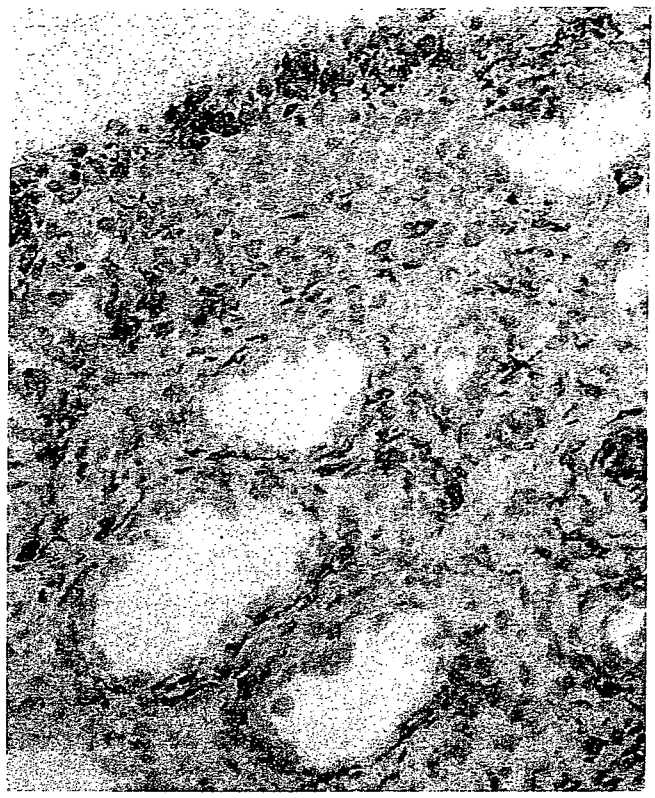

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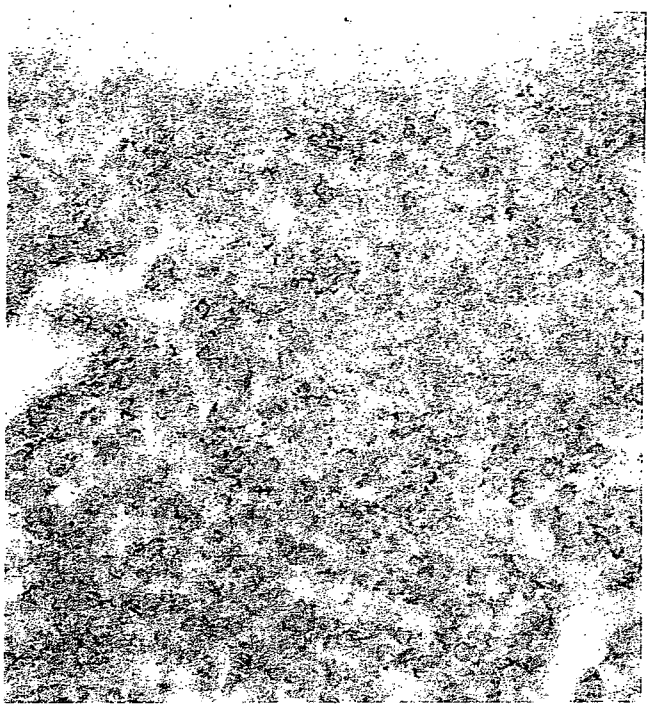

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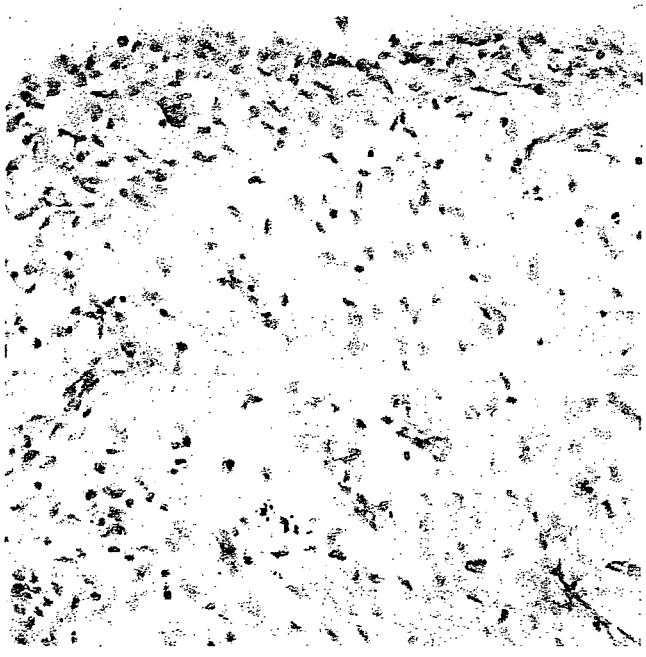

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Figure 3. In situ hybridization of ALX mRNA in synovial tissues from patients with RA or OA. *A*. Expression of ALX mRNA was seen in macrophages and fibroblast-like cells from the lining layer of RA synovium. *B*. Negative control: ALX sense probe staining of RA synovium. *C*. Macrophages in OA synovium are weakly positive for ALX mRNA. *D*. Negative control: ALX sense probe staining of OA synovium. *A-D:* Original magnification ×200.

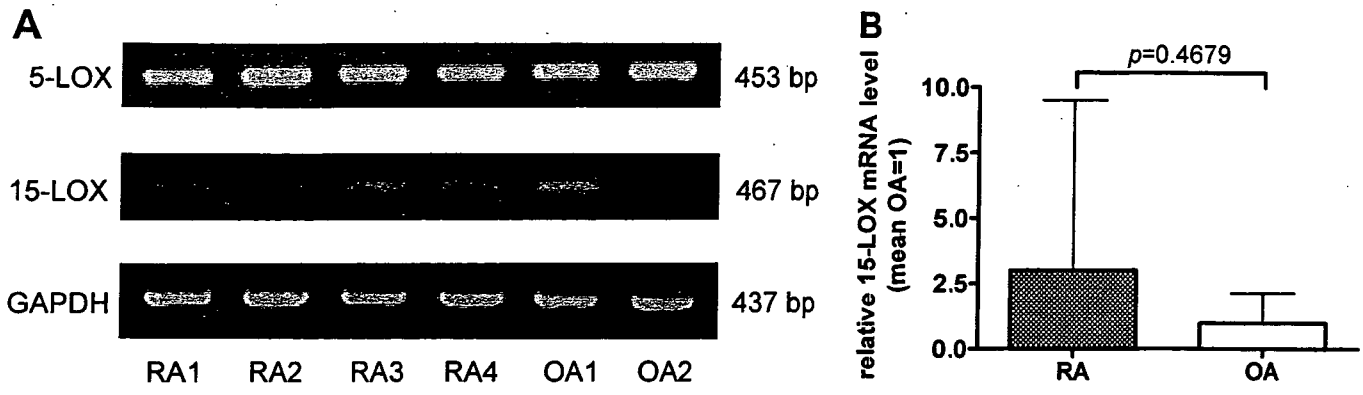

*Figure 4.*A. 5-LOX and 15-LOX mRNA expression in synovial tissues from 4 patients with RA and 2 with OA. 5-LOX and 15-LOX mRNA were detected by RT-PCR in all the samples, but 15-LOX expression was relatively weak compared with that of 5-LOX. B. Comparison of the expression of 15-LOX mRNA in synovial tissues from patients with RA and OA. ALX mRNA expression was stronger in RA syovium (3.01 ± 6.49) than in OA synovium (1.00 ± 1.14, p = 0.4679). Results represent the mean ± SD (n = 20 for RA and n = 10 for OA). mRNA levels are shown relative to the mean value for OA, which was defined as 1.

nomenclatures have clarified human ALX, which was at first recognized as formyl peptide receptor-like 1 (FPRL1)[34,35]. FPRL1 is known to possess high DNA sequence homology (~70%) to human formyl peptide receptor (FPR), which is a low affinity receptor for LXA_4. FPRL2 is also one of the FPR family receptors, but LXA_4 has not been confirmed as a ligand for FPRL2. Although our primer sets for ALX cannot distinguish ALX from FPR or FPRL2, ALX is the only high affinity receptor for LXA_4. ALX transgenic mice showed diminished activation of the proinflammatory transcription factor nuclear factor-kappa B (NF-κB) in the local inflammatory response[36]. Our data suggest that high levels of proinflammatory lipid mediators (PGE_2 and LTB_4) induce production of LXA_4 and 15-epi-LXA_4 for antiinflammation (Figure 1C, 1D, 1E, 1F)[4].

Biosynthesis of leukotrienes is initiated by insertion of molecular oxygen at the carbon-5 position of arachidonic acid. Insertion of molecular oxygen at the carbon-5 and carbon-15 positions by 5-LOX and 15-LOX, respectively, results in the formation of lipoxins. This can occur through oxygenation of leukocyte-derived LTA_4 by platelet lipoxygenase through cell–cell interactions[37]. Several reports have indicated that lipoxygenase can be found in synovial tissues from patients with RA.

