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REVIEW ARTICLE

**Atsushi Hashimoto · Toshimichi Matsui · Sumiaki Tanaka
Akira Ishikawa · Hirahito Endo · Shunsei Hirohata
Hirobumi Kondo · Elena Neumann · Ingo Helmut Tarner
Ulf Müller-Ladner**

Laser-mediated microdissection for analysis of gene expression in synovial tissue

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Abstract In experimental rheumatology, *transcriptomics* is one of the most important methods for investigating the pathogenesis of diseases. The biological material of most studies on rheumatoid arthritis has been bulk rheumatoid synovial tissues, but they are not suitable because they consist of several kinds of cells or structures. Laser-mediated microdissection (LMM) is a useful tool for isolating particular cells from tissue specimen to assess the functions of each cell. The LMM system employs a combination of a microscope and a laser-beam generator to cut out target areas on cryosections. Tissue compartments or even a single viable cell can be isolated using a non-focused laser beam without direct contact to avoid contamination, and this process is called laser pressure catapulting. An ultraviolet-A laser enables target cells to be procured without any influence on the surrounding. This technique has already been used in several studies in rheumatology, and its validity has been confirmed. Combined with other new techniques such as real-time quantitative polymerase chain reaction or microarray analysis, LMM is becoming more important in the analysis of gene expression in rheumatology.

Key words Laser capture microdissection · Laser-mediated microdissection · Rheumatoid arthritis

A. Hashimoto (✉) · T. Matsui · S. Tanaka · A. Ishikawa · H. Endo · S. Hirohata
Department of Rheumatology and Infectious Diseases, Kitasato University School of Medicine, 1-15-1 Kitasato, Sagamihara 228-8555, Japan
Tel. +81-42-778-8745; Fax +81-42-778-9465
e-mail: hashi@med.kitasato-u.ac.jp

H. Kondo
Department of Internal Medicine, Kitasato Institute Medical Center Hospital, Kitamoto, Japan

E. Neumann · I.H. Tarner · U. Müller-Ladner
Justus-Liebig-University of Giessen, Giessen, Germany

E. Neumann · I.H. Tarner · U. Müller-Ladner
Kerckhoff-Klinik, Bad Nauheim, Germany

Introduction

The mainstream of current medical research is moving toward proteomics based on genomics. However, gene analysis is still a substantial part of research, not only with regard to the pathogenesis of diseases but also with regard to the normal development and physiology of cells and tissues. Various methods have been employed for such studies, but analysis has mainly been performed with bulk tissue samples or bulk-cultured cells consisting of heterogeneous populations in spite of several problems. First, bulk tissue contains a variety of cells and only some of them are relevant to any particular study. When performing gene expression analysis to investigate the pathogenesis of diseases, pure samples from the target lesion are essential and a credible result cannot be obtained from bulk tissue samples because of contamination by normal cells. For example, rheumatoid synovium contains several cellular components, such as fibroblasts, macrophages, and lymphocytes, and is composed structurally of a lining layer, a sublining, vessels, and lymphoid follicles, in which cells show various differences in gene expression. Gene expression in each tissue compartment should be analyzed separately for understanding the contribution made by each component of a tissue to the pathogenesis of rheumatoid arthritis (RA).

Second, there are many kinds of cells which are difficult to isolate, to obtain in a differentiated state, or to culture in an artificial environment. Moreover, cells cultured from organs or tissues can alter the genetic profile and other features in response to environmental changes. Therefore, it is necessary to procure particular parts of a tissue or individual target cells for reliable genetic analysis. Laser-mediated microdissection (LMM), also known as laser microdissection (LMD), is a technique that was developed for this purpose, allowing specific cells to be cut out of a tissue easily for analysis with minimal loss of proteins and DNA or RNA.[1] The technique is also called laser capture microdissection (LCM) or laser microbeam microdissection coupled with laser pressure catapulting (LMM/LPC),

