

## Hypoxia appears at pre-arthritic stage and shows co-localization with early synovial inflammation in collagen induced arthritis

C.H. Jeon<sup>1</sup>, J.-K. Ahn<sup>5</sup>, J.-Y. Chai<sup>2</sup>, H.J. Kim<sup>3</sup>, E.-K. Bae<sup>3</sup>, S.H. Park<sup>4</sup>, E.Y. Cho<sup>4</sup>, H.-S. Cha<sup>5</sup>, K.-S. Ahn<sup>3</sup>, E.-M. Koh<sup>5</sup>

<sup>1</sup>Department of Medicine, Soonchunhyang University College of Medicine, Bucheon;

<sup>2</sup>University of Ulsan College of Medicine, Ulsan; <sup>3</sup>Center for Molecular Medicine, Samsung Biomedical Research Institute, Seoul; <sup>4</sup>Department of Pathology, Samsung Medical Center Sungkyunkwan University School of Medicine, Seoul; <sup>5</sup>Medicine, Samsung Medical Center Sungkyunkwan University School of Medicine, Seoul, Korea.

Chan Hong Jeon, MD; Joong-Kyung Ahn, MD; Ji-Young Chai, MD; Hyun Jung Kim; Eun-Kyung Bae; Sang Hui Park, MD; Eun Yoon Cho, MD; Hoon-Suk Cha, MD; Kwang-Sung Ahn, PhD; Eun-Mi Koh, MD.

This work was supported by the Korea Science and Engineering Foundation (KOSEF) grant funded by the Korea government (# R01-2004-000-11007-0).

Please address correspondence and reprints requests to: Dr. E.M. Koh, Department of Medicine, Samsung Medical Center, Sungkyunkwan University School of Medicine, 50 Ilwon-Dong, Kangnam-Ku, Seoul 135-710, Korea. E-mail: eunmi.koh@samsung.com

Received on February 14, 2007; accepted in revised form on December 13, 2007.

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**Key words:** Rheumatoid arthritis, collagen induced arthritis, hypoxia.

## ABSTRACT

**Objective.** *The presence of hypoxia in rheumatoid synovium has been well known, but exact correlation between hypoxia and synovitis is unclear. The aim of our study was to investigate the time and spatial relationship and the correlation of severity between hypoxia and synovitis in pre-arthritic or early stage of inflammatory joint disease.*

**Methods.** *DBA/1J mice were injected intradermally with type II collagen and adjuvant solution to induce arthritis; mice injected with only adjuvant were used as a control group. CIA and control mice were sacrificed weekly after the injection to evaluate serial pathological changes. H&E stain and hydroxyprobe-1 stain were performed to look at the status of inflammation and hypoxia.*

**Results.** *In serial observations of tissue pathology, we could note the inflammation of synovium developing a week after the injection of type II collagen. Hypoxic change, measured by the hydroxyprobe-1 stain, was also identified in synovium as early as 1 week after the collagen injection, prior to clinically evident arthritis. In addition, we could observe that inflammation and hypoxia co-localize in the synovium and there was a positive correlation between the severity of hypoxia and the degree of synovitis.*

**Conclusion.** *Our results demonstrate that hypoxia takes place in synovium at the pre-arthritic stage of disease and have a close spatial relationship and a positive severity correlation with synovitis.*

## Introduction

Hypoxia is an important factor in the pathogenesis of RA, and some details of its role in inflammation and tissue damage have been identified (1, 2). But there is not sufficient information about when hypoxia appears in the synovium or how it is related with pathological and clinical changes. Since we thought that serial observation of the events occurring in synovitis might provide more information for the above questions, we intended to observe the time-sequence of hypoxia and its spatial and severity relationship to the inflammation in synovium of murine collagen induced arthritis (CIA).

## Materials and methods

DBA/1J mice (Charles River Japan Laboratories Inc., Tokyo, Japan), 7 weeks old and weighing 15-19 grams, were used. To induce arthritis, type II collagen (Chondrex Inc., Redmond, WA, USA) emulsified in complete Freund's adjuvant was injected to CIA mice (n=41) intradermally at the base of the tail. A second group of mice (n=12), injected only with adjuvant, served as controls. Disease progression was monitored weekly using a 4-point clinical scoring scale (0, no swelling or redness; 1, swelling/redness of paw or one joint; 2, two joints involved; 3, more than two joints involved; and 4, severe arthritis of the entire paw and joints). The total score for clinical disease activity was the sum of the scores of all 4 paws (maximum score = 16). At the 1st and 2nd week after the injection, each a mouse from arthritis group and control group was sacrificed to see early pathological changes. From the 3rd week after the injection, 5 mice from the arthritis group and 2 mice from the control group were randomly selected and sacrificed weekly. For the identification of hypoxia in synovial tissue, we used hydroxyprobe-1 (1) and all mice were injected intraperitoneally with 0.8mL/20g of hydroxyprobe-1 (Chemicon International, Temecula, CA, USA) 30 minutes prior to death.