Bonnet, *et al* described the expression of 5-LOX and 5-lipoxygenase-activating protein (FLAP) mRNA in cultured human synovial cells[9] and our group has reported the presence of 5-LOX in the synovial lining layer[10]. In our study we demonstrated more highly expressed 15-LOX mRNA in synovial tissues from patients with RA than in those from patients with OA. The route of lipoxin formation depends on the cells and enzymes present and can be modulated by cytokines. IL-4[13] and IL-13[14], which are thought to be negative regulators of the inflammatory response, increase 15-LOX expression and activity as does PGE_2[4], thereby enhancing LXA_4 formation. The generation of lipoxins by both proinflammatory and antiinflammatory mediators may lead to negative feedback inhibition of the inflammatory response and thus protect the host from potentially deleterious neutrophil-induced responses. 15-LOX was more strongly expressed in the synovial lining of patients with RA than that of patients with OA (Figure 4B). In patients with RA, IL-13 mRNA was also detectable in synovial fluid mononuclear cells and synovial tissues, and the IL-13 level in synovial fluids was significantly higher than that reported for IL-4[38]. 5-LOX was constitutively expressed in both RA and OA synovial tissues[10]. These data suggest that 15-LOX induced by IL-13 may be one of the regulators of the production of LXA_4 in inflamed joints. A wide distribution of LXA_4 levels in synovial fluids might depend on that of 15-LOX. Moreover, overexpression of leukotriene B_4 receptor 2 (BLT2) in synovial leukocytes of patients with RA[10] could contribute to the upregulation of IL-13[39].

One of the pathways identified for LXA_4 biosynthesis involves platelet–neutrophil interactions[37]. This pathway has been highlighted as a route of LXA_4 formation within the vasculature that involves 5-LOX in leukocytes and 12-LOX in platelets. There are numerous platelets in synovial joints, so platelets in synovilal fluid might also be involved in LXA_4 formation.

In synovial fluids of patients with RA, 15-epi-LXA_4 was detected at about one-quarter the level of LXA_4. The concentration of 15-epi-LXA_4 in synovial fluids was around 10 times that in plasma (0.25 ± 0.63 ng/ml) in a report by Chiang, *et al*[40]. Acetylation of cyclooxygenase-2 by aspirin could lead to transcellular biosynthesis of epi-lipoxin, the so-called aspirin-triggered lipoxins. Recently, in a randomized, placebo-controlled study of 8 weeks, a clinically relevant dose of aspirin was found to increase antiinflammatory 15-epi-LXA_4. Plasma 15-epi-LXA_4 levels at 8 weeks were significantly greater than those before aspirin treatment[40]. In our study we were not able to detect the relation between aspirin taking and concentrations of 15-epi-LXA_4 in synovial fluids of patients with RA and OA. On the contrary, LXA_4 and 15-epi-LXA_4 levels were higher in OA patients without NSAID compared with those

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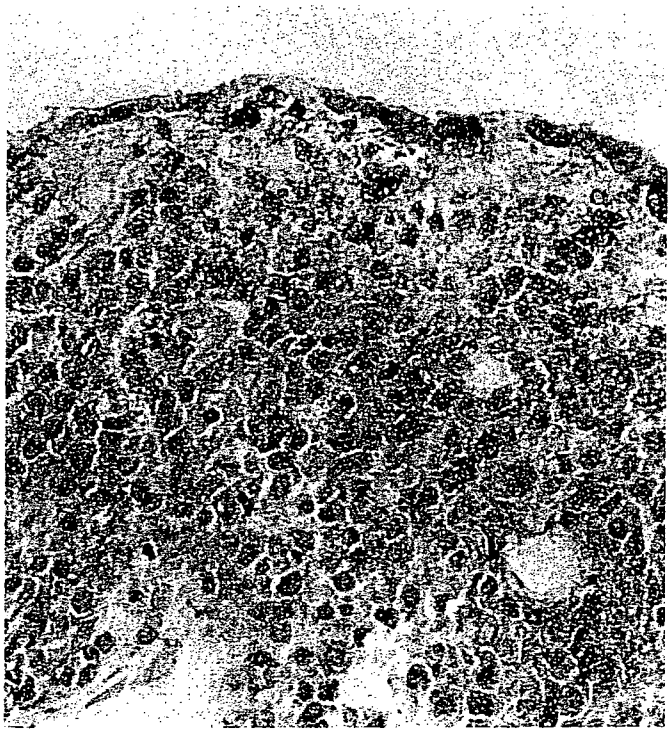

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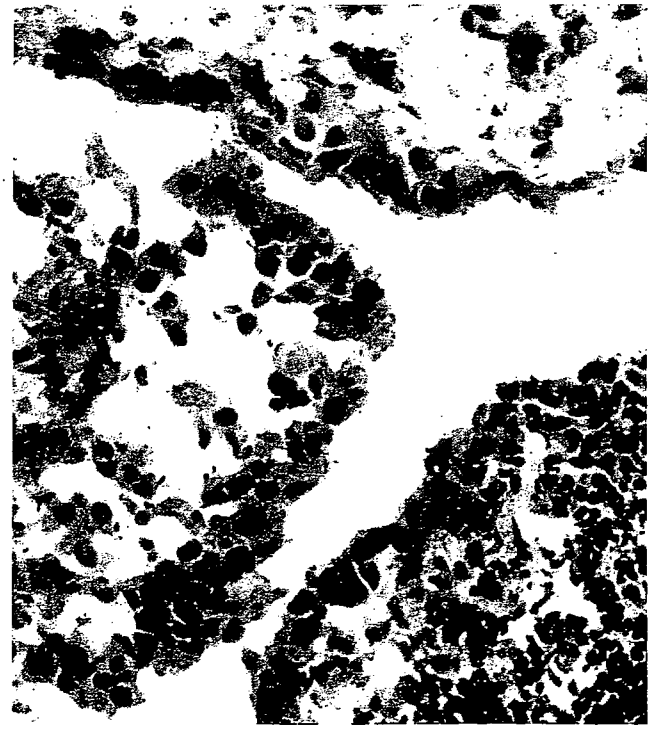

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Figure 5. In situ hybridization of 15-LOX mRNA and immunohistochemical analysis of 5-LOX in synovial tissues from patients with RA. A. Expression of 15-LOX mRNA is seen in macrophages from the lining layer (original magnification ×200). B. Negative control: 15-LOX sense probe staining (original magnification ×100). C. Synovial lining cells stained for 5-LOX (original magnification ×200). D. Distribution of CD68-positive cells. Macrophages show staining by the anti-CD68 antibody (original magnification ×200).

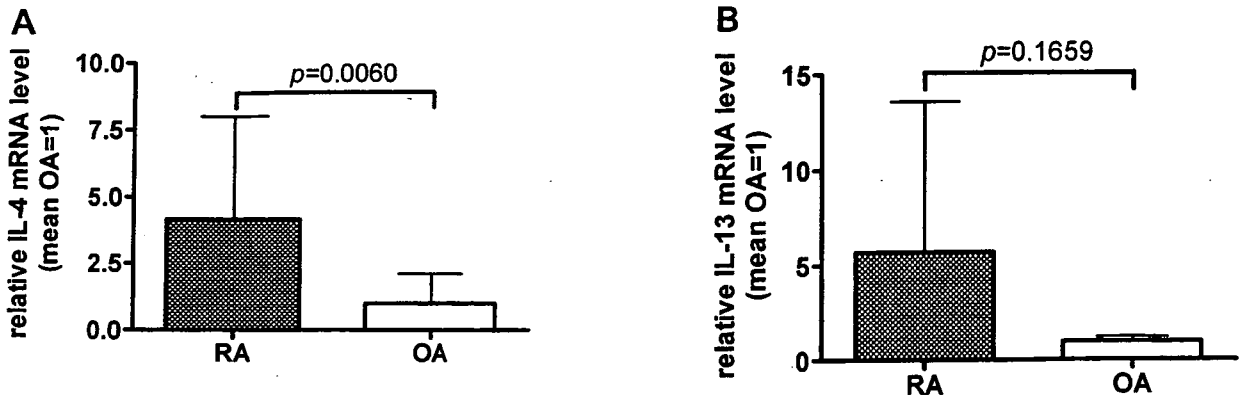

Figure 6. IL-4 and IL-13 mRNA expression in synovial tissues from patients with RA and OA. A. IL-4 mRNA expression was significantly stronger in RA syovium (4.16 ± 3.85) than in OA synovium (1.00 ± 1.10, p = 0.0060). B. IL-13 mRNA expression was also stronger in RA syovium (5.71 ± 7.88) than in OA synovium (1.00 ± 0.69, p = 0.1659). Results represent the mean ± SD (n = 20 for RA and n = 10 for OA). mRNA levels are shown relative to the mean value for OA, which was defined as 1.

treated with NSAID, although 15-epi-LXA_4 levels in synovial fluid can differ from those in plasma. On the other hand HMG-CoA reductase inhibitor drugs, the so-called statins, were recently thought to generate 15-epi-LXA_4[41], only one patient took statin in this study. Regulatory systems of lipoxins, especially 15-epi-LXA_4, remain to be elucidated.

