fatigue [118]. They include polymyositis (PM), dermatomyositis, and inclusion body myositis, but are generally considered to be distinct diseases with different pathophysiological mechanisms. Muscles produce IL-6 [119], and IL-6 has been also shown to play a regulatory role in muscle wasting [120]. Among these inflammatory myopathies, PM appears to be another suitable target disease for tocilizumab. Excessive IL-6 expression has been found in the sera and infiltrating mononuclear cells in the muscles of PM patients [121–123]. Infiltrating cytotoxic T cells are thought to be involved in muscle fiber damage, and IL-6 functions as a helper factor in the induction of cytotoxic T cells [124]. Moreover, in a model of myosin-induced experimental myositis it was shown that control mice developed clinically manifest muscle damage, whereas IL-6-deficient mice showed no clinical or histological signs of muscle damage [125]. In another model of PM, known as C-protein-induced myositis, intraperitoneal administration of anti-IL-6R Ab suppressed the severity of myositis preventatively as well as therapeutically [126]. We tested the efficacy of tocilizumab in two PM patients who had been refractory to corticosteroids and immunosuppressive drugs [127]. Creatine phosphokinase levels of both patients normalized and MR images showed the disappearance of high-intensity zones in the thigh muscles. These findings suggest that tocilizumab may also be effective as a novel drug for refractory PM.

Dermatomyositis is a complement-mediated microangiopathy associated with destruction of capillaries, hypoperfusion, and inflammatory stress on the perifascicular regions, so that the pathology is different from that of PM [118]. Production of IL-6 and type I interferon signature genes was recently proposed as a biomarker for disease activity in childhood dermatomyositis [128], which thus may be another disorder suitable for tocilizumab targeting.

3.4. Takayasu's Arteritis and Giant Cell Arteritis. Vasculitis refers to inflammation where blood vessels are the primary site of inflammation. The pathological consequence of such inflammation is destruction of the vessel wall, which is histologically detected as fibrinoid necrosis. Takayasu's arteritis (TA) and giant cell arteritis (GCA) belong to an entity designated vasculitis syndrome, and involve both large and medium-sized arteries [129, 130]. The pathogenesis of TA and GCA remains unclear, but it is clear that IL-6 is involved in their development [129–133]. Tocilizumab treatment for a 20-year-old woman with refractory active TA improved the clinical manifestations and abnormal laboratory findings [134], and subsequent studies reported that tocilizumab treatment induced a rapid remission in 2 patients with TA and 5 patients with GCA [135]. Surprisingly, two of the patients with GCA went into remission without concomitant use of corticosteroids. Moreover, tocilizumab was also shown to be effective as rescue treatment for three GCA patients for whom the prednisone dose could not be tapered to less than 30 mg/day [136]. Positron emission tomography/CT scans revealed that in two patients generalized large-vessel vasculitis was detected during the active phase, which completely resolved upon a 6-month course of tocilizumab therapy. These reports strongly imply that IL-6 inhibition may serve as an innovative strategy for the treatment of both TA and GCA. However, several studies have suggested that GCA patients with a lesser inflammatory response without an increase in IL-6 expression were at a higher risk of developing ischemic manifestations than were other patients [137], since the angiogenic activity of IL-6 offers protection against ischemia in such GCA patients [138]. These findings indicate that further clinical studies are required to evaluate the efficacy and safety of tocilizumab for GCA and TA.

It is worthy of note that IL-6 has been also implicated in the development of other types of vasculitis syndrome such as polyarteritis nodosa (PAN) and antineutrophil-cytoplasmic-antibody- (ANCA) associated vasculitis [139–142]. However, so far there have been no reports about off-label use of tocilizumab for PAN or ANCA-associated vasculitis.

4. Therapeutic Implications for Other Autoimmune and Inflammatory Diseases

On the basis of excellent results of the efficacy of tocilizumab for Castleman's disease [143, 144] and systemic juvenile idiopathic arthritis [145–147], it has been approved and used as the first-line biologic in Japan. Pilot studies and case reports with off-label use of tocilizumab also indicate the potential indications of this biologic for various other organ-specific autoimmune and chronic inflammatory diseases. These include relapsing polychondritis [148], acquired hemophilia A [149], autoimmune hemolytic anemia [150], adult-onset Still's disease [151–165], Crohn's disease [166], Bechet's disease with posterior uveitis [167], polymyalgia rheumatica [135, 168], remitting seronegative, symmetrical synovitis with pitting edema [169], spondyloarthritides [170–175], graft-versus-host disease [176, 177], TNF-receptor-associated periodic syndrome [178], and pulmonary arterial hypertension complicated with Castleman's disease or mixed connective tissue disease [179–181]. Further clinical trials are essential, however, to evaluate the efficacy and safety of tocilizumab for these diseases.

5. Conclusion

Acute IL-6 synthesis provides a warning signal and protects the host from environmental stress, while its prolonged production causes the onset and progression of various autoimmune diseases. Several clinical trials have verified the efficacy and safety of tocilizumab for RA, systemic juvenile idiopathic arthritis and Castleman's disease, resulting in approval of this innovative biologic for the treatment of these diseases. Case reports of off-label use or pilot studies have also raised the possibility that tocilizumab could become the biological drug of choice for other systemic autoimmune diseases including SLE, systemic sclerosis, polymyositis and large vessel vasculitis. At present, the mechanisms through which tocilizumab exerts its clinical ameliorative effects on phenotypically different autoimmune diseases are not completely understood. IL-6 blockade may suppress autoantibody production or correct the imbalance of autoantigen-specific Th17 and/or Th1 versus Treg. Thus, clarification of

the mechanisms as well as further clinical trials to evaluate the efficacy and safety of tocilizumab for these diseases are important issues.

Conflict of Interests

Toshio Tanaka declares no conflict of interests.

Acknowledgments

The authors thank Professor Tadamitsu Kishimoto, Professor Atsushi Kumanogoh, and Professor Kazuyuki Yoshizaki for valuable discussions, and Dr. Masashi Narazaki, Dr. Yoshihito Shima, Dr. Keisuke Hagihara, Dr. Toru Hirano, Dr. Junsuke Arimitsu, Dr. Sumiyuki Nishida, Dr. Mari Kawai, Dr. Taeko Ishii, Dr. Yusuke Kuwahara, Dr. Atsuyoshi Morishima, Dr. Yoshihiro Hishitani, Dr. Yuji Yoshida, and Dr. Akihiko Nakabayashi for their collaboration with off-label use of tocilizumab for various disorders at Osaka University Hospital. The clinical studies of tocilizumab were supported by a research grant of the Program of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation. Atsushi Ogata has received a consulting fee as a medical adviser from Chugai Pharmaceutical Co., Ltd.

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Mod Rheumatol (2012) 22:298–302
DOI 10.1007/s10165-011-0497-5

CASE REPORT

A case of Behçet's disease treated with a humanized anti-interleukin-6 receptor antibody, tocilizumab

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Received: 25 April 2011 / Accepted: 20 June 2011 / Published online: 12 July 2011

Abstract A 47-year-old female patient with Behçet's disease had been treated with colchicine, prednisolone, cyclosporine A, and infliximab. Because she relapsed, however, treatment with tocilizumab, a humanized anti-interleukin 6 receptor antibody, was started. This treatment suppressed the patient's clinical manifestations, including ocular attacks, for 1 year and improved her visual acuity. This experience indicates that tocilizumab may constitute a therapeutic option for refractory Behçet's disease.