All synovia from mice had been decalcified in 10% EDTA (pH7.5) and cut at a thickness of 4µm. Sections were firstly stained with H&E to evaluate the development of synovitis.

Hydroxyprobe-1 immunostain of synovium was performed according to the manufacturer's instructions using mouse monoclonal anti-hydroxyprobe antibody (Chemicon International, Temecula, CA, USA). Bound antibody was detected using a labeled streptavidin biotin 2 (LSAB 2) kit (Dakocytomation co. Carpinteria, CA, USA), and phosphatase activity was shown using a fast red substrate.

To score the severity of synovitis, we adopted criteria introduced by Koizumi *et al.* (3) modifying the scoring system with selecting the degree of cell proliferation and infiltration by inflammatory cells into the synovial stroma (Table I).

Competing interests: none declared.

**Table I.** Scoring of synovitis in collagen induced arthritis of mouse.

Pathology change	Score
Proliferation of synovial cells	
Slight (2 layers)	1
Moderate (3-4 layers)	2
Marked (>5 layers)	3
Proliferation of synovial stroma	
Inflammatory cell infiltration	
Slight	1
Moderate	2
Marked	3
Synovial granulation tissue	
Slight	1
Moderate	2
Marked	3
Synovial fibrin	2

In developing comparisons of the pathologic changes of synovitis, we used the total score, the sum of each of the items.

We also scored the degree of hypoxia by measuring the extent of hydroxyprobe-stained cell distribution; *i.e.*, synovial lining only, lining to the sublining stroma, and lining to the deep stroma. Scores 0 to 3 were given. A pathology expert reviewed each slide twice, and scored both the synovitis and hypoxia. For the statistical analysis of the relation between degree of synovitis and hypoxia extent, we used Kruskal-Wallis test and Mann-Whitney U-test.

## Results

Clinically detectable swelling and redness was present in the distal joints of the feet starting at week 4. As shown in Figure 1, arthritis, as measured by arthritis score, increased in severity and led to ankle joint deformation by week 7 to 8.

Histological changes were seen beginning even 1 week after the injection preceding the appearance of clinically apparent arthritis (Fig. 1). The early changes in the synovium involved mild proliferation of synovial lining cells. At 4 to 5 weeks after the injection, proliferation of synovial cells was evident and infiltration by mononuclear cells into synovial stroma was noted. At about 7 to 8 weeks after the injection, severe proliferation of synovial cells and a dense infiltration of inflammatory cells, extending into adjacent bone and cartilage was seen.

The onset of hydroxyprobe-1 staining of synovial cells also occurred around 1 week following the injection of collagen before clinically evident arthritis (Fig. 1). Cells of the synovial lining were more intensely stained, but stromal cells were also stained with hydroxyprobe. Extent and density of hydroxyprobe stain gradually increased up to 7 to 8 weeks after injection, colocalizing with the synovitis illustrated by H&E staining.

When we observed the correlation between synovitis and hypoxia by comparing synovitis score among groups with different hypoxia score, synovitis scores were higher in the groups with more severe hypoxia score (2 or 3) than groups with less hypoxia score (1 or less) and it was statistically significant (test for difference between group with score 2 and group with score 0-1,  $p=0.0017$ ; 3 and 0-1,  $p=0.0003$ ; 2-3,  $p=0.0079$ ).

## Discussion

The hypoxic state in inflamed joints has been proved by direct measurements of oxygen tension and other biochemical studies (4, 5).