Biosynthesis of lipid mediators has been observed to show a biphasic pattern during the inflammatory response. *In vivo* analysis of eicosanoid formation in a murine dorsal air-pouch model of inflammation has revealed a distinct time-dependent profile[42]. TNF–induced neutrophil accumulation within the dorsal pouch coincides with an increase of intradorsal PGE_2 levels. A persistent and marked increase of LXA_4 levels occurred, along with a reduction of neutrophil infiltration and PGE_2 production. Our synovial fluid analysis indicated that a patient with high levels of PGE_2 and LTB_4 had high levels of LXA_4 and 15-epi-LXA_4. Lee, *et al* reported that LXA_4 was detected in bronchoalveolar lavage fluid samples from 9 of 12 patients with lung disease and not detected in healthy control samples[43]. Although another report demonstrated that bronchoalveolar lavage fluids in patients with scleroderma lung disease had low levels of LXA_4 and high levels of PGE_2 because of impaired stimulatory effect of PGE_2 on 15-LOX[44], our results support that LXA_4 is basically produced responding to PGE_2 in order to degrade the inflammation. It seems possible that PGE_2 induces a switch in lipid mediator synthesis from predominance of 5-LOX-yielding LTB_4 to predominance of 15-LOX-generating LXA_4, a response that would be paralleled by a reduction of neutrophil infiltration[4]. This leads to the proposal that biosynthesis of inflammatory lipid mediators is biphasic, with a role for eicosanoids in the initiation, progression, and termination of the inflammatory response. The initial phase is coupled to leukotriene biosynthesis, while subsequent prostaglandin formation is coupled to induction of lipoxygenase activity and lipoxin biosynthesis, promoting the resolution of inflammation.

Our study demonstrated that LXA_4 was synthesized in the synovial tissues of patients with RA via a transcellular pathway, which might be regulated by IL-13-induced 15-LOX. LXA_4 and its analog can act as an antiinflammatory negative feedback system for proinflammatory mediators such as LTB_4 and PGE_2 that are involved in the pathogenesis of RA. Activation of lipoxin synthesis, for example by augmentation of 15-LOX, could be a potential new therapeutic approach for RA.

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Histopathology of central nervous system lesions in Behçet's disease

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Abstract

Central nervous system (CNS) involvement in Behçet's disease, usually called neuro-Behçet's syndrome (NB), is one of the most serious complications of the disease. In the present study, we carried out immunohistological examination of biopsied or autopsied brain tissues from 3 patients with different types of NB, acute NB, chronic progressive NB, and NB in a long-term remission. Histopathology of mass lesion in acute NB revealed infiltration of mononuclear cells around small vessels, consisting of CD45RO+ T lymphocytes and CD68+ monocytes with few CD20+ B lymphocytes. Of interest, TUNNEL staining disclosed that most neurons were undergoing apoptosis in the inflammatory lesion. In chronic progressive NB, similar histopathological changes were noted in pons, cerebellum, medulla, internal capsule, and midbrain, although the degree of mononuclear cell infiltration was modest. There were also scattered foci of neurons undergoing apoptosis with formation of a few binucleated neurons. The most prominent feature of NB in a long-term remission was atrophy of basal pons with formation of cystic or moth-eaten lesions, consisting of isomorphic gliosis with viable neurons. There were still scattered foci of perivascular cuffing of T lymphocytes and monocytes. These results emphasize the common features throughout the courses of NB, perivascular cuffing of T lymphocytes and monocytes, irrespective of the clinical phenotypes. More importantly, it is suggested that soluble factors produced by infiltrating cells, including IL-6, might play a role in the induction of apoptosis of neurons in NB.

Keywords: Behçet's disease; Central nervous system; Histopathology; Cerebrospinal fluid; IL-6

1. Introduction

Behçet's disease is a chronic relapsing inflammatory disease of unknown etiology, presenting recurrent aphthous stomatitis, uveitis, genital ulcers, and skin lesions. The predominant histopathological features in the inflamed tissues are infiltration of lymphocytes and monocytes, and sometimes polymorph nuclear leukocytes, around small veins without microscopic changes in the vessel walls. Thrombophilia or thrombophlebilis involving small and large veins is also common, whereas arteritis is rare. In these regards, Behçet's disease is unique compared with other vasculitides [1].

Central nervous system (CNS) involvement in Behçet's disease, usually called neuro-Behçet's syndrome (NB), is one of the most serious complications of the disease. CNS involvement in Behçet's disease is either caused by primary neural parenchymal lesions or is secondary to major vascular involvement [2,3]. The latter type is rarely complicated with the parenchymal lesions and should be called vasculo-Behçet's disease [2]. Of parenchymal CNS involvement, involvement of the brainstem was the most common, whereas spinal cord involvement, hemispheric involvement and meningoencephalitis also occurred [3–5]. Although cerebrospinal fluid analysis was performed in only a fraction of patients, most patients with brainstem disturbance showed abnormalities, including leukocytosis and an increase in protein concentration [3].

Although the role of small vessel vasculitis, mainly venular involvement, in the pathogenesis of NB has been suggested [6], the precise sequelae have not been delineated due to the paucity of autopsy studies. In addition, it remains unclear whether there are any differences in the histopathological characteristics among various clinical patterns of NB, including acute NB and chronic progressive NB [4,5,7]. In the present study, we carried out immunohistological examination of biopsied or autopsied brain tissues from 3

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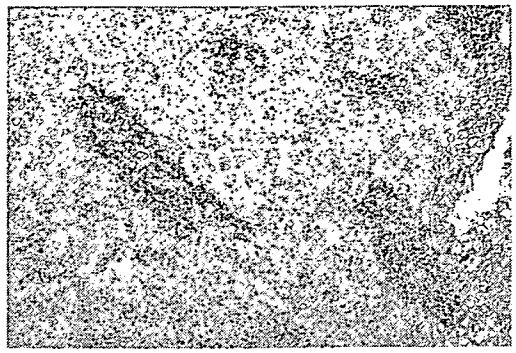

Fig. 1. Histopathology of the mass lesion in acute NB (biopsy). H&E staining. Original magnification: ×25.

patients with different types of NB, one with acute NB, another who died of chronic progressive NB, and the other who died of myocardial infarction during remission of NB.

2. Patients and methods

2.1. Patients

Brain tissue was obtained from 3 patients with different types of NB.

2.1.1. Patient 1 (acute NB)

A 54-year-old Japanese man with Behçet's disease developed convulsion with subsequent left homonymous hemianopsia in 1989. CAT scan showed mass lesion with surrounding brain edema, which was hypovascular on cerebral angiography, in the right occipital lobe. A diagnosis of brain tumor was strongly suspected and open biopsy was performed on April 11, 1990. He was diagnosed as NB by biopsy, and improved by treatment with corticosteroid.

2.1.2. Patient 2 (chronic progressive NB)

A 38-year-old Japanese man developed progressive cerebellar ataxia, dysarthria, bladder–bowel incontinence, and dementia in 1974. Treatment with high doses of prednisolone started in December 1977. However, his neurological symptoms did not improve, and he died of aspiration pneumonia in July 1978.

2.1.3. Patient 3 (NB in a long-term remission)

A 62-year-old Japanese man had developed acute NB (headache and dysarthria) and had been successfully treated with low doses of prednisolone for 15 years before he developed acute myocardial infarction on November 24, 2001. He received aorto-coronary bypass operation in August 2002, but he died of congestive heart failure on September 2, 2002. After he developed myocardial infarction, there was no evidence for exacerbation of NB until he died of congestive heart failure.

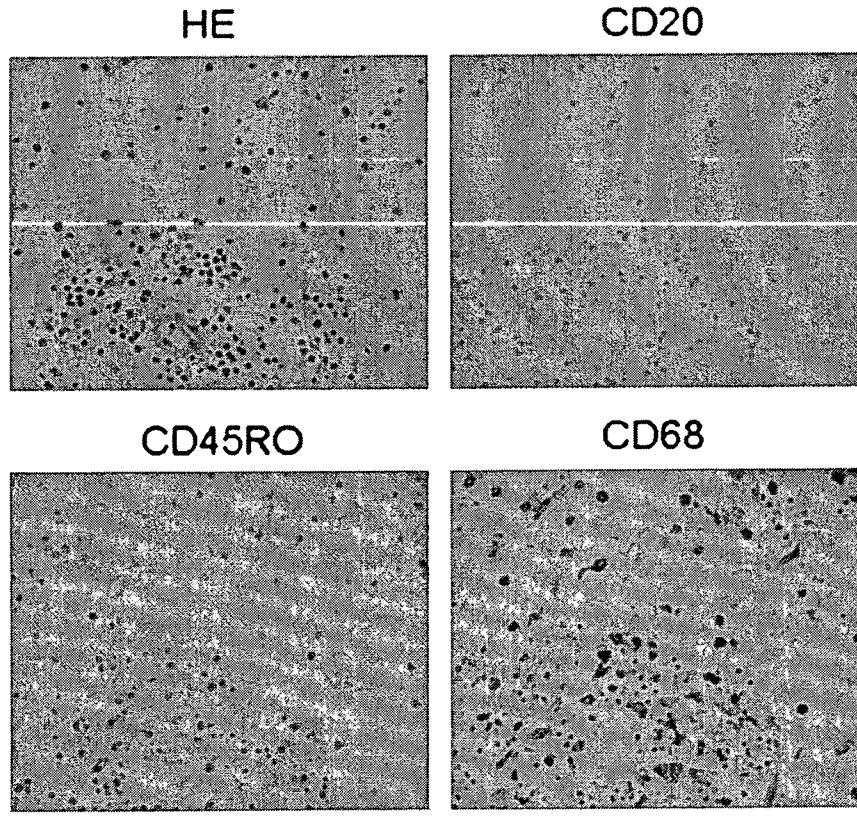

Fig. 2. Immunohistological examination of the infiltrated mononuclear cells in acute NB (biopsy). CD20 (L26), CD45RO (UCHL-1), CD68 (KP-1). Original magnification: ×50.