Keywords Behçet's disease · IL-6 · Tocilizumab

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Introduction

Behçet's disease (BD) is a systemic inflammatory disease, characterized by recurrent oral and genital ulcers, skin and ocular lesions, and other manifestations including neurological, gastrointestinal, and vascular involvements [1]. In BD patients, recurrent ocular attacks, especially of posterior uveitis, eventually lead to irreversible visual loss at a reported frequency rate of about 25% [1]. Patients with BD with refractory central nervous system (CNS) involvement (neuro-BD) often suffer irreversible loss of cognitive function in conjunction with various neurological disturbances. These disease manifestations and the irreversible loss of function have a major negative impact on patients' quality of life (QOL), while severe involvement of the CNS, gastrointestinal tract, and blood vessels can be life-threatening, especially for adolescent male patients. Several treatment regimens using immunosuppressants such as cyclosporine A and azathioprine, interferon α (IFNα), and anti-tumor necrosis factor α (anti-TNFα) agents have been developed and their effectiveness for refractory BD has been proven [1–4]. For patients resistant to these therapeutic regimens, however, no other effective therapies are available at present.

The pathogenesis of BD is not yet fully understood, but it is known that genetic factors as well as environmental factors interact with each other, leading to the activation of immunological and inflammatory responses [1]. As for the genetic background, a higher than normal positivity for HLA-B51 is well known to be associated with the occurrence of BD, as are genetic polymorphisms in several genes, including those for interleukin-1 (IL-1); intracellular adhesion molecule-1 (ICAM-1); endothelial nitric oxide synthetase (eNOS); Mediterranean fever (MEFV), IL-10, and IL-12/IL-23 receptor β1 [1, 5, 6]. Most patients with

BD show hypersensitivity to the oral streptococcal bacterium *Streptococcus sanguinis*. Molecular mimicry between heat shock proteins in bacteria and humans is thought to activate T cells such as Th1 and Th17 to produce distinct cytokines leading to neutrophil activation [1]. In fact, several studies have shown that serum levels of cytokines including IL-2, IL-6, IL-8, IL-10, IL-12, IL-17, IL-18, IFNγ, and TNF α are elevated in patients with active BD [1, 7]. The production of IL-6 from the T cells, monocytes, and peripheral blood mononuclear cells (PBMCs) of BD patients is reportedly enhanced in vitro [8, 9]. In addition, IL-6 is the only cytokine which is elevated in the cerebrospinal fluid of patients with the chronic progressive type of neuro-BD [10, 11]. These findings indicate that IL-6 may have a pathologic role in BD and could thus be a candidate target molecule for the treatment of BD. In the study reported here, we used tocilizumab, a humanized anti-IL-6 receptor antibody [12], for a patient with BD who had been refractory to conventional therapeutic drugs.

Case report

A 35-year-old woman was diagnosed with BD in 1997 on the basis of the presence of recurrent oral and genital ulcers, erythema nodosum, and uveitis, which met the classification criteria for BD established by the Japanese Ministry of Health and Welfare [13]. Because there was no evidence of involvement of the CNS, gastrointestinal tract, or blood vessels, the patient was initially treated with colchicine and prednisolone (20 mg/day). Although cyclosporine A was added in combination with colchicine and prednisolone, the patient's severe posterior uveitis persisted and her visual acuity deteriorated (right eye from 1.2 to 0.2, left eye from 1.2 to 0.15). In August 2007, administration of infliximab, a monoclonal chimeric antibody against TNFα, was started, at a dose of 5 mg/kg, followed by a regular treatment schedule (at weeks 2 and 6, and then every 8 weeks). The infliximab treatment resulted in almost complete disappearance of the clinical manifestations, including the oral and genital ulcers, erythema nodosum, and uveitis, indicating that the disease had gone into remission. In spite of the continuous treatment with infliximab, however, the disease flared up again in December 2008, and the patient presented with recurrent oral ulcers, erythema nodosum, and uveitis, and her visual acuity in the right eye had worsened even more (from 0.2 to 0.1).

Because the disease activity could not be controlled with colchicine, prednisolone, cyclosporine A, and infliximab, we considered azathioprine or tocilizumab as a therapeutic option. The ethics committee of Osaka University Hospital approved the use of tocilizumab for this patient, and her written informed consent was obtained. Infliximab was discontinued in April 2009, and monthly intravitreal injections of triamcinolone were continued until the administration of tocilizumab was instituted, and this regimen resulted in a transient amelioration of her visual acuity. In June 2009, when the patient was 47 years old, treatment with tocilizumab (8 mg/kg, every 4 weeks) was started as monotherapy; that is, without the intravitreal injections of triamcinolone or the concomitant use of colchicine, cyclosporine A, or prednisolone. The tocilizumab was paid for with a grant from the Program for Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation. The patient's serum concentration of IL-6 before the beginning of the treatment was 5.2 pg/ml (normal value <4). The clinical effect of tocilizumab was assessed by using the European Behçet's disease current activity form (BDCAF) [14], which assesses headache, oral ulceration, genital ulceration, erythema nodosum, and arthralgia on a scale of 1 to 4 (0, no symptoms; 1, symptoms for 1 week; 2, symptoms for 2 weeks; 3, symptoms for 3 weeks; 4, symptoms for 4 weeks). Change of visual acuity was also monitored. In addition, QOL was assessed with the short-form 36-item health survey, version 2 (SF-36v2) [15] before the treatment and at the 6 and 12th tocilizumab infusions. The SF-36v2 assesses physical health in terms of physical functioning (PF), role physical (RF), body pain (BP), and general health perceptions (GH), and mental health in terms of vitality (VT), social functioning (SF), role emotional (RE), and mental health (MH).

During the tocilizumab treatment, the clinical manifestations such as genital ulcers, erythema nodosum, and uveitis were attenuated and these manifestations remained silent for 12 months. Changes in the BDCAF score and visual acuity are shown in Fig. 1. The frequency and severity of ocular attacks decreased, along with improvement in visual acuity (right eye from 0.2 to 0.3, left eye from 0.4 to 0.4). Occasional administration of low-dose prednisolone (5 to 15 mg/day for 2 days) was required for mild ocular attacks, but after the 6th infusion of tocilizumab even such occasional use of oral steroids became unnecessary. The patient became entirely free of genital ulcers and erythema nodosum, although oral ulcers were observed occasionally, but they were few in number. The overall severity of her symptoms became milder than ever before. The SF-36v2 scores, especially for physical functioning, role physical, vitality, social functioning, and role emotional also improved dramatically, so that she could perform her daily living activities without difficulties (Fig. 2). The serum level of IL-6 was elevated from 5.2 to 232 pg/ml at 4 weeks after the first infusion of tocilizumab, but decreased to 22 pg/ml just before the 12th infusion.