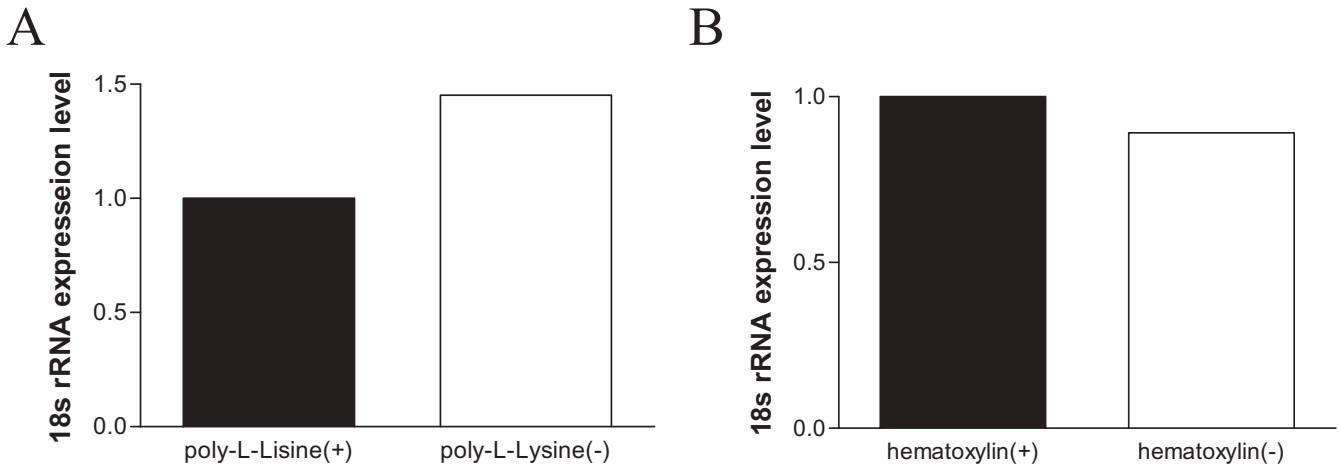

Fig. 1. The amounts of RNA of housekeeping gene (18s rRNA) from the microdissected samples with or without poly-L-lysine coated slides were measured with real-time quantitative polymerase chain reaction (PCR). The same vessels in serial sections of synovial tissues of patients with rheumatoid arthritis (RA) on coated or non-coated slides were obtained with laser-mediated microdissection (LMM). The total RNA of the vessels was extracted and the cDNA was synthesized, which was applied to real-time quantitative PCR analysis using the Lightcycler system and SYBR Green detection. Poly-L-lysine coating of slides may reduce the total RNA obtained from microdissected samples (**a**). Similarly, the effect of hematoxylin staining was analyzed. The reduction caused by hematoxylin staining was not detected (**b**). The relative mRNA levels were normalized against the values of samples with the process of poly-L-lysine (**a**) or hematoxylin (**b**), which were set as 1

especially when the system allows the isolation of specific cells dissected from a tissue sample on a slide. Before this technique was developed, cells had to be extracted manually.[2]

The LMM technique has already been widely applied to various fields of medical research such as oncology,[3,4] nephrology,[5] endocrinology,[6] dermatology,[7] and rheumatology, as described in this article, and it facilitates the analysis of gene expression in every field of research.

LMM technique

Frozen tissue samples (especially, synovial tissues from patients with RA) have been generally analyzed using LMM in many rheumatological experiments, although a single viable cell can be removed from a mixed culture.[8] Today there are various LMM systems, such as that produced by Arcturus Engineering (Mountain View, CA, USA) or that from Leica Microsystems (Bensheim, Germany). The former system was developed from the original LCM system invented by the National Cancer Institute of the National Institutes of Health (Bethesda, MD, USA), and it detaches target cells from a tissue sample by focally melting the polymer membrane on which the tissue is mounted.[1] The latter is the only system that employs an upright microscope, and target cells are extracted into a tube set below the stage utilizing gravity without contact.[9] Our group has also employed the use of the system by P.A.L.M. Microlaser Technologies (Wolfratshausen, Germany).[10] In brief, fresh tissue samples are embedded in optimal cutting temperature (OCT) compound and frozen in liquid nitrogen. Cryosections (5–8 μm thick) are made using a cryostat. The sections are mounted on glass slides coated with a polyethylene naphthalate (PEN) film.