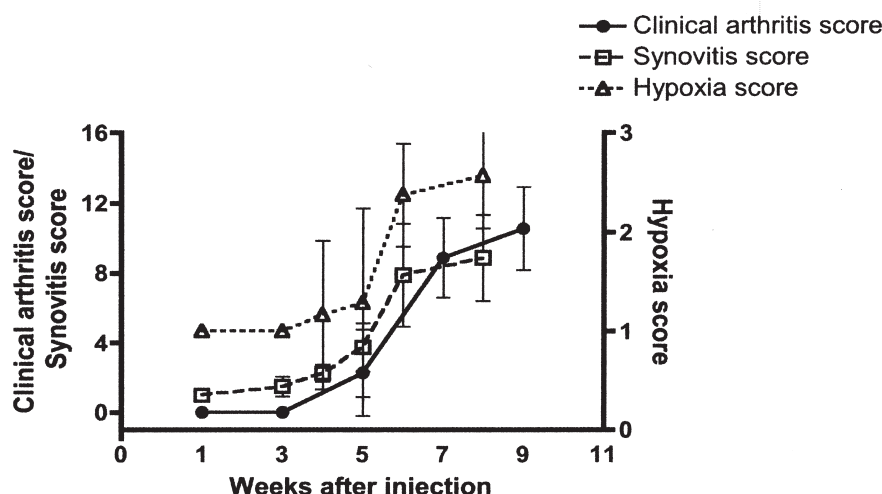
The most prominent effects of hypoxia in arthritis are associated with angiogenesis. Tissue hypoxia in the rheumatoid joint results in increased VEGF mRNA stability and enhanced VEGF gene transcription through the binding of HIF-1 and HIF-2 and these transcription factors contributing to neovascularization are over-expressed in the lining and stromal cells of rheumatoid synovium (6).

Hypoxia promotes the production of matrix MMPs and oxygen radicals thus damaging joints. Rheumatoid synovial fibroblasts cultured in hypoxic conditions increase the expression of MMP-1 and MMP-3, with a decreasing expression of TIMP-1 (7) and repeated hypoxia-reoxygenation cycles produced by joint movement in dysfunctional synovial vessels may contribute to the development of reactive oxygen species (6).

Hypoxia is also related with activation and maintenance of inflammatory cells. Hypoxia influences myeloid cell function via HIF (8, 9) and delay neutrophil apoptosis (10), which may interrupt the resolution of inflammation.

Hypoxia in rheumatoid synovium is thought to result from the following factors: the rapid rate of synovial proliferation exceeding the rate of angiogenesis (11), increased metabolic demand due to inflammation (12), and the increase in intra-articular pressure in the affected joint which diminishes the perfusion pressure (13).

Although possible causes of hypoxia have been suggested, so far few studies



**Fig. 1.** Progression of clinical arthritis, synovitis score, and hypoxia score. Clinically apparent arthritis was found after 4 weeks following the injection of collagen and the arthritis score showed a gradual increase, peaking at the 9<sup>th</sup> week. Control mice showed no detectable arthritis. Microscopic synovial inflammation was found 1 week after the injection of collagen and hypoxic change was also found as soon as the synovitis appeared. The synovitis score and the hypoxia score were both increased gradually. Values are given as mean  $\pm$  standard deviation.

have looked at when hypoxia appears and where it is located in the synovium. In serial observation of joint pathology, we could note the development of synovitis by 1 week after the collagen injection, prior to clinically evident arthritis. Hypoxic change, measured by hydroxyprobe-1 stain also appeared 1 week after the injection, as the microscopic synovitis take place and it was more noticeable in the lining layer of the synovium, colocalizing with the sites of inflammation. As the synovitis became worse, the extent of hydroxyprobe stain was also increased. Based on the above findings, we could postulate that hypoxia develops even in the pre-clinical stage of synovitis and, reciprocally affecting each other, worsened inflammation and hypoxia may contribute to the establishment and progression of disease.

The result of our experiment is different from a previous study which looked at the onset of hypoxia in CIA using microelectrode to measure intra-articular oxygen tension (4). In that experiment, hypoxia was detected when the clinical arthritis had established and there were no differences of joint oxygen tension between normal and pre-arthritis mice. We think that the discrepancy of result could happen due to factors as follow. If the hypoxic change in pre-arthritis or very early stage CIA is localized in site of inflammation without even distribution, it might not be detected well by microelectrode. Furthermore, if the amount of oxygen tension change in pre-arthritis stage were too little, it would not be detected by probe or could fail to have statistical significance. There have been a few reports of lack of correlation between oxygen electrode measurement and histological

marker of hypoxia including hydroxyprobe (14, 15). In our experiment, by using hydroxyprobe stain, we could detect very subtle hypoxic changes and could also get information about the location of hypoxia in the synovium.