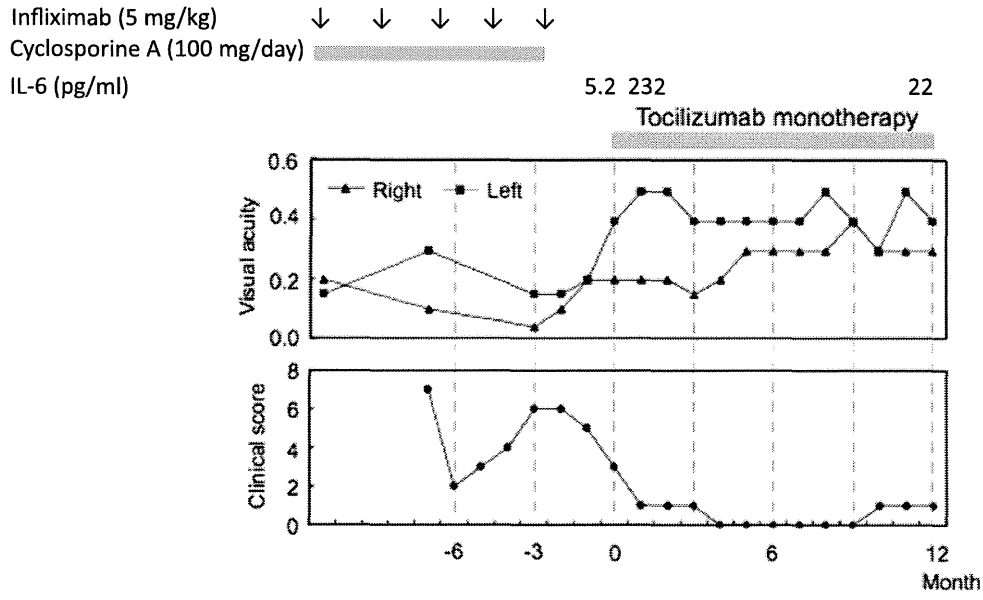

Fig. 1 Clinical effect of toclizumab on visual acuity and Behçet's disease current activity form (BDCAF). Changes in visual acuity, summed score for headache, oral ulceration, genital ulceration, erythema nodosum, and arthralgia entered on the BDCAF, and serum interleukin-6 (*IL-6*) level are shown

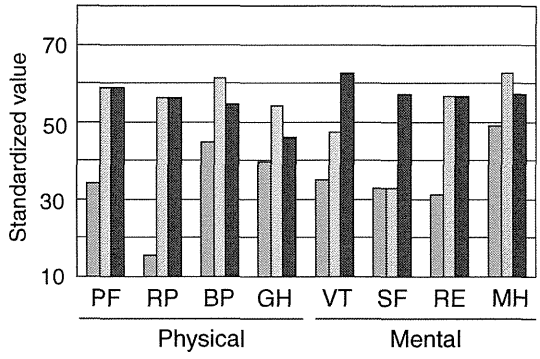

Fig. 2 Clinical effect of tocilizumab on quality of life (QOL). Changes in QOL were assessed with the short-form 36-item health survey (version 2) before tocilizumab administration (*left bars*) and at the 6th (*middle bars*), and 12th infusions (*right bars*). Standardized values (mean = 50 with standard deviation = 10) are shown. *PF* physical functioning, *RP* role physical, *BP* body pain, *GH* general health perceptions, *VT* vitality, *SF* social functioning, *RE* role emotional, *MH* mental health

There were no adverse events, except for a transient increase in the serum low-density lipoprotein (LDL)-cholesterol level (115 mg/dl at baseline, 154 mg/dl at the 6th infusion, and 123 mg/dl at the 12th infusion).

Discussion

In the case reported here, tocilizumab monotherapy for the patient resulted in a 12-month continuation of low disease activity of BD, which had relapsed during combined treatment using colchicine, prednisolone, cyclosporine A, and infliximab. The European League Against Rheumatism recommends that for refractory eye involvement in BD either cyclosporine A or infliximab should be used in combination with azathioprine and corticosteroids; alternatively IFNα with or without corticosteroids could be used [3]. Anti-TNFα agents and IFNα reportedly constitute the most potent therapies for refractory BD complicated by uveitis [1–4, 16, 17]. Infliximab for the treatment of refractory BD was approved in Japan in 2007, while IFNα has not yet been approved for this purpose. The disease activity in our patient was initially satisfactorily controlled by infliximab combined with colchicine, cyclosporine A, and prednisolone, but flared up 15 months after the start of infliximab administration. After intravitreal injections of triamcinolone, her visual acuity recovered somewhat. Tocilizumab administration resulted in further attenuation of the BD clinical manifestations, not only showing attenuation of the uveitis but also attenuating the oral ulcers and erythema nodosum. Because the effect of intravitreal triamcinolone injections is thought to be limited to ocular lesions and to last for only a few months [18], we considered that the systemic clinical improvement, as well as the improvement in QOL, observed in our patient during the 12-month tocilizumab treatment period was due to the direct effect of this agent. The serum IL-6 level depends on the balance between IL-6 production and elimination, and its level during tocilizumab treatment represents the actual endogenous production of IL-6, which correlates with the true level of disease activity [19]. The serum concentration of IL-6 in our patient at baseline was 5.2 pg/ml and appeared to be not so high. But when measured at 4 weeks after the first injection of tocilizumab, the serum IL-6 level had increased to 232 pg/ml, indicating high endogenous production of IL-6. The level decreased to 22 pg/ml just before the 12th infusion of tocilizumab, suggesting the efficacy of this agent. To the best of our knowledge, this is the first case to substantiate the efficacy of tocilizumab for the treatment of BD.

Tocilizumab has been approved as a biological drug for the treatment of rheumatoid arthritis, systemic and polyarticular juvenile idiopathic arthritis, and Castleman's disease [12]. Recent case reports and pilot studies have reported the efficacy of tocilizumab for the treatment of various other autoimmune and inflammatory diseases [20]. The ameliorative effect of tocilizumab in our patient raises the possibility that BD could be another target disease for tocilizumab. The mechanism by which IL-6 blockade can lead to the suppression of disease activity in a patient with refractory BD remains unknown. However, it is known that tocilizumab inhibits the proinflammatory activities of IL-6 and also affects the function of effector T cells. One study found that the serum IL-17 level was higher in active BD than that in remission, which suggests a role for Th17 cells in the development of BD [7]. It has been reported that IL-6, together with transforming growth factor β (TGF-β), induces the differentiation of naïve T cells into Th17 cells, while IL-6 inhibits TGF-β-induced regulatory T cell (Treg) differentiation [21]. Dysregulation of IL-6 production thus causes imbalance in the Th17/Treg ratio. In fact, it was demonstrated that the blockade of IL-6 signaling in a murine model of autoimmune uveoretinitis suppressed the severity of uveoretinitis through Th17 and/or Th1 inhibition or Treg induction [22–24]. Whether tocilizumab can affect effector T cell function in BD patients remains to be determined. In conclusion, IL-6 blockade may constitute an optional treatment strategy for refractory BD with uveitis, although further clinical studies are required to elucidate the efficacy and safety of tocilizumab for BD.

Acknowledgments This work was supported by the Program for Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation.

Conflict of interest Tadamitsu Kishimoto holds a patent for tocilizumab and receives royalties for Actemra. Atsushi Ogata has received a consulting fee as medical adviser and speaking fees from Chugai Pharmaceutical Co., Ltd. Toshio Tanaka has received speaking fees from Chugai Pharmaceutical Co., Ltd. The other authors declare no conflicts of interest.

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Joint Bone Spine 79 (2012) 85–87

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Case report

Psoriatic arthritis in two patients with an inadequate response to treatment with tocilizumab

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ARTICLE INFO

Article history:
Accepted 23 June 2011
Available online 1 October 2011

Keywords:
Psoriatic Arthritis
Tocilizumab

ABSTRACT

Psoriatic arthritis (PsA) is considered as one of the seronegative spondylarthropathies. Like rheumatoid arthritis (RA), the increased production of interleukin (IL)-6 suggests a pathogenic role of IL-6 in PsA. However, whether humanized anti-IL-6 receptor antibody such as tocilizumab (TCZ) might be effective for PsA as well as RA has yet to be determined. We report herein two cases of PsA treated using TCZ. Although, TCZ treatment resulted in disappearance of serum CRP in both patients, arthritis and skin lesions were not improved despite 6-month administration of TCZ. In contrast, tumor necrosis factor (TNF) inhibitor proved effective against arthritis and skin lesions in these patients. Collectively, these findings not only indicate that IL-6 has distinct pathological roles in RA and PsA, but also suggest that TNF inhibitor therapy (but not TCZ) is effective for arthritis and skin lesions of PsA.