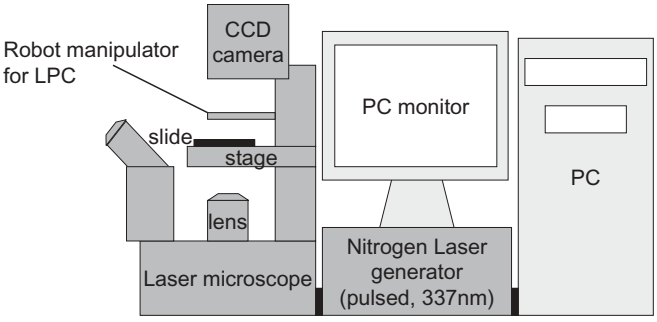

Fig. 2. The LMM system consists of a laser microscope, a laser generator, and a PC with a monitor. Target regions in a tissue on a slide are viewed and marked on the PC monitor through a charge-coupled device camera. A laser beam is generated in the generator and goes upward through the lens, penetrating and cutting the tissue along the outlines of marked areas automatically with the moving stage

To facilitate the attachment of cryosections to the slide, the PEN film can be coated with 0.1% poly-L-lysine which causes ionic charge, but this process can reduce the yield of RNA (Fig. 1a). Glass slides coated with PEN films are available from several companies. The film on the slide allows a microdissected area to be peeled off without disintegration of the cells. The slides are fixed in 5% acetic acid/95% ethanol or in other solutions such as 75% ethanol or 100% methanol. If necessary, the slides can be stained with stains such as hematoxylin, toluidine blue, or the HistoGene Staining Kit (Arcturus) after fixation for detecting particular cells prior to microdissection.[11,12] The loss of RNA due to staining with hematoxylin is negligible (Fig. 1b). Even immunohistochemical staining can be performed to distinguish particular cells for mRNA analysis using LMM.[13,14] The slides should be used for analysis as soon as possible, or stored at –80°C until use to avoid the loss of RNA.