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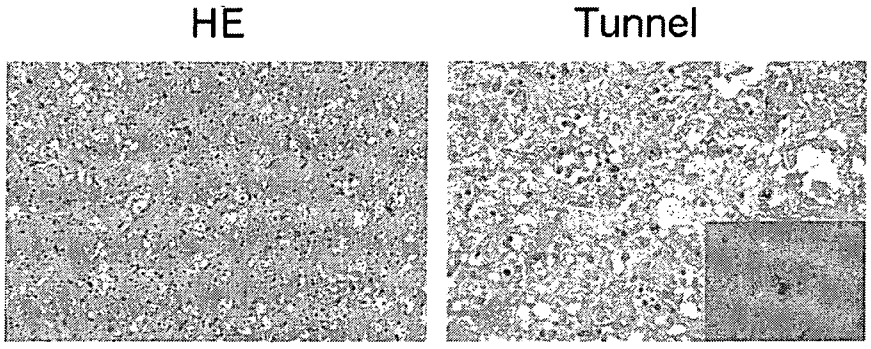

Fig. 3. TUNNEL staining of the mass lesion in acute NB (biopsy). Original magnification: ×25 (×50 in lower right).

2.2. Light microscopy

Brain tissues were fixed in formalin and embedded in paraffin. Sections were subjected to a variety of stain procedures, including hematoxylin and eosin, Klüver-Barrera, TUNNEL, and immunohistological stainings for B cells (L26, CD20), T cells (UCHL1, CD45RO), and monocytes (KP-1, CD68).

3. Results

3.1. Histopathology in patients with active NB (Patient 1 and Patient 2)

Histopathology of the mass lesion in Patient 1 (acute NB) revealed infiltration of mononuclear cells around small vessels, consisting of mononuclear cells and very few polymorph nuclear leukocytes in the brain parenchyma (Fig. 1). Immunohistological examination revealed that the infiltrating mononuclear cells consisted mostly of CD45RO+ T lymphocytes and CD68+ monocytes with very few CD20+ B lymphocytes (Fig. 2). Of interest, TUNNEL staining disclosed that most neurons were undergoing apoptosis in the inflammatory lesions. Some neurons undergoing apoptosis are binucleated (Fig. 3).

In Patient 2, who died of chronic progressive NB during treatment with high doses of steroid, similar histopathological changes were noted, although the degree of mononuclear cell infiltration was modest. Infiltration of mononuclear cells around small vessels was observed in pons, cerebellum, medulla, internal capsule, and midbrain (Fig. 4). The mononuclear cells consisted mostly of CD45RO+ T lymphocytes and CD68+ monocytes/macrophages with very few CD20+ B lymphocytes (Fig. 5). Of note, there were so called foam-cells [8] surrounding small vessels, which consisted of CD68+ cells. There were also scattered foci of neurons undergoing apoptosis with binucleation (Fig. 6). In addition, scattered foci of demyelination and gliosis were observed in hippocampus, pons and internal capsule.

3.2. Histopathology in a patient with clinically inactive NB (Patient 3)

The most prominent feature was atrophy of basal pons with formation of cystic or moth-eaten lesions (Fig. 7). The lesions in pons consisted of isomorphic gliosis, suggesting that the lesions were formed gradually by repeated minor attacks. In addition, there were viable neurons that were negative for TUNNEL staining within the lesions with isomorphic gliosis (Fig. 8), obviating the possibility that the pontine lesions might

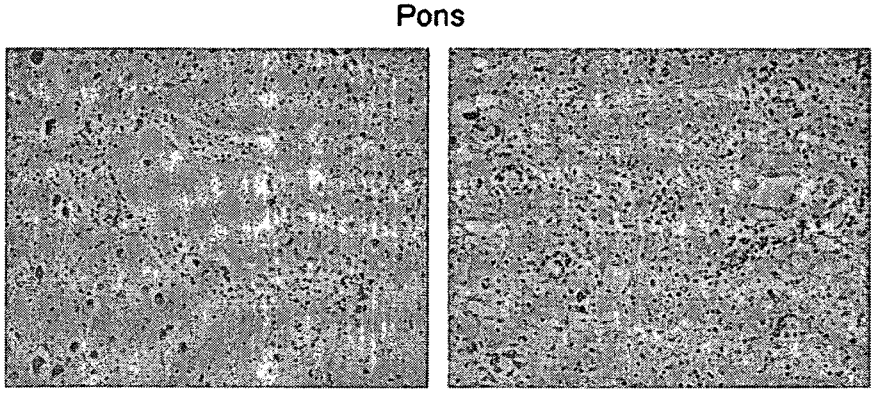

Fig. 4. Histopathology of the pontine lesion in chronic progressive NB (autopsy). H&E staining. Original magnification: ×25.

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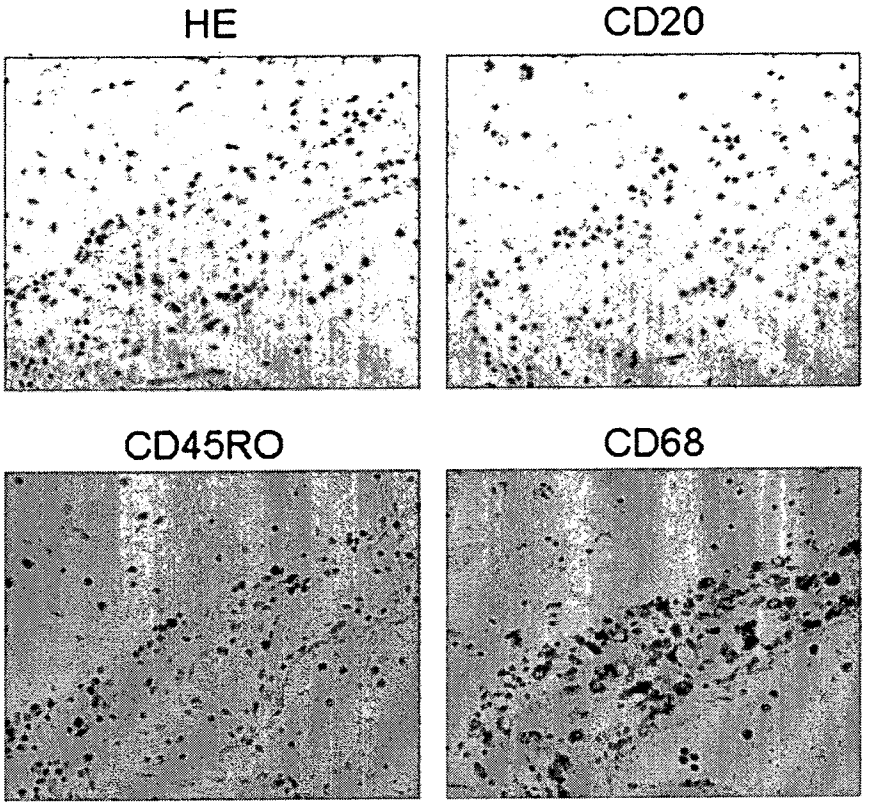

Fig. 5. Immunohistological examination of the infiltrated mononuclear cells in chronic progressive NB (autopsy). CD20 (L26), CD45RO (UCHL-1), CD68 (KP-1). Original magnification: ×50.

result from ischemia due to arterial occlusion. It was therefore suggested that repeated inflammatory attacks might be responsible for these lesions. Finally, there were still foci of mild infiltration of CD45RO+ T lymphocytes and CD68+ monocytes around small vessels (Fig. 9).