1. Introduction

Psoriatic arthritis (PsA) is a common inflammatory arthritis that is associated with psoriasis. Like rheumatoid arthritis (RA), increased production of interleukin (IL)-6 is well known in psoriasis and PsA [1,2].

Most reports have demonstrated correlations between serum levels of IL-6 and disease severity of PsA [3], mouse with epidermal over expression of IL-6 (K14-IL-6 transgenic mouse) exhibits phenotype of psoriasis [4]. The transcription factor signal transducer and activator of transcription 3 (STAT3) is up regulated in psoriasis and IL-6 induces STAT3 phosphorylation and are also thought to be potential therapeutic targets [5].

Characteristic features differ between PsA and RA. In PsA, peripheral arthritis evolves with a distinct joint pattern, potentially involving the distal interphalangeal joints. Dactylitis with enthesitis involving the entire digit is a characteristic feature. Furthermore, articular damage as assessed by radiographic erosion is more common than in RA and typically shows an asymmetric pattern in PsA. Despite these differences in characteristic features, therapeutic options including tumor necrosis factor (TNF) inhibitors and methods of assessing disease activity are mostly held in common. This suggests that IL-6 may play a similar role in the inflammatory arthritis in both RA and PsA.

A humanized anti-IL-6 receptor antibody, tocilizumab (TCZ), was recently approved for RA patients and efficacy of TCZ in patients with RA has been demonstrated [6]. PsA is included in the category of seronegative spondylarthritides, which are defined by the absence of rheumatoid factor and include diseases such as ankylosing spondylitis (AS) and reactive arthritis (ReA). Variable efficacy of TCZ has also been reported in patients with ReA [7] and AS [8–10], since the efficacy is variable pending of the evaluation. However, whether TCZ is effective for PsA as well as RA has not been determined. We therefore tried using TCZ in patients with PsA. Severity of inflammation was evaluated using erythrocyte sedimentation rate (ESR), and levels of C-reactive protein (CRP) and matrix metalloproteinase (MMP)-3. Severity of arthritis was evaluated by two measures of composite disease activity: Disease Activity Score including the 28-joint count (DAS28) and Clinical Disease Activity Index (CDAI). Severity of psoriatic skin was evaluated using the Psoriasis Area-and-Severity Index (PASI).

2. Case 1

A 35-year-old man was diagnosed with psoriasis in July 2002 and developed complications of arthritis in April 2004, particularly in bilateral knee and shoulder joints. Since prednisolone (5 mg/day) and cyclosporine (5 mg/kg/day) proved ineffective for improving arthritis, methotrexate was started at 6 mg/week, then the anti-TNF antibody Infliximab (IFX) was started at 3 mg/kg in November 2006. Although, arthritis and eruptions initially showed marked improvements, symptoms exacerbated in June 2008. Informed consent from the patient and approval by the ethics committee of Osaka

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doi:10.1016/j.jbspin.2011.06.011

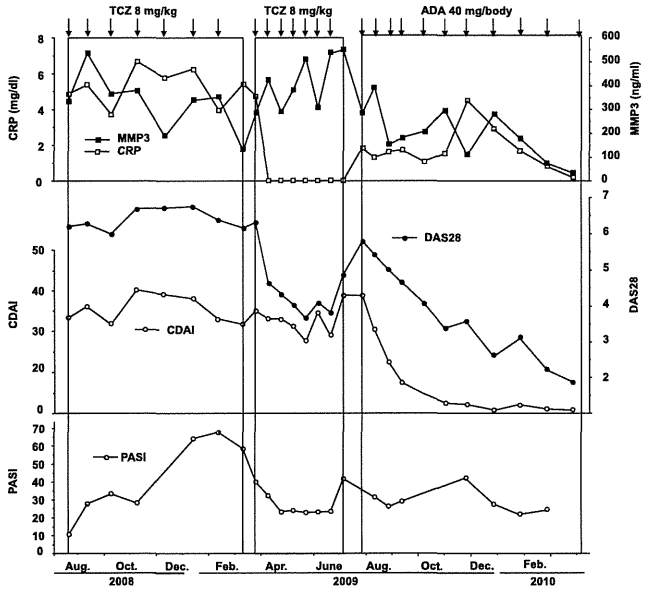

Fig. 1. Changes in C-reactive protein (CRP) level, matrix metalloproteinase (MMP)-3 level, Disease Activity Score including the 28-joint count (DAS28), Clinical Disease Activity Index (CDAI) and Psoriasis Area-and-Severity Index (PASI) score in case 1 during tocilizumab and adalimumab therapy.

University Hospital were obtained for TCZ treatment, which was then started at 8 mg/kg every 4 weeks in July 2008. IL-6 concentration was 77.6 pg/mL (normal, <4 pg/mL) at the time of starting TCZ. Clinical course is shown in Fig. 1. Before TCZ treatment, DAS-28 was 6.44, CDAI was 30.8 and PASI was 51.3. After seven infusions of TCZ, CRP was not improved (7.20 mg/dL to 5.71 mg/dL) suggesting that the 4-week interval infusion of TCZ was not sufficient to inhibit IL-6 function in this patient. Two-week interval infusion was then performed from April 2009 to June 2009. ESR and CRP then showed complete normalization. However, clinical symptoms remained unimproved and MMP-3 level tended to increase (290 mg/dL to 555 mg/dL). DAS28 was slightly improved (6.32 to 4.85), but this was attributed to ESR normalization (54.2 mm/hour to 4.0 mm/hour) by TCZ. If ESR was not included, clinical assessment by CDAI was unimproved (35.0 to 38.7). Skin disease activity as evaluated by PASI showed no improvement (40.5 to 42.1). Administration of TCZ was therefore suspended and injection of adalimumab (ADA) was started in June 2009. Although, ESR and CRP level increased (18 mm/hour and 1.31 mg/dL, respectively), clinical symptoms of arthritis, DAS28, CDAI and MMP3 levels were significantly improved (2.19, 1.6 and 73.5 mg/dL, respectively) by February 2010. Although, skin disease activity as assessed by PASI did not improve rapidly, significant improvement was achieved using ADA by April 2010 (42.1 to 16.5).