We think the major limitation of our study is the small number of mice used in the experiment. For mice were sacrificed weekly after the collagen injection, data in a more detailed time frame such as daily or hourly could not be obtained. Furthermore, although we could see synchronism and co-localization of hypoxia and synovitis in mice before clinical arthritis began, we do not have enough information on the factors connecting the hypoxia and synovitis at this stage. To identify in more detail the events related to hypoxia, we believe that different approaches may be needed, for example, looking for genes or proteins that are turned on or off under hypoxia using tools such as DNA chip analysis.

In conclusion, we were able to see that hypoxia appears before clinically evident arthritis and has a close time and spatial relationship with starting synovitis in CIA and we think that a more detailed study on the role of hypoxia in early stage inflammation might give more information about the pathogenesis of RA.

## References

- PETERS CL, MORRIS CJ, MAPPI PI, BLAKE DR, LEWIS CE, WINROW VR: The transcription factors hypoxia-inducible factor 1alpha and Ets-1 colocalize in the hypoxic synovium of inflamed joints in adjuvant-induced arthritis. *Arthritis Rheum* 2004; 50: 291-6.
- STEINBRECH DS, LONGAKER MT, MEHRARA BJ *et al.*: Fibroblast response to hypoxia: the relationship between angiogenesis and matrix regulation. *J Surg Res* 1999; 84: 127-33.
- KOIZUMI F, MATSUNO H, WAKAKI K, ISHII Y, KURASHIGE Y, NAKAMURA H: Synovitis in rheumatoid arthritis: scoring of characteristic histopathological features. *Pathol Int* 1999; 49: 298-304.
- ETHERINGTON PJ, WINLOVE P, TAYLOR P, PALEOLOG E, MIOTLA JM: VEGF release is associated with reduced oxygen tensions in experimental inflammatory arthritis. *Clin Exp Rheumatol* 2002; 20: 799-805.
- JAMES MJ, CLELAND LG, ROFE AM: Determinants of synovial fluid lactate concentration. *J Rheumatol* 1992; 19: 1107-10.
- TAYLOR PC, SIVAKUMAR B: Hypoxia and angiogenesis in rheumatoid arthritis. *Curr Opin Rheumatol* 2005; 17: 293-8.
- CHA HS, AHN KS, JEON CH, KIM J, SONG YW, KOH EM: Influence of hypoxia on the expression of matrix metalloproteinase-1, -3 and tissue inhibitor of metalloproteinase-1 in rheumatoid synovial fibroblasts. *Clin Exp Rheumatol* 2003; 21: 593-8.
- MURDOCH C, MUTHANA M, LEWIS CE: Hypoxia regulates macrophage functions in inflammation. *J Immunol* 2005; 175: 6257-63.
- CRAMER T, YAMANISHI Y, CLAUSEN BE *et al.*: HIF-1alpha is essential for myeloid cell-mediated inflammation. *Cell* 2003; 112: 645-57.
- CROSS A, BARNES T, BUCKNALL RC, EDWARDS SW, MOOTS RJ: Neutrophil apoptosis in rheumatoid arthritis is regulated by local oxygen tensions within joints. *J Leukoc Biol* 2006; 80: 521-8.
- STEVENS CR, BLAKE DR, MERRY P, REVELL PA, LEVICK JR: A comparative study by morphometry of the microvasculature in normal and rheumatoid synovium. *Arthritis Rheum* 1991; 34: 1508-13.
- TAYLOR PC: Serum vascular markers and vascular imaging in assessment of rheumatoid arthritis disease activity and response to therapy. *Rheumatology (Oxford)* 2005; 44: 721-8.
- STEVENS CR, WILLIAMS RB, FARRELL AJ, BLAKE DR: Hypoxia and inflammatory synovitis: observations and speculation. *Ann Rheum Dis* 1991; 50: 124-32.
- JANKOVIC B, AQUINO-PARSONS C, RALEIGH JA *et al.*: Comparison between pimonidazole binding, oxygen electrode measurements, and expression of endogenous hypoxia markers in cancer of the uterine cervix. *Cytometry B Clin Cytom* 2006; 70: 45-55.
- NORDSMARK M, LONCASTER J, AQUINO-PARSONS C *et al.*: Measurements of hypoxia using pimonidazole and polarographic oxygen-sensitive electrodes in human cervix carcinomas. *Radiother Oncol* 2003; 67: 35-44.