4. Discussion

It has been considered that CNS lesions in Behçet's disease are caused by vasculitis with venous predominance [8–10]. However, definite vasculitis is not so frequently observed [11]. Instead, most frequently observed abnormalities include small softening lesions with perivascular cellular infiltration of lymphocytes and sudanophilic foam-cells [6,8]. Consistently, in the present study, the lesions in acute NB showed marked perivascular cuffing of mononuclear cells around the small vessels. Of note, the results in the current studies have revealed that the mononuclear cells infiltrating around the small vessels were predominantly T lymphocytes and monocytes without B lymphocytes. More importantly, the neurons in the lesions

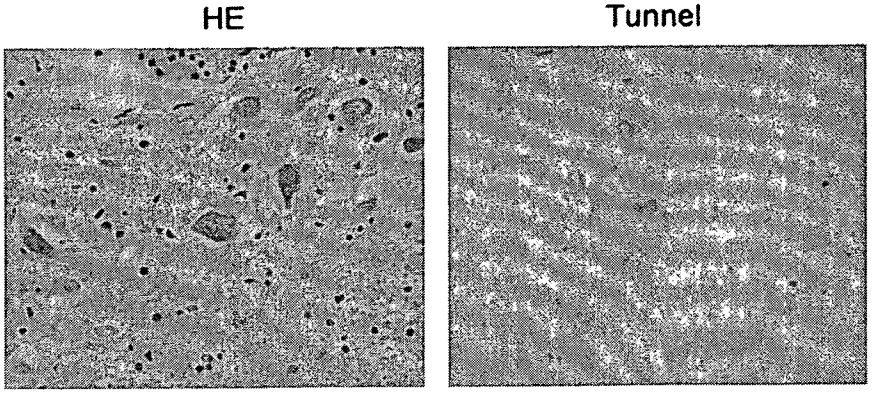

Fig. 6. TUNNEL staining of the mass lesion in chronic progressive NB (autopsy). Original magnification: ×25.

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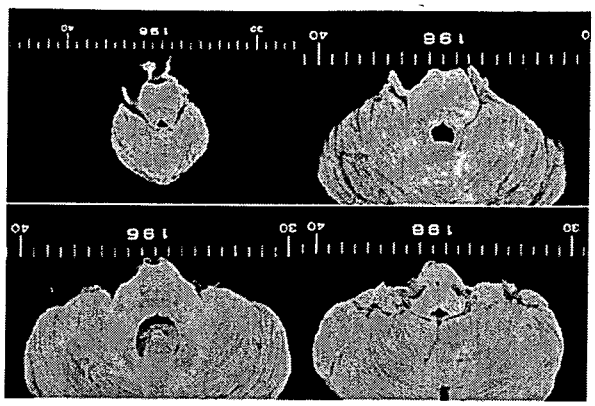

Fig. 7. Macroscopic findings of brainstem and cerebellum in NB in a long-term remission (autopsy).

were undergoing apoptosis. It is therefore suggested that soluble factors, including proinflammatory cytokines, produced by infiltrating T lymphocytes and monocytes might play a role in the induction of apoptosis of neurons. In this regard, we have disclosed that IL-6 levels were markedly elevated in cerebrospinal fluid from patients with NB [7]. Further studies to identify the precise mechanisms by which proinflammatory cytokines are produced within the CNS would be important for a complete understanding of the pathogenesis of NB.

We have recently disclosed that NB can be classified into acute NB and chronic progressive NB [7,12]. Thus, acute NB is characterized by acute meningoencephalitis, which responds well to corticosteroid and is usually self-limiting [7]. By contrast, chronic progressive NB is characterized by intractable, slowly progressive neuro-behavior changes and ataxia, along with persistent marked elevation of CSF IL-6 (usually more than 20 pg/ml) [1,6,7]. MRI findings may show only atrophy of brain stem and cerebellum, in contrast with the acute NB. The majority of patients with parenchymal CNS involvement had only single attacks, whereas one-third of patients underwent further attacks [3–5]. These patients correspond to acute NB in our classification [1]. Of note, previous studies also reported the presence of patients who underwent progressive deterioration leading to disability (primary or secondary progressive course) [3,4]. These patients are considered to be the same as chronic progressive NB in our classification [1]. These two types of NB are currently considered to represent different stages rather than be independent clinical entities [12]. In fact, most patients with chronic progressive NB had history of attacks of acute NB prior to the development of progressive neuropsychological symptoms [7]. Moreover, we have recently experienced some patients who displayed prolonged elevation of CSF IL-6 activity following acute NB [7]. Accordingly, the results in the current studies disclosed that the histopathological characteristics of chronic progressive NB are comparable to those of

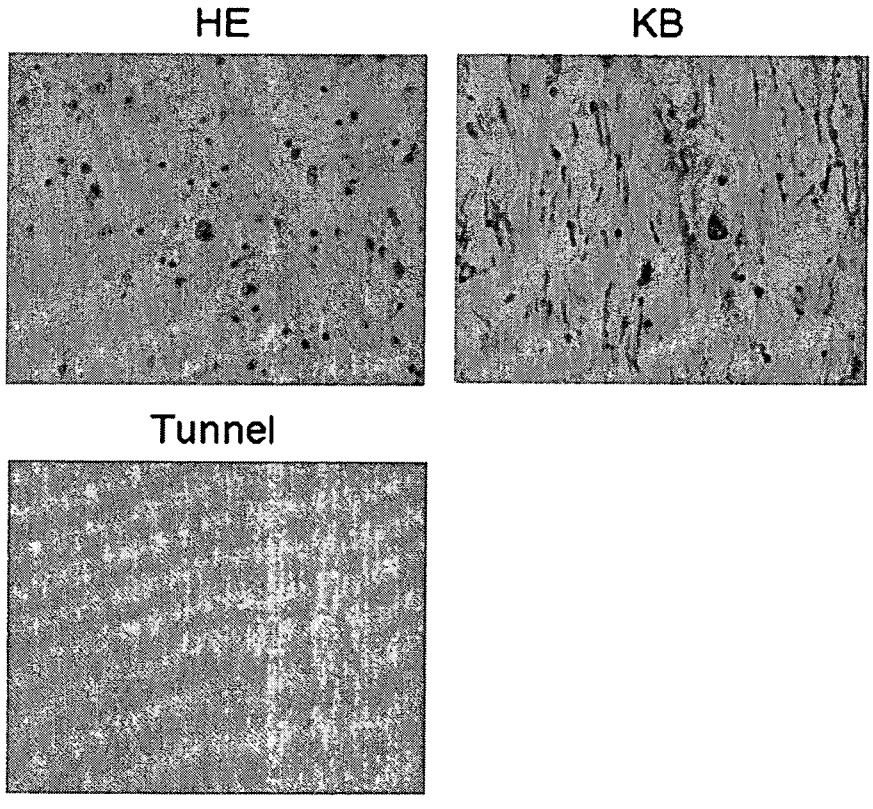

Fig. 8. Histopathology of the pontine lesion in NB in a long-term remission (autopsy). KB: Klüver-Barrera staining. Original magnification: ×25.

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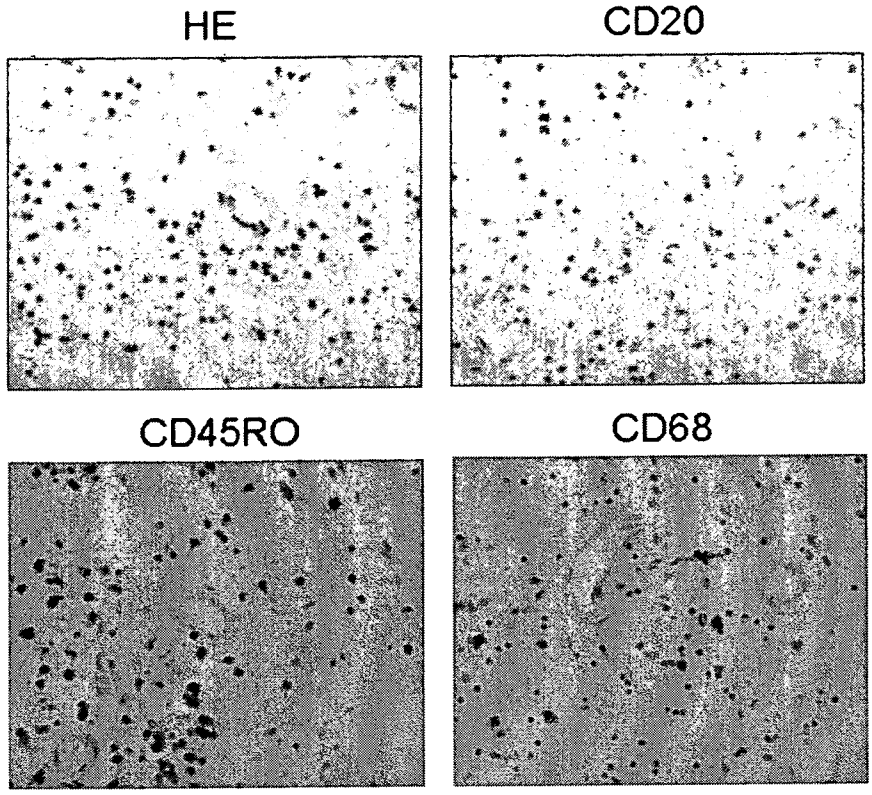

Fig. 9. Immunohistological examination of the infiltrated mononuclear cells in NB in a long-term remission (autopsy). CD20 (L26), CD45RO (UCHL-1), CD68 (KP-1). Original magnification: ×50.

acute NB. Thus, in the Patient 2 in the current studies, there were also foci of perivascular cuffing of mononuclear cells, consisting of T lymphocytes and activated monocytes/macrophages, with neurons undergoing apoptosis.