3. Case 2

A 28-year-old man was diagnosed with psoriasis in 1991 and developed complications with arthritis in 2002. Since response to DMARDs (disease-modifying antirheumatic drugs) including sulfasalazopiridine (1000 mg/day) and methotrexate (6 mg/week) was inadequate, he joined the Japanese phase III study of ADA in 2006. Arthritis and eruptions were significantly improved during ADA treatment. However, symptoms showed flare-up after the 1-year ADA therapy. In September 2008, he joined a Japanese phase III clinical study of Ustekinumab (fully human monoclonal immunoglobulin [IgG1] antibody targeting the interleukin [IL]-12/23 shared P40 subunit). CRP and MMP-3 levels and

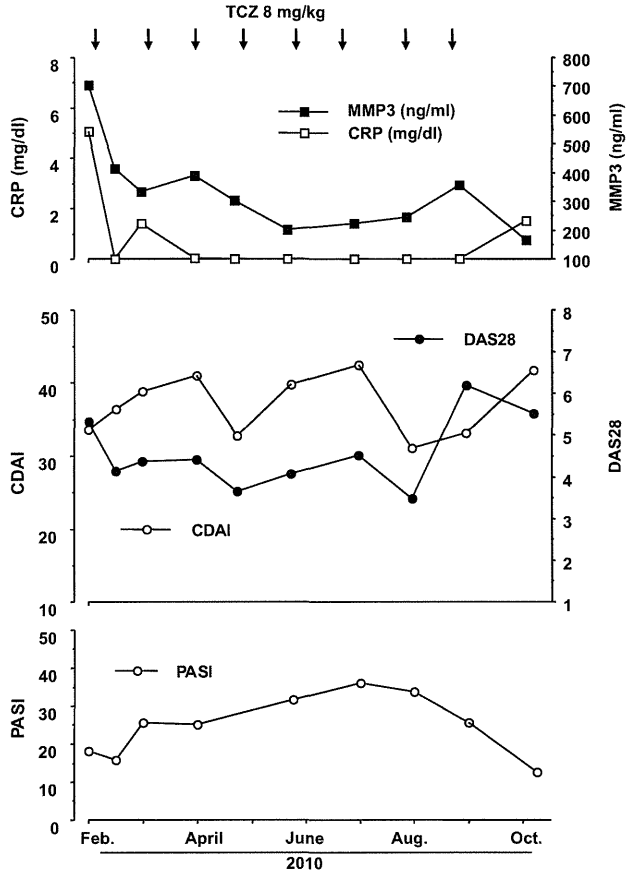

Fig. 2. Changes in C-reactive protein (CRP) level, matrix metalloproteinase (MMP)-3, Disease Activity Score including the 28-joint count (DAS28), Clinical Disease Activity Index (CDAI) and Psoriasis Area-and-Severity Index (PASI) score in case 2 during tocilizumab therapy.

symptoms improved slightly during Ustekinumab therapy. After the 1-year Ustekinumab therapy, symptoms again flared up. CRP level increased to 5.08 mg/dL and MMP-3 level increased to 704 ng/mL. Informed consent from the patient and approval by the ethics committee of Osaka University Hospital were obtained for TCZ treatment. TCZ was then started at 8 mg/kg every 4 weeks in February 2010, at 47 years old. Clinical course is shown in Fig. 2. Inflammatory markers of ESR and CRP showed complete normalization (21 mm/hour to 1.2 mm/hour and 5.08 mg/dL to 0 mg/dL, respectively). However, clinical symptoms as evaluated by CDAI and PASI remained unimproved after seven infusions of TCZ (28.5 to 33.2 and 18.1 to 25.6, respectively). DAS28 was slightly improved (5.33 to 3.48), but this was attributed to ESR normalization by TCZ. Administration of TCZ was therefore suspended in August 2010.

4. Discussion

Although, the characteristics of arthritis differ between PsA and RA, most therapeutic options for these diseases are similar. The differences in mechanism of arthritis between RA and PsA are thus unclear. We report herein the inadequate response to TCZ in PsA, suggesting a different pathogenic role of IL-6 in RA and PsA. In our patients, TCZ normalized CRP levels. Since CRP is induced by IL-6, we can monitor the efficacy of TCZ in suppressing IL-6 by monitoring CRP. IL-6 function thus appeared to be completely suppressed. However, arthritis and skin manifestations of PsA

were unimproved, suggesting that inhibition of IL-6 function was insufficient to improve arthritis in PsA. In contrast, use of ADA as a TNF inhibitor just after TCZ therapy was effective for arthritis in case 1. Although, inflammatory markers (CRP level and ESR) increased, clinical symptoms were improved. In case 2, TNF inhibitor could not be started just after TCZ suspension as the cost proved prohibitive. However, the patient had a history of adequate response to ADA. Unfortunately, ADA could not be continued, again due to financial reasons. Disease activity flared up after suspension of ADA, suggesting that TNF is more important than IL-6 in the pathogenesis of PsA.

During TCZ treatment, the method of evaluating arthritis is important. In RA, TCZ improved CRP and ESR completely and rapidly. However, clinical improvement with TCZ was slow. Improvements of inflammatory markers and clinical symptoms thus do not occur in parallel. We sometimes observe patients in whom arthritis does not improve even if CRP levels normalize. Improvement of inflammatory markers may not match improvement of arthritic symptoms during TCZ therapy. Disease activity has recently been seen to be reduced by TCZ irrespective of the measure of composite disease activity [11]. In our case, DAS28 overestimated the efficacy of TCZ due to the effect of TCZ on ESR and the high weighting for ESR in the DS28.

The exact reason for the different efficacy of TCZ in RA and PsA is unclear. We hypothesized that this may be related to different pathogenic roles of IL-6 in RA and PsA. The traditional model of pathogenesis for psoriasis and PsA hypothesizes that chronic inflammation occurs as a result of T-cell-directed autoimmunity against a common skin and joint autoantigen. However, recent imaging, histological and genetic studies have challenged this view. Clinically unrecognized enthesitis is commonly seen in early PsA and induces frequent microdamage. Tissue repair at normal enthesis attachment sites in healthy joints has resulted in the proposal of a new model of PsA pathogenesis embracing the concept of auto-inflammation [12]. Based on observations of the cytokine dependency of arthritis models, type II collagen-induced arthritis (CIA) requires both IL-6 and TNF [13], whereas anti-type II collagen antibody-induced arthritis (CAIA) requires only TNF, not IL-6 [14]. This suggests that IL-6 is necessary for the production of antibodies specific for joint components (autoimmune phase) and TNF is necessary for the generation of arthritis (inflammation phase) [15]. TCZ may inhibit only inflammatory markers, not arthritis directly. The arthritic improvement effects of TCZ may thus depend on immune modulation rather than inflammatory suppression. TCZ might therefore be ineffective in auto-inflammatory arthritides like PsA.

Our observations suggest that the pathogenic role of IL-6 differs between RA and PsA. Inhibition of IL-6 functions is not indispensable for the treatment of PsA. The present observations are limited by the small number of patients, and larger clinical studies are necessary to confirm the efficacy of TCZ in PsA.

Disclosure of interest

AO received consultant fee from Chugai Pharmaceutical Co. Ltd. NU, IK. AK and TT have no conflict interest.

Funding: This study was supported by the Program for Promotion of Fundamental Studies in Health Sciences from the National Institute of Biomedical Innovation.

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Neuroscience Letters 534 (2013) 85–89

Contents lists available at SciVerse ScienceDirect

Neuroscience Letters

journal homepage: www.elsevier.com/locate/neulet

Proteomic analysis of the hippocampus in Alzheimer's disease model mice by using two-dimensional fluorescence difference in gel electrophoresis

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HIGHLIGHTS

- We perform the proteome for $APP_{E693\Delta}$-transgenic mice. Methods are two-dimensional fluorescence difference in gel electrophoresis and mass spectrometry techniques. The expression of 14 proteins are changed in the brain. Aβ oligomers contribute to the expression of proteins.

