(Fig. 6A-B)** and also in control bone marrow culture stimulated with RANKL and M-CSF (both 20ng/ml) **(Fig. 6C)**. However, the number of TRAP positive, immature osteoclasts strongly increased **(Fig. 6A)** in coculture with Id-2 transfected RASF compared to bone marrow cells cocultured with mock transfected RASF **(Fig. 6B)** (185 ± 23 versus 49 ± 25, respectively, mean ± SEM per 4 randomly selected fields, $p < 0.05$). After 14 days of culture, mature multinuclear TRAP positive osteoclasts were present in bone marrow cocultured with Id-2 transfected RASF **(Fig. 6D),** but not with mock transfected RASF **(Fig. 6E)**. On the other hand, the osteoblast and bone formation marker, alkaline phosphatase (AP), remained unchanged in the

bone marrow cell compartment **(Fig. 6F)**. Taken together, these data show that overexpression of Id-2 in RASF favors the development of osteoclasts from bone marrow precursors, while not affecting the development of osteoblasts.

Next, we looked at the mechanisms by which the Id-2 overexpressing RASF mediates the differentiation of osteoclasts from bone marrow precursors. Interestingly, coculture of Id-2 transfected RASF with bone marrow cells significantly increased the expression of the pro-osteoclastogenic protein RANKL in the bone marrow compartment as compared to mock-transfected RASF **(Fig. 6F)**. In parallel, there was a significant downregulation of the RANKL inhibitor OPG in the bone marrow compartment upon coculture with Id-2 transfected RASF **(Fig. 6F)**. In addition, the effect of Id-2 overexpressing RASF on RANKL/OPG ratio in osteoblast cells was confirmed by coculture of Id-2 and mock transfected RASF with the osteoblast-like cell line Saos-2 in a transwell system. Indeed, Saos-2 cell cocultured with RASF overexpressing Id-2 showed an upregulation of RANKL and, in parallel, downregulation of OPG expression compared to cells cultured with mock RASF **(Supplement Fig. 2A)**. Since a similar effect on osteoblasts was obtained with cancer cells overexpressing PTHrP (27) we hypothesized that PTHrP might be a crucial mediator of the Id-2 induced osteoclastogenesis. Indeed, the development of TRAP-positive osteoclasts was strongly inhibited by adding anti-PTHrP antibodies **(Supplement Fig. 2B)** in coculture of RASF with bone marrow cells.

In addition, exposure of RASF to 1% hypoxia for 48h induced PTHrP expression by 2.7 ± 0.4 ($p < 0.05$) compared to normoxic controls. This indicates that hypoxia, which triggers Id-2, is able to induce the Id-2 downstream molecule PTHrP in RASF.

Discussion

Evidence from animal studies as well as indirect evidence from human studies suggest that the RA synovium is hypoxic due to inflammation and synovial hyperplasia (1, 4, 37). Using the hypoxia marker EF-5, we were able to show the distribution of hypoxic areas in arthritic joints. Interestingly, hypoxic cells were found mostly at sites of synovial invasion into bone. The invasion of synovial cells into cartilage and bone is the anatomical prerequisite for local erosion and destruction of joints, which is the most characteristic feature of RA. Notably, the avascular cartilage and the mineralized bone themselves are hypoxic tissues (38, 39). Thus, our data provide the evidence that during the invasion of synovial cells into cartilage and bone, these cells are exposed to an increasing level of hypoxia.

In agreement with previous studies, hypoxic cells were also found in inflammatory infiltrates in the synovium (40-42) These findings are an important confirmation for the landmark studies performed by Cramer et al (4), who showed that the hypoxia responsive transcription factor HIF-1α is essential for the initiation and perpetuation of inflammatory responses driven by myeloid-lineage cells. Recently, Hamada et al. further supported that concept by showing that hypoxia triggers a release of pro-inflammatory protein HMGB-1 from RASF (41).