Previous studies revealed that binucleated neurons were often observed in the lesions of NB [9]. In the present study, binucleated neurons were also found in acute NB as well as in chronic progressive NB. More importantly, these binucleated neurons were positive for TUNNEL staining. It is therefore most likely that neurons undergoing apoptosis might form binucleated neurons. It has been shown that various factors cause apoptosis and binucleation in a variety of cells through the cytokinesis-blocking [13,14]. It is therefore suggested that some inflammatory assault might cause apoptosis and binucleation of neurons in NB. Further studies to explore the precise natures of such assault would be important.

Although the current studies showed that acute NB and chronic progressive NB have the common histopathological features, the mechanism of persistent inflammation in the CNS remains unclear. In this regard, it has been recently disclosed that HLA-B51 and cigarette smoking, and especially their combination, are risk factors for chronic progressive NB [15]. It is therefore likely that certain substances in cigarettes might be immunogenic in the context of HLA-B51, resulting in the persistent activation of immune responses within the CNS in Behçet's disease [15].

It is currently unclear which factors are responsible for the induction of apoptosis of neurons in NB. It has been shown that the concentration of IL-6 is markedly elevated in CSF from patients with acute NB and chronic progressive NB in relation to their disease activities [7]. Thus, CSF IL-6 was decreased when the disease activities were successfully suppressed [16]. Of note, previous studies also demonstrated that proinflammatory cytokines, including IL-6, play important roles in the damages of neurons [17–19]. Accordingly, there has been a growing appreciation of the destructive potential of elevated levels of IL-6 in the CNS. Thus, IL-6 has been found to cause neuronal degeneration and cell death in various disorders [20]. Moreover, recent studies have demonstrated that IL-6 mediates spinal cord neural injury through the signaling pathway from JAK/STAT activation to iNOS expression to poly (ADP-ribose) polymerase activation and cell death [21]. It is therefore most likely that high amounts of proinflammatory cytokines, especially IL-6, might be important for the induction of apoptosis of neurons in NB.

Perivascular infiltration of sudanophilic foam-cells has been described as one of the characteristic features of NB [8]. Accordingly, in the present study, perivascular cuffing of foam-cells was observed in acute NB as well as in chronic progressive NB. More importantly, it turned out that these cells were CD68+ cells. It is therefore suggested that these

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foam-cells might be activated macrophages, which phagocyte damaged white matters (myelin).

The long-term course of NB after remission has not been explored. Patient 3 in the current studies represents a patient of NB who had been in a long-term remission after successful treatment. However, there still remained foci of perivascular cuffing of T lymphocytes and monocytes in this patient, suggesting that small inflammatory attacks still took place in NB during the remission phase.

Previous studies revealed that the most frequent CNS finding in NB was a preference for involvement of brainstem-diencephalon and pontobulbar region [2]. Koçer et al suggested that the anatomic variability of venous anatomic arrangements at different levels of the CNS might explain such predilection [10]. Consistently, in Patient 3 of our study with a long-term course of NB showed atrophy of brainstem and cerebellum, as has been reported previously [2]. On the other hand, arterial involvement resulting in CNS vascular disease is rare in Behçet's disease [6]. Of note, the reservation of viable neurons within the parenchymal lesions with isomorphic gliosis in our patient with a long course of NB obviates the possibility that the lesions might result from ischemia due to vasculitis of arteries. Rather, these findings suggest that relapsing-remitting small attacks with perivascular cuffing of mononuclear cells might be repeated during the long course in this patient.

In summary, the current studies have disclosed the characteristics of histopathology in 3 patients with different phases of NB, including acute phase, chronic progressive phase, and remission phase. The common features throughout the courses of NB appeared to be relapsing-remitting attacks of inflammation with perivascular infiltration of T lymphocytes and activated monocytes/macrophages. The magnitude and the duration of the inflammation might determine the clinical features and outcome. Finally, it is suggested that the production of huge amounts of proinflammatory cytokines, such as IL-6, might induce neuronal dysfunction and apoptosis. Further studies to delineate the triggers for the inflammation attacks as well as the factors for their perpetuation would be important for a complete understanding of the pathogenesis of NB.

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REVIEW ARTICLE

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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

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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),

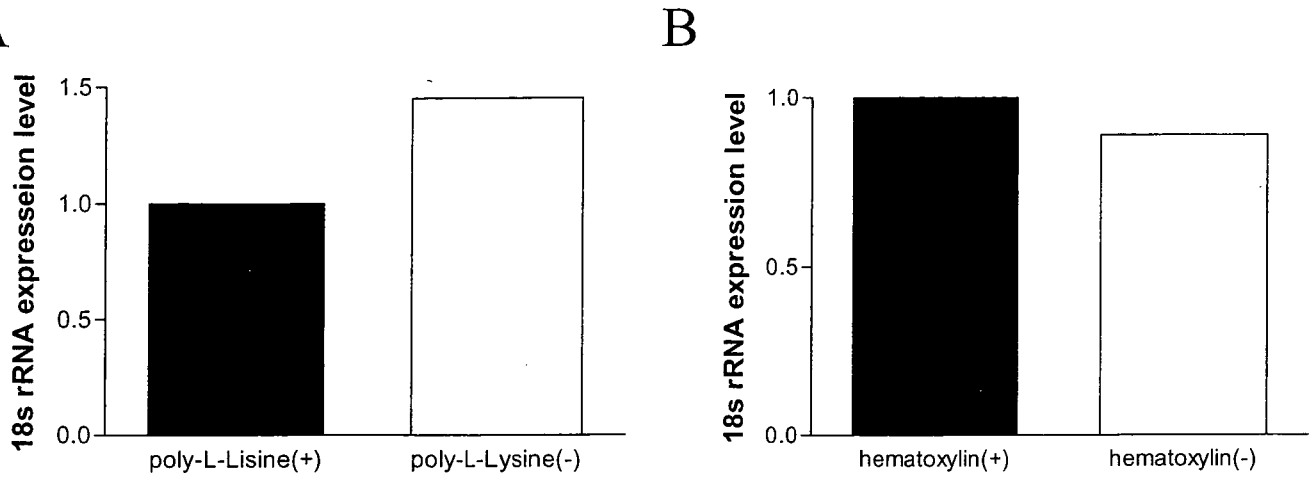

Fig. 1. The amounts of RNA of housekeeping gene (18s rRNA) from the microdissected samples with or without poly-L-lysine coated slides were measured with real-time quantitative polymerase chain reaction (PCR). The same vessels in serial sections of synovial tissues of patients with rheumatoid arthritis (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.

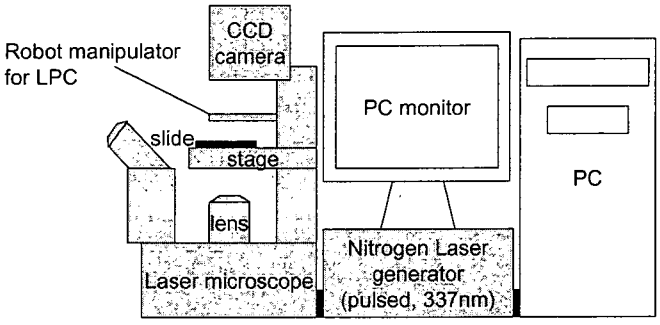

Fig. 2. The LMM system consists of a laser microscope, a laser generator, and a PC with a monitor. Target regions in a tissue on a slide are viewed and marked on the PC monitor through a charge-coupled device camera. A laser beam is generated in the generator and goes upward through the lens, penetrating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

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 (5–8μm thick) are made using a cryostat. The sections are mounted on glass slides coated with a polyethylene naphthalate (PEN) film. 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.