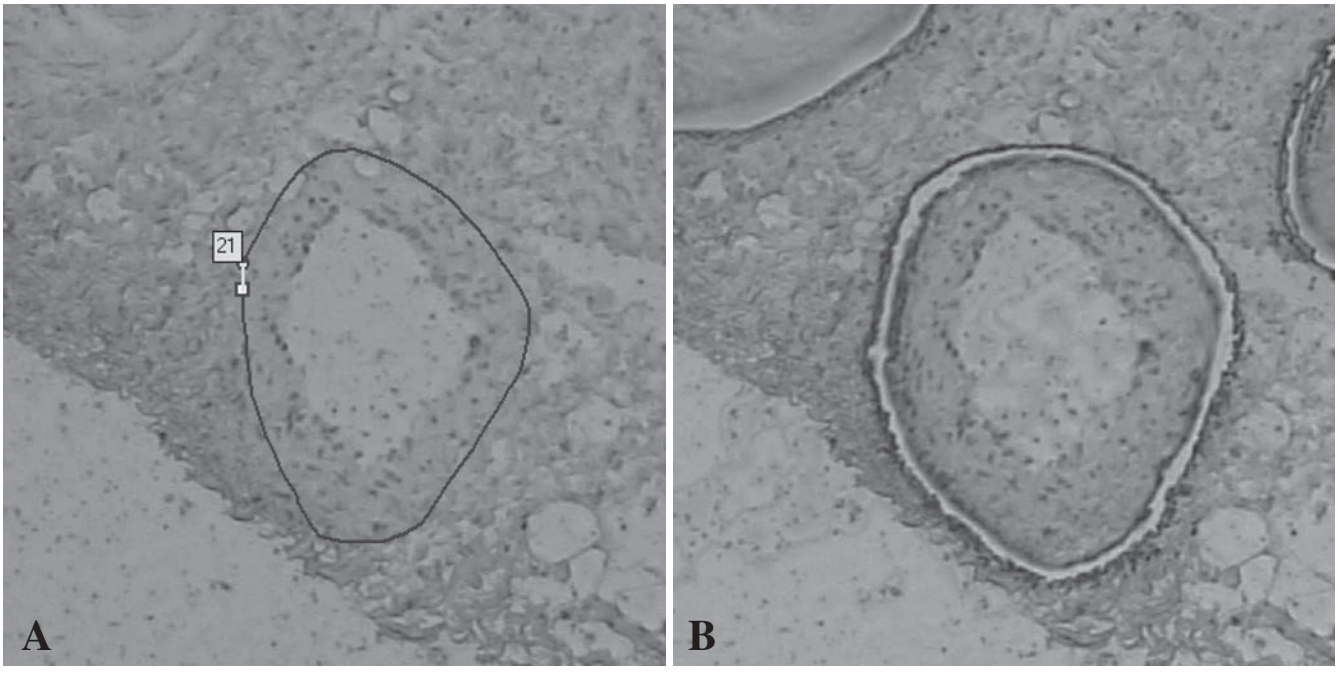

Fig. 3. A cryosection of synovial tissue of RA is mounted on a slide with a membrane film and set on the LMM microscope. The sample is observed on the PC monitor and a target area is marked (**a**). Afterward, the defined line is cut by a laser beam (**b**) (×100)

The P.A.L.M. MicroLaser system consists of a laser microscope with a charge-coupled device camera, a laser generator, and a computer (PC) with monitor for general control and operation (Fig. 2). A slide is set on the stage of the laser microscope and target regions are viewed on the PC monitor, marked by an operator and cut out by a laser beam as the stage is moved automatically (Fig. 3). Recently, infrared lasers which cause thermal tissue damage have been replaced by pulsed ultraviolet (UV)-A lasers in most LMM systems because the UV-A laser does not cut with heat transformation but with a photochemical process. The phenomenon of ablative photo-decomposition occurs only at the focal point of the laser, and the surrounding tissues or cells remain completely intact. Therefore, this process is called cold ablation.[15,16]

Microdissected cells are lifted up vertically by a non-focused laser beam and are collected in a tube by a robot manipulator (Fig. 4). This method is called laser pressure catapulting and it is a useful technique that enables non-contact preparation of samples with an LMM system, which is important to avoid contamination. However, larger pieces of tissue can also be collected by hand with a 27-gauge needle after microdissection. Following collection of the desired samples, the extraction of DNA, RNA, or protein can be performed.

RNA is extracted for subsequent analysis of gene expression using techniques such as real-time quantitative polymerase chain reaction (PCR), differential display, and microarray analysis. Unlike the conventional PCR method, the relative or absolute amount of RNA can be accurately and easily measured using real-time quantitative PCR.[17] One of the most popular real-time quantitative PCR methods employs SYBR Green fluorescent dye. The quantity of