ARTICLE INFO

Article history:
Received 4 August 2012
Received in revised form 13 October 2012
Accepted 6 November 2012

Keywords:
Proteome
Amyloid β oligomers
Alzheimer's disease
Hippocampus
2D-DIGE

ABSTRACT

We previously identified the E693Δ mutation in amyloid precursor protein (APP) in patients with Alzheimer's disease (AD) and then generated APP-transgenic mice expressing this mutation. As these mice possessed abundant Aβ oligomers from 8 months of age but no amyloid plaques even at 24 months of age, they are a good model to study pathological effects of amyloid β (Aβ) oligomers. The two-dimensional fluorescence difference in gel electrophoresis (2D-DIGE) technology, using a mixed-sample internal standard, is now recognized as an accurate method to determine and quantify proteins. In this study, we examined the proteins for which levels were altered in the hippocampus of 12-month-old $APP_{E693\Delta}$-transgenic mice using 2D-DIGE and liquid chromatography–tandem mass spectrometry (LC–MS/MS). Fourteen proteins were significantly changed in the hippocampus of $APP_{E693\Delta}$-transgenic mice. Actin cytoplasmic 1 (β-actin), heat shock cognate 71 kDa, γ-enolase, ATP synthase subunit β, tubulin β-2A chain, clathrin light chain B (clathrin) and dynamin-1 were increased. Heat shock-related 70 kDa protein 2, neurofilament light polypeptide (NFL), stress-induced-phosphoprotein 2, 60 kDa heat shock protein (HSP60), α-internexin, protein kinase C and casein kinase substrate in neurons protein 1 (Pacsin 1), α-enolase and β-actin were decreased. Western blotting also validated the changed levels of HSP60, NFL, clathrin and Pacsin 1 in $APP_{E693\Delta}$-transgenic mice. The identified proteins could be classified as cytoskeleton, chaperons, neurotransmission, energy supply and signal transduction. Thus, proteomics by 2D-DIGE and LC–MS/MS has provided knowledge of the levels of proteins in the early stages of AD brain.

1. Introduction

AD is neuropathologically characterized by abnormal accumulation of extracellular amyloid plaques and intracellular neurofibrillary tangles throughout cortical and limbic regions. Although the current amyloid cascade hypothesis [6] and tau hypothesis [15] provide frameworks for studying AD pathogenesis. Recently, diverse lines of evidence suggest that Aβ peptides play more important roles in AD pathogenesis [13,16,20]. Especially, soluble oligomers of Aβ could be a cause of synaptic and cognitive dysfunction in the early stages of AD. To address the relationship between Aβ oligomers and pathological features of AD, we generated APP transgenic mice expressing the E693Δ mutation, which enhanced Aβ oligomerization without fibrillization [25]. It might provide a clue for elucidating AD pathology caused by Aβ oligomers to analyze the $APP_{E693\Delta}$-transgenic mice.

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http://dx.doi.org/10.1016/j.neulet.2012.11.010

One of the most utilized approaches in proteomics to quantify and identify proteins is two dimensional gel electrophoresis (2DE) and mass spectrometry (MS) [5]. Proteomic approaches were most widely based on methods using differential expression on 2D-PAGE gels, or more recently 2D chromatography, followed by mass spectrometry protein identification. Compared to these conventional analyses, 2D-DIGE has higher reproducibility and sensitivity because of its internal standard design which minimizes gel-to-gel variation, improves spot matching, reduces number of gels needed, and permits quantitative analysis of small sample amounts.

In this study, we studied the altered expression of proteins in the hippocampus of $APP_{E693\Delta}$-transgenic mice using 2D-DIGE and LC–MS/MS approach. This approach revealed that the levels of at least 14 proteins were altered in the hippocampus of 12-month-old $APP_{E693\Delta}$-transgenic mice. These findings suggest that Aβ oligomers might cause synaptic and cognitive dysfunction by affecting the expression of these proteins in the hippocampus.

2. Experimental procedures

2.1. Materials

Sodium dodecyl sulfate, urea, thiourea, CHAPS, dithiothreitol, iodoacetamide, bromophenol blue, and RNase A and DNase I for SDS-PAGE or 2DE were all obtained from Wako Pure Chemical Industries (Osaka, Japan). Source information for all other assay reagents and materials are incorporated into their respective assay methods described below.

2.2. Animal subjects

Transgenic mice expressing human APP_{695} with the APP E693Δ mutation under the mouse prion promoter were used [25]. Heterozygous human $APP_{E693\Delta}$-transgenic mice and age-matched non-transgenic littermates were sacrificed at 12 months of age, and their hippocampi were isolated on an ice-cold plate. Animal care and handling were performed strictly in accordance with the Guidelines for Animal Experimentation at Kobe Gakuin University and Himeji Dokkyo University. Every effort was made to minimize the number of animals used and their suffering.

2.3. Protein labeling with CyDyes

Equal amounts of total protein from 4 hippocampi of $APP_{E693\Delta}$-transgenic mice or age-matched non-transgenic littermates were separately pooled. Protein samples were labeled with CyDyes (GE Healthcare, Piscataway, NJ), as per manufacturer's instructions. In brief, 50 μg of total protein from each sample was mixed in a tube and labeled with Cy2 minimal dye, and 50 μg protein taken from the mix was used as an internal standard on each gel for the three subsequent 2DE and image analysis. In parallel, 50 μg protein from each sample was labeled with either Cy3 or Cy5, and the dyes scrambled within each group to avoid possible dye bias. As a result, one replicate was Cy3 labeled proteins and another replicate was Cy5 labeled proteins. Two replicates (Cy3 and Cy5 labeled samples) were mixed, divided and applied each three independent gels. The sample volumes were adjusted to 18 μL with labeling buffer (7M urea, 2 M thiourea, 4% CHAPS, 30 mM Tris), followed by addition of 1 μL dye (working solution) to each individual sample. The samples were left on ice for 30 min in the dark, followed by adding 1 μL of 10 mmol/L lysine to stop the reaction.

2.4. 2D electrophoresis and image analysis

One sample from each of the CyDye groups was mixed together and adjusted to final concentrations of 1% DTT, 1% IPG buffer at a total volume of 350 μL with lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS) and was used to 24 cm pH 4–7 IPG strips (non-linear; GE Healthcare, Piscataway, NJ) overnight. First dimension isoelectric focusing (IEF) was carried out with IPGphor II (GE Healthcare, Piscataway, NJ). Second dimension SDS-PAGE was performed by mounting the IPG strips onto 20 × 26 cm 12.5% DIGE gels (GE Healthcare, Piscataway, NJ) using Ettan DALT six Large Electrophoresis System (GE Healthcare, Piscataway, NJ) and running the gels at 16 mA/gel for the initial hour and 25 mA/gel at 25 °C constantly until bromophenol blue reached the bottom of the gel. The lysates were labeled at the ratio of 50 μg proteins: 400 pmol Cy3 or Cy5 protein-labeling dye (GE HealthcareBiosciences) in dimethylformamide according to the manufacturer's protocol.

In summary, three analytical gels were completed in total, running 25 μg of pooled reference sample labeled with Cy2, along with two samples (25 μg each), one labeled with Cy3 and the other labeled with Cy5. Gels selected for picking were stained with Deep purple (GE Healthcare, Piscataway, NJ). Approximately 1100 spots were matched across all three analytical gels. The analytical gel was picked using an automated robotic system, Ettan Spot picker (GE Healthcare, Piscataway, NJ). The pick list was created based on the Deep purple image. 2 mm gel plugs were picked, washed, reduced and alkylated, and then digested with trypsin, and the resulting peptides were extracted. Gel trypsinization was performed as previously described [24].

2.5. LC/MS/MS identification

Trypsinized peptides were analyzed by nano LC/MS/MS on a ThermoFisher LTQ Orbitrap XL. In brief, 30 mL of hydrolysate was loaded onto a 5 mm 675 mm ID C12 (Jupiter Proteo, Phenomenex) vented column at a flow-rate of 10 mL/min. Gradient elution was conducted on a 15 cm by 75 mm ID C12 column at 300 nL/min. A 30 min gradient was employed. The mass spectrometer was operated in a data-dependent mode, and the six most abundant ions were selected for MS/MS. Mass spectrometry results were searched using Mascot (www.matrixscience.com). Samples were processed in the Scaffold algorithm using DAT files generated by Mascot. Parameters for LTQ Orbitrap XL data require a minimum of two peptide matches per protein with minimum probabilities of 90% at the protein level.