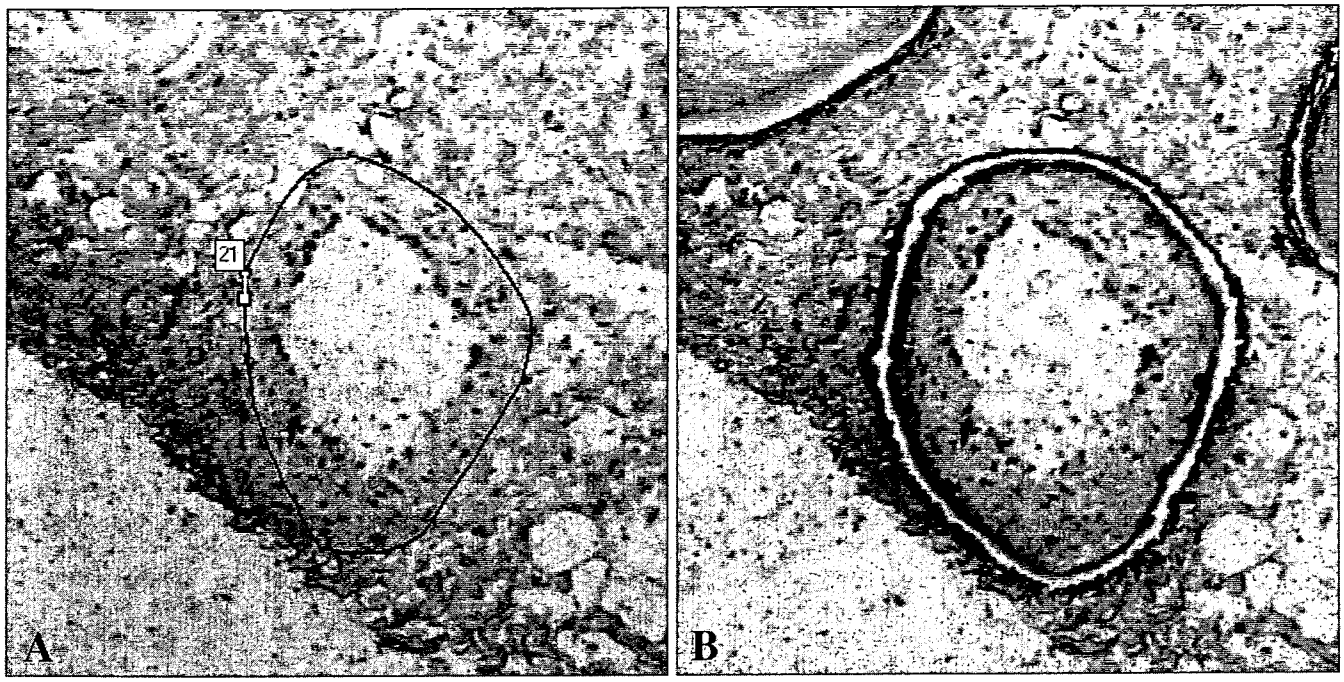

Fig. 3. A cryosection of synovial tissue of RA is mounted on a slide with a membrane film and set on the LMM microscope. The sample is observed on the PC monitor and a target area is marked (**a**). Afterward, the defined line is cut by a laser beam (**b**) (×100)

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

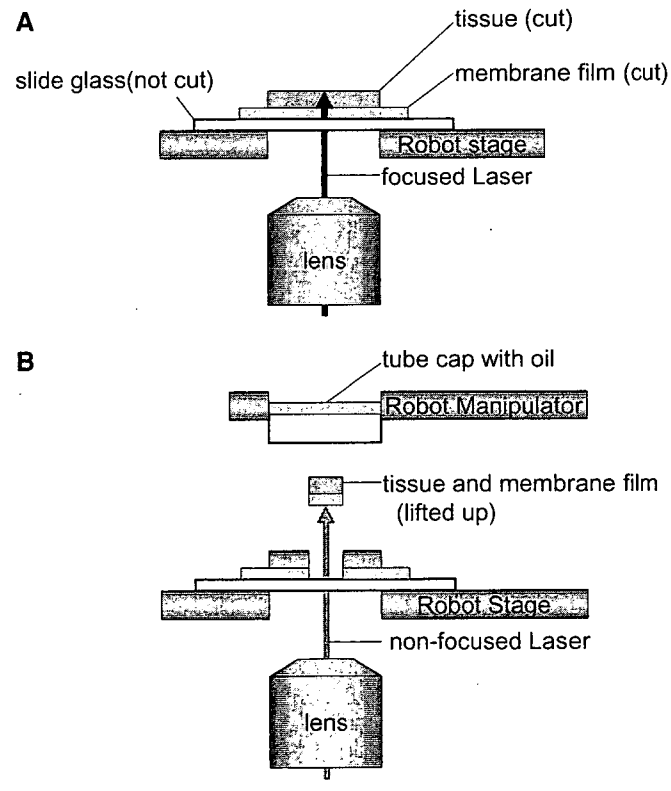

Fig. 4. Side view. A focused laser beam, which comes through a lens under the robot stage, does not cut a glass slide but does cut a transparent membrane film and a tissue with "cold ablation" (**a**). Microdissected cells with a membrane film are lifted up vertically by a non-focused laser and collected 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.

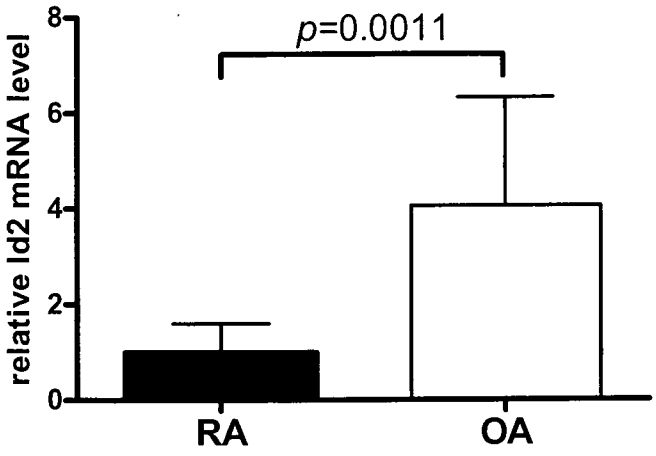

Fig. 5. Relative mRNA levels of Id2 in synovial vessels of RA or osteoarthritis (OA). The Id2 mRNA level of synovial vessels of OA was fourfold higher than that of RA. Values are normalized with endogenous control (18s rRNA) and the mean value of relative mRNA level of RA is set as baseline (reference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

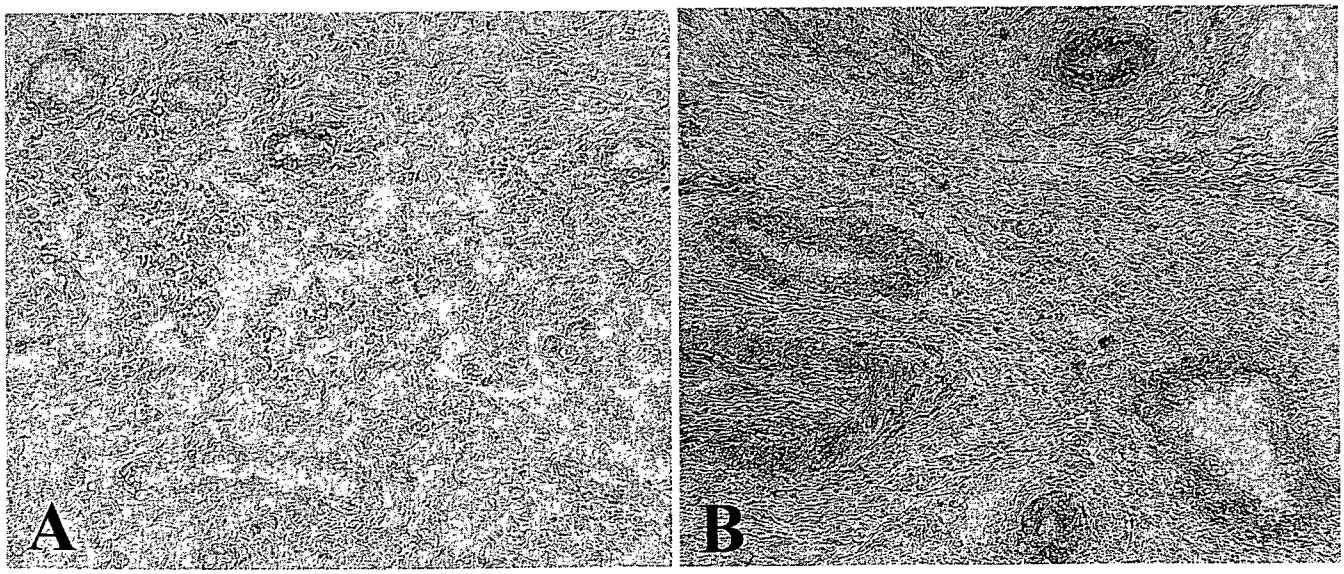

Fig. 6. Id2 protein expressed in synovial tissues of RA or OA detected with immunohistochemistry using a specific antibody. Strong positive staining of Id2 was seen in synovial vessels of OA (**b**) compared with those of RA (**a**). These stainings of Id2 corresponded to the levels of Id2 mRNA expression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

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Potential New Therapeutic Options for Involvement of Central Nervous System in Behçet's Disease (Neuro-Behçet's Syndrome)

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Abstract: Neuro-Behçet's syndrome consists of acute type and chronic progressive type (primary progressive and secondary progressive). Attacks of acute type neuro-Behçet's syndrome are sometimes self-limiting. However, when the neurological manifestations are progressive and severe, administration of corticosteroid is necessary. In addition, infliximab and interferon alpha might also be effective in acute type neuro-Behçet's disease. There are no drugs which have been demonstrated to be effective in preventing the occurrence of attacks of acute type neuro-Behçet's disease. Colchicine, low dose of steroids and various immunosuppressive drugs have been used anecdotally for this purpose. As to chronic progressive neuro-Behçet's syndrome, one should realize that corticosteroids are not effective. Cyclophosphamide is not effective, either. Low dose methotrexate (MTX) has been shown to be beneficial for the treatment of chronic progressive neuro-Behçet's syndrome by an open clinical trial. Thus, low dose MTX has been shown to decrease cerebrospinal fluid IL-6 levels without progression of neuropsychological manifestations, although there are a fraction of patients who do not adequately respond to MTX. Preliminary results indicate that infliximab has a beneficial effect in such patients with MTX-resistant chronic progressive neuro-Behçet's syndrome.