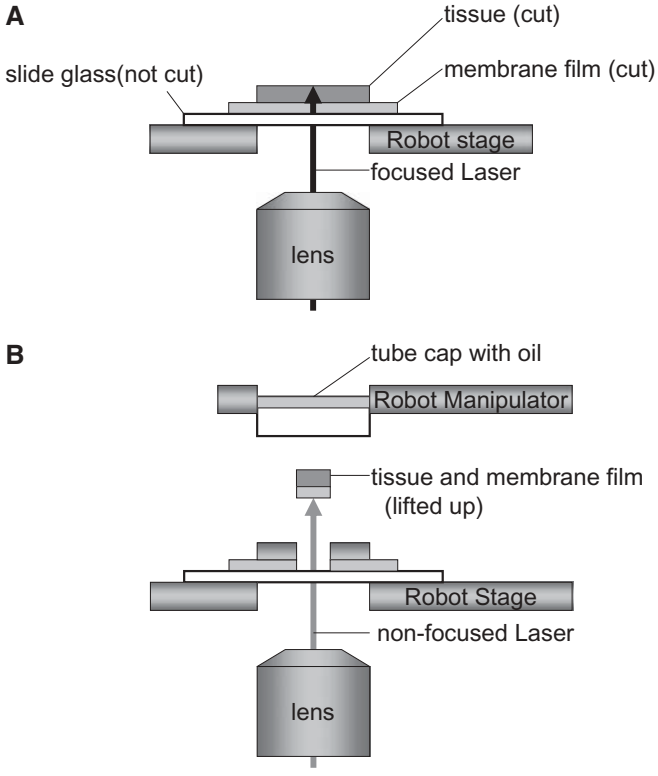

Fig. 4. Side view. A focused laser beam, which comes through a lens under the robot stage, does not cut a glass slide but does cut a transparent membrane film and a tissue with "cold ablation" (**a**). Microdissected cells with a membrane film are lifted up vertically by a non-focused laser and collected in a tube cap which is sighted and fixed upside down with a robot manipulator. The lifted sample is caught with oil in the bottom of the cap. This system is called laser pressure catapulting (**b**)

the PCR product is measured during every cycle by detecting the fluorescence which is generated when SYBR Green binds to double-stranded DNA synthesized during the PCR reaction.[18,19] Differential display, also called fingerprinting, is a technique to detect the differential expression of mRNA employing processes such as arbitrary primed PCR and gel electrophoresis.[20] Microarray analysis is a recently developed tool for the assessment of gene expression, and its high throughput facilitates gene expression profiling.[21] In brief, a microarray is composed of a glass slide, a plastic chip, or a nylon membrane on which single-stranded cDNA clones of various genes or a large set of oligonucleotides are fixed in a high-density array. The mRNA expression in a sample is detected with the binding of labeled single-stranded cDNA synthesized from sample mRNA to complementary sequences on the microarray.

The number of cells necessary for gene analysis depends on various factors such as the level of gene expression, the type of cells, treatment of sample, and the methods of isolating, amplifying, and analyzing mRNA. For example, Judex et al.[22] reported that 600 cells obtained with LMM from the synovial lining and sublining of RA patients provided sufficient mRNA to carry out differential display analysis without mRNA amplification, whereas our group needed 5000–8000 cells from the synovial vessels of patients with RA or osteoarthritis (OA) to quantify mRNA for every target gene accurately by real-time quantitative PCR.[23] In contrast, only 10–20 macrophages, dendritic cells, or T cells from RA synovium with immunohistochemical staining provided enough material for real-time polymerase chain reaction (RT-PCR).[24]

A critical weak point of LMM is its low yield of biological starting materials, especially RNA, because sample cells were obtained from a limited area of thin cryosections (5–8μm thick) and every preparative procedure such as fixation or staining can degrade RNA. Then some methods were invented to amplify RNA especially for microarray assays. The switch mechanism at the 5′ end of RNA templates (SMART) with reverse transcription is a common protocol. Another protocol employs a bacterial RNA polymerase promoter sequence such as the T7 RNA polymerase, with which single-cell gene expression profiling is possible.[25–28]

Application of LMM for rheumatology

Laser-mediated microdissection has been applied thus far in the field of rheumatology. Judex et al.[22] demonstrated the differential expressions of fibronectin 1, ciz-1, and thrombospondin 4 in cells between the lining layer and the sublining in RA synovium with LMM followed by nested RNA arbitrarily primed-PCR and differential display, and the result was confirmed by in situ hybridization at the mRNA level and immunohistochemical staining at the protein level. This is the first report of applying the LMM technique for a study in rheumatology. These procedures were also detailed in another report.[29]

Fractalkine (neurotactin, CX3CL1) is a chemokine thought to be concerned with monocyte chemotaxis and angiogenesis in the rheumatoid synovium. Fractalkine receptor (CX3CR1) was found in different cell types of RA synovium.[24] Each type of cells such as macrophages (CD68+), dendritic cells (CD1a+), and T cells (CD3+) was individually distinguished with immunohistochemical staining and thereafter microdissected and collected separately. Thus, using LMM, fractalkine receptor mRNA expression in each type of cells in RA synovium was detected with RT-PCR.