This study and the previous data (37, 41, 42) showing that cells in the inflamed synovium are exposed to severely reduced oxygen levels have a direct impact on virtually all in vitro studies relevant to joint destruction and inflammation in RA. Thus, cell culture studies performed under normoxic conditions clearly do not reflect the hypoxic situation occurring in vivo in RA patients. Indeed, the influence of hypoxia-driven pathways has not been taken into account in the

majority of in vitro experiments in the past. In our screening experiment series we were able to identify a number of genes induced by hypoxia in synovial fibroblasts.

The inhibitor of differentiation-2 (Id-2) was the most intriguing finding because of its role in the regulation of transcription. We demonstrated that hypoxia strongly up-regulates the expression of Id-2 in RASF but not in skin fibroblasts. The selective induction of Id-2 in RASF suggests that this protein might have a specific role in the response of synovial fibroblasts to the hypoxic environment which occurs in RA synovium. Using HIF-1α-/- MEF, as well as HIF-1α silencing in RASF, we showed that hypoxia is able to induce the expression of Id-2 independently of HIF-1α signaling. In contrast, we observed a partial inhibition of Id-2 expression in cells transected with HIF-2α siRNA suggesting that that member of HIF family might be, at least partially, involved in hypoxia induced Id-2 expression in RASF. The molecular mechanisms of Id-2 induction by hypoxia might be cell-specific and the HIF-1α -independent induction might be unique for fibroblasts (43).

The in vivo relevance of these experiments was confirmed by our immunohistochemistry studies showing that Id-2 was expressed at higher amounts in synovial tissues of RA patients than either in OA synovium or normal synovium.

One of the most interesting findings of our study was the strong expression of Id-2 in RASF at sites of synovial invasion into bone in both human RA tissues as well as in CIA mice. The co-expression of the hypoxia marker EF-5 and Id-2 at sites of invasion indicates that hypoxia is indeed a potent inducer of Id-2 in vivo. Based on the observation that Id-2 was expressed by fibroblasts invading bone and in close proximity to TRAP positive osteoclasts, we hypothesized that Id-2 contributes to bone degradation. Indeed, we found that overexpression of Id-2 in RASF triggered the expression of several factors promoting osteoclastogenesis by

stimulating the differentiation of osteoclasts such as M-CSF (25) and PTHrP (27). In addition, BMP-2, which was also found to be induced by Id-2 in RASF, has a dual role in bone turnover and can be a potent inducer of osteoclast differentiation and survival in the presence of M-CSF or RANKL (29). Most interestingly, PTHrP was not only induced by Id-2 overexpression, but also directly by hypoxia and reversely reduced upon Id-2 silencing in RASF, indicating that this factor might be direct mediator of Id-2 responses.

The biological relevance of the potent osteoclastogenesis-promoting effects of RASF overexpressing Id-2 has been proven by the coculture assays of Id-2 transfected RASF with bone marrow cells. The development of osteoclasts from bone marrow precursor cells was strongly increased in the presence of Id-2 overexpressing RASF compared to controls, while the formation of osteoblasts remained unchanged. Thus, the small induction of OPG in RASF overexpressing Id-2 was not biologically meaningful and its inhibitory effect was apparently overcome by the stronger effects of the several factors promoting osteoclastogenesis. In addition, in the cocultures of Id-2 overexpressing RASF with bone marrow cells or osteoblasts, we observed an enhanced expression of RANKL accompanied by a decreased expression of OPG by bone marrow stromal cells or osteoblasts. These data suggest that the osteoclastogenesis promoting effects of RASF overexpressing Id-2 is mediated via changing the ratio of RANKL to OPG in the stromal cells. Interestingly, PTHrP, which we identified as an important downstream molecule of Id-2 in RASF, has been shown to favor the development of osteoclasts by increasing the expression ratio of RANKL to OPG in stromal cells (27). Indeed, in our coculture experiments, neutralization of PTHrP inhibited osteoclast development. Moreover, the PTHrP gene promoter contains functional E-box-like elements (44) as well as Ets binding sites (45) that

can be regulated by Id family members. Thus, these data indicate that PTHrP might mediate, at least partially, the Id-2 induced osteoclastogenesis.