Keywords: IFN-α, colchicine, cyclosporin A, methotrexate, MRI, infliximab, cerebrospinal fluid, IL-6.

1. INTRODUCTION

Behçet's disease is a chronic relapsing inflammatory diseases of unknown etiology, presenting recurrent aphthous stomatitis, uveitis, genital ulcers, and skin lesions. The predominant histopathological features in the inflamed tissues are infiltration of lymphocytes and monocytes, and sometimes polymorph nuclear leukocytes, through small veins without microscopic changes in the vessel walls. Thrombophilia or thrombophlebilis involving small and large veins is also common, whereas arteritis is rare. In these regards, Behçet's disease is unique compared with other vasculitides [1].

Behçet's disease is characterized by the recurrent episodes of remission and exacerbation of various symptoms, whereas chronic sustained inflammation in certain tissues is rare [1]. Recurrent uveitis attacks usually result in the loss of vision that affects profoundly the activity of daily life of the patients. The involvement of the vascular system, of the intestinal system, and of the central nervous system (CNS) is usually life-threatening, and requires aggressive therapy.

The CNS involvement in Behçet's disease is either caused by primary neural parenchymal lesions (neuro-Behçet's syndrome) or is secondary to major vascular involvement [2, 3]. The latter type is rarely complicated with the parenchymal lesions and should be called vasculo-Behçet's disease [2]. This vasculo-Behçet's disease type generally has a better prognosis compared with the parenchymal type [2].

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The most commonly involved area in neuro-Behçet's syndrome is the brain stem, but spinal cord lesions, cerebral hemisphere lesions and meningoencephalitis also occur frequently [3, 4]. Among a variety of signs and symptoms, pyramidal tract signs are most common [2, 3]. The etiology and pathogenesis of neuro-Behçet's syndrome still remain unclear. In addition, factors determining prognosis and appropriate treatment have not been delineated. However, recent investigations have made significant progress in these areas. Moreover, increasing attention has been paid to the effect of anti-tumor necrosis factor alpha (TNF-α) therapy in this disease [1]. The present article overviews an update on the neuro-pathogenesis, clinical manifestation, and treatment of neuro-Behçet's syndrome, suggesting potential new therapeutic options.

2. CLINICAL MANIFESTATIONS

Headache is the most common neurological symptom seen in Behçet's disease [4, 5]. Although headache is seen in venous sinus thrombosis and uveal inflammation [5], it is also accompanied in the majority of neuro-Behçet's syndrome [5]. Previous studies revealed that the most frequent CNS finding in neuro-Behçet's syndrome was a preference for involvement of brainstem-diencephalon and pontobulbar region [2]. Consistently, motor symptoms, cerebellar symptoms, brainstem symptoms and dysarthria are frequently seen (Table 1) [4]. It should be noted that behavioral symptoms are seen in approximately 10%.

The differential diagnosis of neuro-Behçet's syndrome include many CNS diseases, among which multiple sclerosis was one of the leading misdiagnoses. MRI findings indicate that the major lesion is located in the brainstem-diencephalon-basal ganglion region in neuro-Behçet's syndrome [6]. However, the predominant lesion may be in the periventricular white matter in some cases, where it will be

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difficult to discriminate from multiple sclerosis. In such cases, cerebrospinal fluid (CSF) pleocytosis with polymorphonuclear predominance [6], the absence of more than two oligoclonal IgG bands [6], and the elevation of CSF IL-6 [1] may indicate neuro-Behçet's syndrome. Within patients of Behçet's disease, differential diagnosis should include isolate headache syndromes, cardiogenic embolic stroke, astrocytoma, meningioma, and syringomyelia [4]. Although headache is the most common symptoms in neuro-Behçet's syndrome [4, 5], it is also a common symptom in Behçet's disease independent from neurologic involvement [5]. In addition to careful clinical evaluation, the diagnostic values of CSF and magnetic resonance imaging (MRI) should be prospectively studied.

Table 1. Neurological Symptoms in 164 Patients with Behçet's Syndrome

Headache	101	(61.6%)
Motor symptoms	88	(53.7%)
Cerebellar symptoms other than dysarthria	49	(29.9%)
Brainstem symptoms other than dysarthria	48	(29.3%)
Dysarthria	37	(22.6%)
Behavioral symptoms	20	(12.2%)
Sensory symptoms	18	(11%)
Alteration of consciousness	12	(7.3%)
Cognitive symptoms	4	(2.4%)
Others (seizures, peripheral neuropathy, optic neuritis, etc)	16	(9.8%)

The 164 patients include 20 patients with venous sinus thrombosis 124 patients with neuro-Behcet's syndrome. (modified from ref. [4]).

We have recently disclosed that neuro-Behçet's syndrome can be classified into acute type and chronic progressive type [7]. Acute neuro-Behçet's syndrome is characterized by acute meningoencephalitis with or without focal lesions, presenting high-intensity areas in T2-weighted images or fluid attenuated inversion recovery (FLAIR) images on MRI scans [7] (Fig. **1**). Cyclosporin A is frequency associated with acute type neuro-Behçet's syndrome, at least among the Japanese patients [8]. Acute type neuro-Behçet's syndrome responds to corticosteroid therapy, and is usually self-limiting, although recurrence of the attacks is sometimes seen. It should be noted, however, that there are still patients with high degree of permanent damage or disability due to acute type attacks [4, 6, 9].

By contrast, the chronic progressive type of neuro-Behçet's syndrome is characterized by intractable, slowly progressive neurobehavior changes, ataxia and dysarthria, leading to severe disability and deteriolation (Table **2**) [1, 4]. The neurobehavior changes include cognitive dysfunction, euphoria, loss of insight, disinhibition, indifference to their disease, psychomotor agitation or retardation, with paranoid attitudes and obsessive concerns [4]. These symptoms should not be confused with psychosis associated with the use of corticosteroid or other therapy. Of note, the patients with the chronic progressive type of neuro-Behçet's syndrome show persistent marked elevation of CSF IL-6 (>20 pg/ml) with

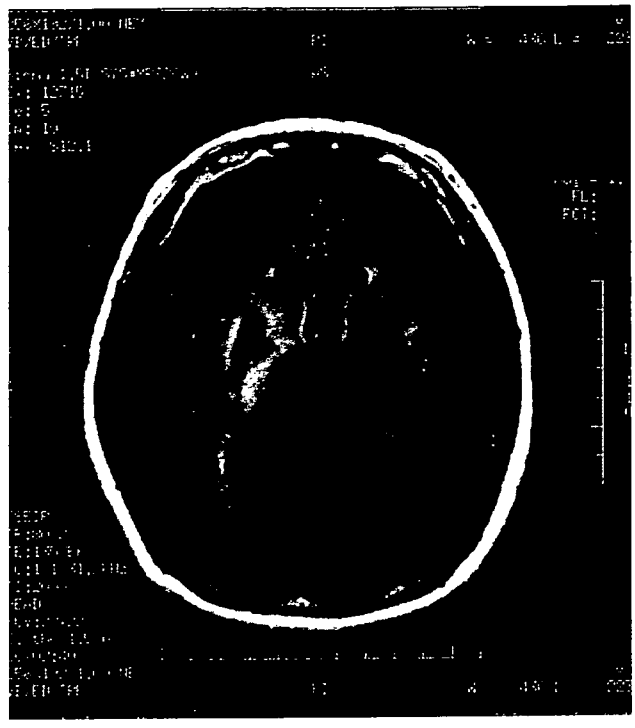

Fig. (1). Axial fluid attenuated inversion recovery (FLAIR) brain MRI of a patient with acute type neuro-Behçet's syndrome, showing high density lesions in the right internal and external capsules.

Table 2. Clinical Features of Chronic Progressive Type Neuro-Behçet's Syndrome: Summary of 11 Cases

Neurological manifestations	
Dementia/psychosis	11 cases
Ataxia	10 cases
Dysarthria	9 cases
Urinary incontinence	7 cases
Myoclonus	1 cases
MRI findings	
Atrophy	
Brainstem/cerebellum	10 cases
Cerebrum	4 cases
Scattered T2 high lesions	5 cases

very modest increase in cell numbers and total protein (Fig. **2**) [10]. No significant elevation of serum IL-6 was noted in patients with chronic progressive neuro-Behçet's syndrome. The MRI findings may show only atrophy of brain stem and cerebellum in the chronic progressive neuro-Behçet's syndrome (Fig. **3**). Most patients (approximately 90%) in our series with the chronic progressive type of neuro-Behçet's syndrome were HLA-B51-positive, and they had history of attacks of acute type neuro-Behçet's syndrome prior to the development of progressive neuropsychological symptoms [10]. Recently, it has been disclosed that HLA-B51 and cigarette smoking, and especially their combination, are risk fac-