Currently, the principal manner for the analysis of gene expression profiling in rheumatology is to procure pure groups of cells from rheumatoid synovial tissue and analyze them with microarrays. Whereas whole synovial tissues of RA have been used for analysis with microarrays in several studies, Tsubaki et al.[30] applied LMM to obtain the synovial lining tissues in early RA. In this study, samples were clustered into two groups on the basis of their gene expression profile and the grouping correlated with the histological evaluation of each sample. The different expression profiles of several candidate genes between these two groups suggested differences in the pathogenesis of synovitis and could be employed for diagnostic and prognostic studies of early RA. In another study, Tsubaki et al.[31] procured cells from the synovial lining, sublining, vascular, and lymphoid follicular regions separately in RA synovial tissues and analyzed mRNA expression of a chemokine receptor CXCR3 in each region using RT-PCR. The mRNA expression was confirmed with immunohistochemical staining at the protein level, and the result suggested that plasma cells expressing CXCR3 in early RA synovium is recruited via the ligand, Mig/CXCL9, which is produced by fibroblasts mainly in synovial sublining regions. It is notable that they picked several distinct regions individually from a cryosection and detected mRNA expression in each region because this style of analysis could not be carried out without the LMM technique.

Recently, angiogenesis has been recognized as a crucial factor to develop and perpetuate rheumatoid synovitis.[32] Our group directed our attention to angiogenesis in RA synovium for the understanding of pathogenesis of RA.[23] We analyzed the expressions of several genes including those concerned with angiogenesis in synovial vessels of RA and OA which were procured with LMM. Of the seven genes analyzed with real-time quantitative PCR, two genes showed a significant differential expression between RA and OA synovial vessels. Id2, the inhibitor of differentiation and promoter of proliferation, was highly expressed in OA vessels than that of RA. It was confirmed with real-time quantitative PCR at the mRNA level (Fig. 5) and with immunohistochemistry at the protein level (Fig. 6). In contrast, the expression of vascular endothelial growth factor receptor-1 (VEGFR-1; Flt-1) was higher in RA vessels than in OA. These results were supported by immunohistochemical staining, and it was exhibited that small synovial vessels in synovial tissue can be a material of mRNA analysis.

Laser-mediated microdissection is also applied for the analysis of diseases other than RA. Plasma cells in synovial

lesions of patients with Lyme disease were identified with immunofluorescent staining using an anti-CD138 antibody, procured with LMM, and the mRNA was analyzed with RT-PCR.[14] In this study, fast and careful procedures of fixation, immunofluorescent staining, and development were adopted to prepare LMM samples, especially for the avoidance of degradation of RNA. In brief, the sample slides were fixed in acetone at 4°C for 4 min, exposed to primary and secondary antibody for 10 min and 5 min on a cold block, respectively, followed by procurement of target cells with LMM.

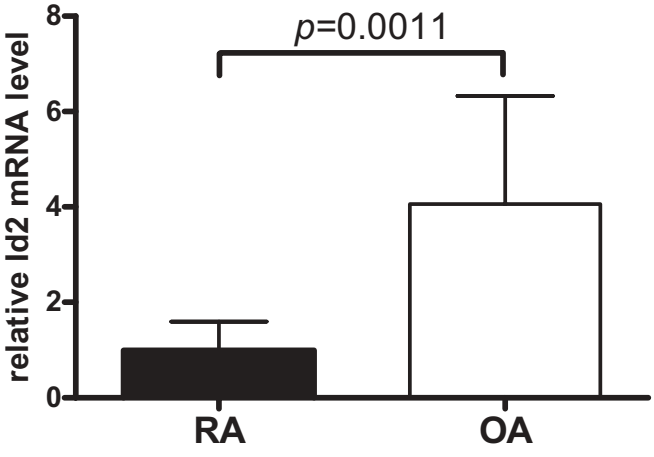

Fig. 5. Relative mRNA levels of Id2 in synovial vessels of RA or osteoarthritis (OA). The Id2 mRNA level of synovial vessels of OA was fourfold higher than that of RA. Values are normalized with endogenous control (18s rRNA) and the mean value of relative mRNA level of RA is set as baseline (reference value = 1) Source: Hashimoto et al.[23]

Immunoglobulin V regions expressed in plasma cells of Lyme arthritis were amplified with RT-PCR, and the analysis of nucleotide sequence illustrated the repertoire and mutational status of antibodies, suggesting the pathogenesis of chronic arthritis through the potential cross activity of antigens.