2.6. Western blotting

Approximately 25 μg of protein from mouse hippocampus was applied to a 12.5% acrylamide gel and SDS-polyacrylamide gel electrophoresis was performed at 17.5 mA/gel for 2 h in second dimension. The gels were transferred onto PVDF membranes (Pall Corporation, Pensacola, FL, USA), in a trans-blot electrophoresis transfer cell (Nihon Eido, Tokyo, Japan). Western blotting was performed by using monoclonal antibodies against β-actin (diluted 1:1000, Cell Signaling, USA) and clathrin (diluted 1:250, Abcam, USA), polyclonal antibodies HSP60, NFL, voltage-dependent anion-selective channel protein 1 (VDAC) (diluted 1:1000, Cell Signaling, USA) and Pacsin 1 (diluted 1:500, Millipore, USA). Peroxidase-conjugated antibody (diluted 1:5000, Abcam, USA) was used as secondary antibody. The reaction was detected by chemiluminescence with ECL reagents (Pierce Biotechnology, USA). A semi quantitative analysis based on optical density was performed by ImageJ software (available at http://www.rsbweb.nih.gov/ij).

3. Results and discussion

The 2D-DIGE gels of the hippocampi from wild type and $APP_{E693\Delta}$-transgenic mice pools were shown as Fig. 1. Two replicates of each pooled sample were run, labeling one replicate with

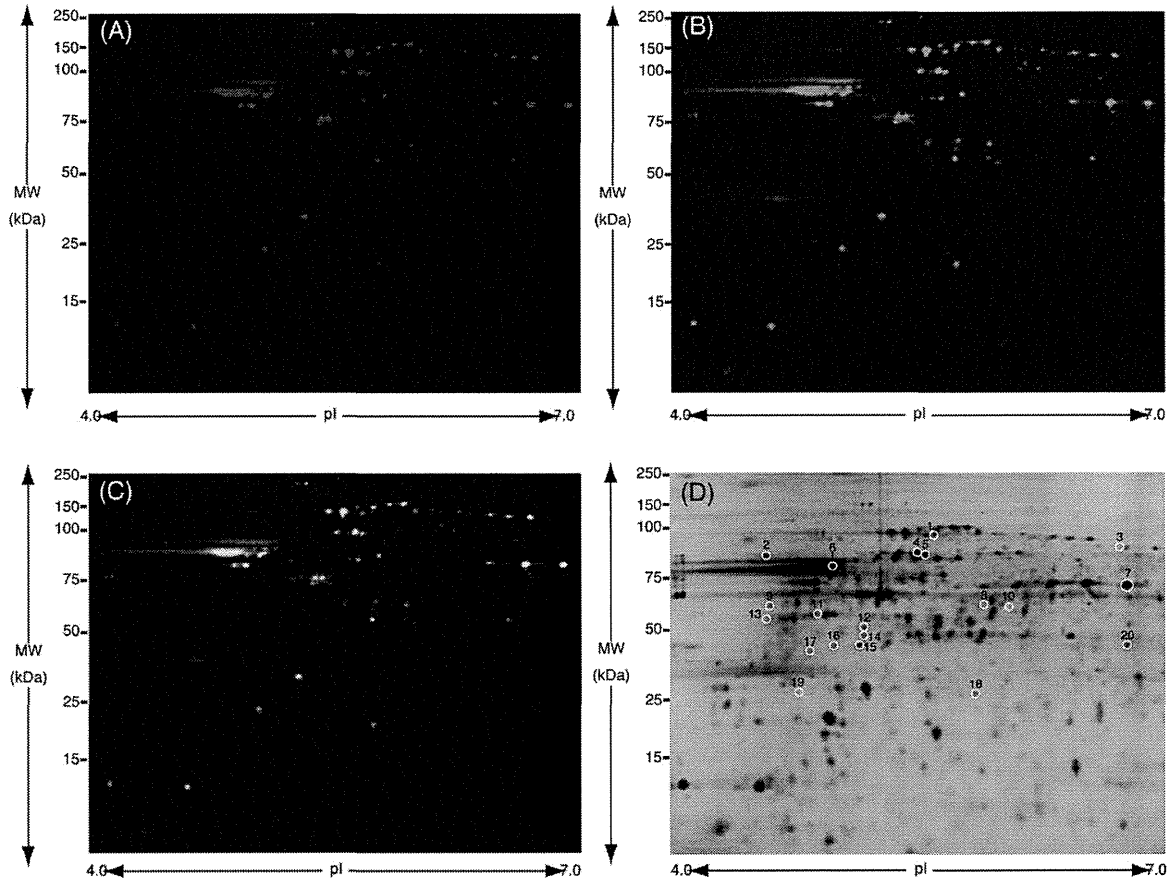

Fig. 1. 2D-DIGE gel image of fluorescence-labeled hippocampal proteins of non-transgenic and $APP_{E693\Delta}$-transgenic mice. (A) Analysis of the proteome of non-transgenic mice hippocampi with Cy3 Dye. (B) $APP_{E693\Delta}$-transgenic mice hippocampi with Cy5 Dye. (C) Merged. (D) Fourteen protein spots identified from non-transgenic and $APP_{E693\Delta}$-transgenic mice hippocampi by LC/MS/MS. Black numbers with white circles indicate proteins that are listed in Table 1.

Cy3 (Fig. 1A) and one replicate with Cy5 (Fig. 1B), resulting in three analytical gels. The 2D-DIGE comparative analysis of the wild type and $APP_{E693\Delta}$-transgenic mice revealed significant 74 spots (Fig. 1C). These spots were investigated by LC–MS/MS (Fig. 1D). Finally, fourteen proteins were identified as shown in Table 1. These proteins are classified into several groups that are involved in cytoskeletal, chaperone, energy metabolic, vesicle transport and signaling proteins (Table 2).

Spot nos. 1, 3 and 4 were identified as heat shock-related 70 kDa protein 2, stress-induced-phosphoprotein 1 and HSP60, respectively. The stress-induced-phosphoprotein 1 is the co-chaperone and thought of the function in regulation of interaction with Hsp70 and Hsp90 [10]. HSP60 is the chaperonin which is implicated in mitochondrial protein import and macromolecular assembly and may facilitate the correct folding of imported proteins [9]. The amounts of heat shock-related 70 kDa protein 2, stress-induced-phosphoprotein 1, and HSP60 were significantly decreased. On the contrary, spot no. 9 which was identified as heat shock cognate 71 kDa protein was significantly increased. This protein is also the chaperone and acts as a repressor of transcriptional activation [8]. Thus, Aβ oligomers might contribute to changing the expression of the chaperons.

Spot nos. 8, 10–12 and 16 were identified as actin, and spot nos. 15 and 17 were identified as tubulin β-2A chain. Actin is one of the major cytoskeletal proteins in neurons, and the dynamics of its assembly are involved in many aspects of cell motility, vesicle transport, and membrane turnover [14]. Actin itself is known to link with Aβ, which enhances the neurotoxicity induced by tau-mediated actin filament formation [4]. The four spots of actin but not no. 12 and those of tubulin were significantly increased. Thus, Aβ oligomers might lead to increasing the amounts of actin and tubulin.