It has to be emphasized that the effect of Id-2 on cell-differentiation often depends on the cell-type and origin of cells (6-10, 46). Thus, while this study shows that Id-2 promotes the synovial fibroblast-dependent osteoclastogenesis in rheumatoid arthritis, the role of Id-2 in osteoclasts of RA synovium requires further investigation. Recently, it has been shown that Id family members can function as negative regulators of RANKL induced osteoclast differentiation from bone marrow precursors in vitro. However, that inhibition could be overcome by high dose of RANKL or the presence of stromal cells (47).

In summary, we demonstrated that synovial cells are exposed to reduced oxygen levels in inflammatory infiltrates, mostly at sites of synovial invasion into bone in rheumatoid arthritis. Differential screening experiments and confirmation by Real-time PCR and immunoblotting showed that Id-2 is consistently induced by hypoxia. Id-2 was upregulated in synovial biopsies of patients with RA, and was particularly expressed by synovial fibroblasts at sites of synovial invasion into bone. Overexpression of Id-2 in RASF induced the expression of factors promoting osteoclastogenesis and strongly stimulated the differentiation of osteoclasts from bone marrow precursors. Taken together, our data show that Id-2 induced by hypoxia may contribute to joint destruction in patients with RA by promoting synovial fibroblast-dependent osteoclastogenesis.

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Figure legends

Figure 1: The hypoxia marker EF-5 accumulates in the synovium of CIA mice

Figure 1 A-F, Immunohistochemistry with ELK3-51 antibodies to detect EF-5 cell protein adducts. Sections of ankle joints in hind paws are shown. **Figure 1A**, Section from healthy control mouse**. Figure 1B-E**, Sections from CIA mice showing strong accumulation of EF5 at sites of inflammation **(C, enlarged picture,** open arrows, brown signal) at sites of synovial invasion into bone (closed arrows) **(Fig. C-E)**. **Figure 1F**, Section from CIA mouse incubated with isotype matched antibodies for control. The stainings are representative for four CIA mice and three control mice. A-B original magnification, x 10; C-D, F original magnification, x 25; E original magnification x 40. B, bone; S, synovium; C, cartilage.

Figure 2: Hypoxia induces the expression of Id-2

Figure 2A, Hypoxia induces the expression of Id-2 mRNA. Rheumatoid arthritis synovial fibroblasts (RASF, n=5) were cultured in 21%, 1% or 0.3% oxygen for 48h followed by total RNA extraction and SYBR Green Real-time PCR. Values are the mean fold change ± SEM compared to normoxic controls of 3 independent experiments. *$p<0.05$, RASF treated with 1% or 0.3% oxygen versus RASF treated with 21% oxygen. **Figure 2B,** Hypoxia increases Id-2 protein. RASF were cultured as described in Figure 2A followed by protein isolation and Western blot for Id-2 and nucleoporin p62 as a loading control. The Western blot is representative of two independent experiments. **Figure 2C,** Hypoxia induces the expression of Id-2 independently of HIF-1α signaling in mouse embryonic fibroblasts (MEF). HIF-1α+/+ MEF and HIF-1α-/- MEF were treated as described in Figure 2A. Values are the mean ± SEM of three independent experiments. *$p<0.05$, MEF cultured in 21% oxygen versus MEF cultured in 1%

oxygen. **Figure 2D**, Hypoxia induces the expression of Id-2 independently of HIF-1α in RASF **Figure 2E,** Expression of Id-2 triggered by hypoxia is partially mediated by HIF-2α. *$p<0.05$, RASF cultured in 21% oxygen versus RASF cultured in 1% oxygen.