Conclusion

Although the LMM technique was originally invented and developed mainly in the field of oncology, it has been popularized into a variety of fields in medical science, and the instrumentations and procedures used in LMM have progressed over the years. Despite the cost, it is certain that the LMM technique is necessary to develop new dimensions of research, as mentioned above, not only in the field of rheumatology. Although the LMM technique has been utilized mainly for the analysis of synovial tissues in rheumatology research, currently, a single cell or living cells can also be isolated with the LMM technique. Not only RNA or DNA but also proteins can be analyzed, especially for the investigation of proteomics, which can reveal the functions of each cell in the disease. On the technical side, better preparative methods, including staining without loss of yield, should be established. The procedures for cutting and capturing cells should be further automated to save the work of operators and to obtain materials accurately and objectively. Future studies of gene expression analysis need technologies for obtaining enough nucleic acid from limited amount of material and high-throughput precise assays such as microarrays; LMM plays an important role in this.

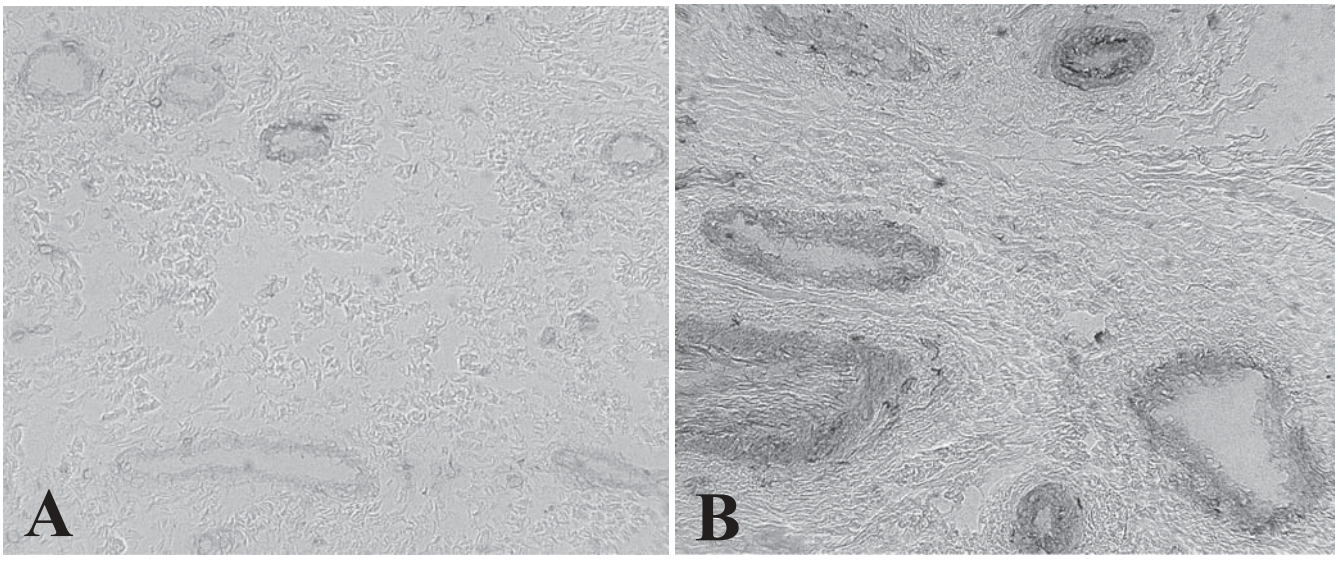

Fig. 6. Id2 protein expressed in synovial tissues of RA or OA detected with immunohistochemistry using a specific antibody. Strong positive staining of Id2 was seen in synovial vessels of OA (**b**) compared with those of RA (**a**). These stainings of Id2 corresponded to the levels of Id2 mRNA expression in synovial vessels of RA and OA (×100)

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