Spot nos. 5 and 2 were identified as α-internexin and NFL, respectively, which are known as neuronal intermediate proteins [2,18]. The amounts of α-internexin and NFL were significantly decreased. Thus, the decreased amounts of NFL and internexin might raise neural dysfunction in the hippocampus of AD.

Spot nos. 7 and 13 were identified as α-enolase. Spot nos. 14 and 19 were identified as γ-enolase and ATP synthase subunit β, respectively. Enolase is a multifunctional protein as glycolytic enzyme, belonging to a novel class of surface proteins [11]. ATP synthase is a key role enzyme that provides energy for the cell to use through the synthesis of ATP [1]. The amount of α-enolase was significantly decreased, but the amounts of γ-enolase and ATP synthase subunit β were significantly increased. Interestingly, the levels of α-enolase and ATP synthase subunit α mitochondrial proteins significantly increased in the hippocampus of J20 Tg mice with amyloid deposition [19]. The amyloid deposit enhanced the expression of energy metabolic proteins [22]. Combined with our findings, both Aβ oligomers and amyloid deposition might play an important role in the change of energy metabolic proteins as α-enolase, γ-enolase and ATP synthase subunit β.

Spot no. 20 was identified as dynamin. Dynamin, a well studied neuron-specific mechanochemical GTPase, pinches off synaptic vesicles, freeing them from the membrane and allowing them to re-enter the synaptic vesicle pool to be refilled for future release

Table 1
Identified proteins from differentially expressed in the hippocampus of$APP_{E693\Delta}$-transgenic mice when compared to non-transgenic littermates.

Spot no.	Protein ID	Fold (APP/WT)	*t*-Test	Accession	Coverage	#Peptides	Predicted MW (kDa)	Calc. pI	Score
1	Heat shock-related 70 kDa protein 2	−1.32	0.040	P14659	26.22	23	69.6	5.67	625.70
2	Neurofilament light polypeptide	−1.48	0.002	P08551	39.96	43	61.5	4.64	1004.84
3	Stress-induced-phosphoprotein 1	−1.44	0.002	Q60864	16.21	9	62.5	6.80	157.49
4	60 kDa heat shock protein	−1.36	0.013	P63038	52.71	71	60.9	6.18	1916.39
5	Alpha-internexin	−1.34	0.023	P46660	42.66	39	55.7	5.27	1119.47
6	Protein kinase C and cascin kinase substrate in neurons protein 1	−1.48	0.023	Q61644	28.34	15	50.5	5.24	356.92
7	Alpha-enolase	−1.32	0.000	P17182	34.33	24	47.1	6.80	474.21
8	Actin, cytoplasmic 1	1.51	0.003	P60709	25.87	14	41.7	5.48	231.79
9	Heat shock cognate 71 kDa protein	1.35	0.015	P63017	12.54	16	70.8	5.52	319.85
10	Actin, cytoplasmic	1.34	0.004	P60709	24.27	13	41.7	5.48	279.37
11	Actin, cytoplasmic 1	1.38	0.022	P60709	15.47	7	41.7	5.48	243.14
12	Actin, cytoplasmic 1	−1.56	0.013	P60709	22.67	12	41.7	5.48	131.57
13	Gamma-enolase	1.33	0.005	P17183	20.05	13	47.3	5.11	237.25
14	ATP synthase subunit beta	1.40	0.047	P56480	23.60	18	56.3	5.34	356.19
15	Tubulin beta-2A chain	1.31	0.021	Q13885	14.83	13	49.9	4.89	313.07
16	Actin, cytoplasmic 1	1.47	0.002	P60709	6.93	3	41.7	5.48	97.01
17	Tubulin beta-2S chain	1.44	0.009	Q13885	11.46	5	49.9	4.89	118.50
18	Clathrin light chain B	1.68	0.005	P09497	8.30	3	25.2	4.64	95.06
19	ATP synthase subunit beta	1.46	0.013	P06576	16.64	16	56.5	5.40	283.06
20	Dynamin-1	1.40	0.006	Q05193	9.61	13	97.3	7.17	242.16

Mass spectrometry protein identification of 2D-DIGE spots of interest and statistical analysis using *t*-test between wild type mice and $APP_{E693\Delta}$-transgenic mice gels ($P < 0.05$). The proteins of mouse hippocampus were separated by 2DE and identified by LC MS/MS, following in-gel digestion with trypsin. The spots representing identified proteins are indicated in Fig. 1D and are designated with their ID accession numbers of Swiss Prot database. Score relates to the probability assignment. Score and sequence coverage were calculated by MASCOT search engine (http://www.matrixscience.com).

Table 2
Functions regulated by proteins that showed an altered expression in $APPE_{693\Delta}$-transgenic mouse hippocampus.

Function	Identified protein	Up/down
Cytoskeletal and their interacting proteins	Neurofilament light polypeptide	Down
	Alpha-internexin	Down
	Actin, cytoplasmic 1	Up/down
	Tubulin β-2A Chain	Up
Chaperone and their interacting proteins	Stress-induced-phosphoprotein 1	Down
	60 kDa heat shock protein	Down
	Heat shock cognate 71 kDa protein	Down
Energy metabolic proteins	Alpha-enolase	Down
	Gamma-enolase	Up
	ATP synthase subunit beta	Up
Vesicle transport and recycling	Dynamin-1	Up
	Clathrin light chain B	Up
Signaling proteins	Protein kinase C and casein kinase substrate in neurons protein 1	Down

The analysis of proteins function was done by using MOTIF (http://www.genome.jp/tools/motif/).

[12]. The amount of dynamin was significantly increased. Our findings in $APP_{E693\Delta}$-transgenic mice without plaque deposition are consistent with previous findings that protein levels of dynamin were increased in Tg2576 mice with plaque deposition [21], suggesting that the release of neurotransmitter is affected by dynamin increased irrespective of AD stage. Also, spot no. 6 was identified as Pacsin 1. The Pacsin 1 is colocalized, oligomerized and bound with dynamin, and both proteins participate in synaptic vesicle endocytosis [17]. The amount of Pacsin 1 was significantly increased. Taken together, Pacsin 1 and dynamin enhanced by Aβ oligomers

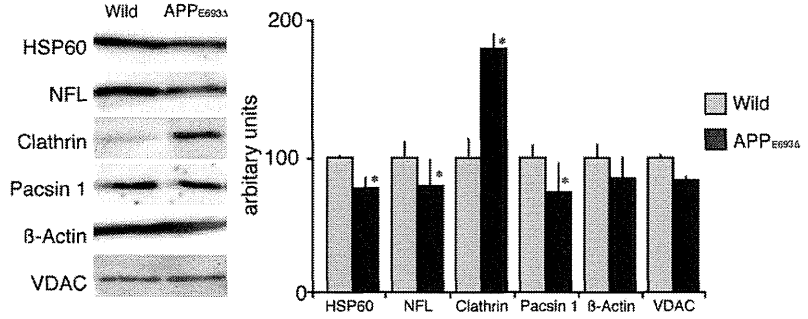

Fig. 2. Differentially expressed proteins validated by Western blotting for the hippocampus of non-transgenic and $APP_{E693\Delta}$-transgenic mice. (A) The levels of HSP60, NFL, clathrin, Pacsin 1, β-actin and VDAC in individual samples of each group were detected. (B) Graphical representation of the semi quantitative analysis (mean ± SEM of O.D. of bands). Data are presented as mean ± SEM ($n = 4$) *t*-test; *$P < 0.05$ vs. $APP_{E693\Delta}$-transgenic mice.