Figure 3: Id-2 is overexpressed in rheumatoid arthritis synovium

Figures 3A-B, RA synovial tissues incubated with anti-Id-2 antibodies showing strong expression of Id-2 in the sublining layer (A) and in the lining layer (B, open arrows). **Figure 3C**, Osteoarthritis synovial tissue incubated with anti-Id-2 antibodies showing that the expression of Id-2 protein is limited to blood vessels (brown signal, closed arrows). **Figure 3D**, RA synovial tissue incubated with control IgGs. Original magnification x 20; L, lining layer; SBL, sublining layer.

Figure 4: Id-2 is expressed by synovial fibroblasts at sites of synovial invasion into bone.

Figure 4A and D, RA synovial tissue incubated with anti- Id-2 antibodies showing strong expression of Id-2 at sites of invasion into bone (brown signal). **Figure 4B**, Section of synovial tissue from CIA mouse incubated with anti- Id-2 antibodies showing strong expression of Id-2 at sites of invasion into bone. **Figure 4C**, RA tissue specimen stained with anti-Id-2 antibodies (blue signal) and anti-vimentin antibodies (red signal). **Figure 4E**, RA synovial tissue stained for TRAP (red signal). SF, synovial fibroblasts; OC, osteoclasts; S, synovium; B, bone. Bottom left corner inserts at A-C represent control tissue incubated with appropriate IgGs. Data are representative of 3 RA patients and 3 CIA mice with similar results. Original magnification x 20.

Figure 5: Id-2 triggers the expression of genes involved in bone turnover by rheumatoid arthritis synovial fibroblasts.

Figure 5A, RASF transfected with Id-2 expressed increased levels of Id-2 protein. The Western blot for Id-2 and α-tubulin is representative of three independent experiments with similar results. **Figure 5B,** Overproduction of Id-2 in RASF triggers the expression of M-CSF, PTHrP, BMP-2 and OPG. Total RNA from transfected RASF (n=3, 5 clones) was isolated and Real-time PCR was performed. Data are expressed as mean fold increase ± SEM compared to mock transfected cells of three independent experiments, *$p<0.05$, Id-2 versus mock transfected cells. **Figure 5C**, Decreased levels of Id-2 protein in RASF transfected with Id-2 siRNA. RASF were transfected with control siRNA or Id-2 siRNA, cultured for 48 followed by Western blotting with Id-2 and α-tubulin antibodies. **Figure 5D,** Inhibition of Id-2 in RASF leads to downregulation of the expression of PTHrP and BMP-2. RASF (n=3) were transfected with Id-2 siRNA or control siRNA. 48h later, total RNA was isolated and Real-time PCR was performed. Data are expressed as mean (% of control siRNA transfected control) ± SEM of two independent experiments**.***$p<0.05$, Id-2 siRNA versus control siRNA transfected cells.

Figure 6: Coculture of Id-2 transfected rheumatoid arthritis synovial fibroblasts (RASF) with bone marrow cells promotes the development of osteoclasts

Figure 6 A-B, TRAP staining of bone marrow cells (donors, n=3) cocultured with Id-2 overexpressing RASF (n=3) **(A)** or mock transfected RASF (n=3) **(B)** for 7 days. **C**, Bone marrow cells cultured in the presence of RANKL and M-CSF (both 20ng/ml) for 7 days. Bottom left corner insert represents control not stained with TRAP substrates. **D-E,** TRAP staining of bone marrow cells cocultured with Id-2 overexpressing RASF (n=3) **(D)** or mock transfected

RASF (n=3) **(E)** for 14 days**.** *p<0.05, number of osteoclasts (mean ± SEM of two independent experiments) in cocultures of bone marrow cells with Id-2 transfected RASF versus mock transfected RASF. Blue staining indicates nuclei; violet signal indicates TRAP expression. Original magnification x 20. **Figure 6F,** Coculture of Id-2 transfected RASF with bone marrow cells increases the expression of RANKL in bone marrow cells. RNA from bone marrow cells was isolated on day 4 of coculture and SYBR Green Real-time PCR for AP and TaqMan Real-time PCR for RANKL and OPG were performed. Data are expressed as mean fold change ± SEM compared to mock-transfected cells. *p<0.04 Id-2 transfected RASF versus mock-transfected cells.