Abatacept may be effective and safe in patients with amyloid A amyloidosis secondary to rheumatoid arthritis

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Abstract

Objective

To examine the efficacy and safety of abatacept (ABT) in patients with amyloid A (AA) amyloidosis secondary to rheumatoid arthritis (RA), and to speculate about the immunologic association of ABT with AA amyloid deposit regression.

Methods

We administered ABT to 70- and 65-year-old Japanese women with RA and AA amyloidosis. We quantified serum cytokine concentrations and analysed regulatory T lymphocytes (Treg cells) via flow cytometry. We also studied AA amyloid deposits via histopathology and immunohistochemistry.

Results

ABT improved rheumatoid inflammation and AA amyloidosis, one case showing clinical remission and the other demonstrating incomplete recovery of nephrosis but stable kidney function. Serum levels of interleukin-6 and tumour necrosis factor α decreased to baseline in the first 6 months of treatment, but serum interleukin-2 concentrations did not change. $CD4^+CD25^{++}FoxP3^+$ Treg cells gated on T lymphocytes and $CD4^+$ T lymphocytes decreased to baseline in the first 3 treatment months. One case showed complete regression of AA amyloid fibrils in serial upper gastrointestinal biopsies, but the other case still had AA amyloid deposits despite ABT-induced normalised rheumatoid inflammation, with polymorphonuclear leukocytes and macrophages infiltrating tissues containing AA amyloid.

Conclusion

ABT demonstrated efficacy and safety in AA amyloidosis secondary to RA and affected Treg cells and inflammatory cytokines. Because the gradual decrease in Treg cells population coincided with AA amyloid deposit regression during ABT therapy, AA amyloid fibril turnover in these patients may involve an immunologic mechanism. Phagocytes seemed to have an important role in AA amyloid fibril regression, which suggests an immunologic interaction.

Key words

rheumatoid arthritis, AA amyloidosis, abatacept, Treg cells, cytokines

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Introduction

Rheumatoid arthritis (RA) is a chronic debilitating systemic autoimmune disease characterised by joint inflammation and destruction, with T lymphocytes having a central role in RA pathogenesis (1). Reactive amyloid A (AA) amyloidosis is one of the most severe extraarticular complications of RA (2, 3) and AA amyloidosis secondary to RA is thus a difficult-to-manage condition and is a rare but important complication (4). However, AA amyloidosis seems not to be irreversible because AA deposits have regressed in experimental AA amyloidosis when inflammation ceased (5, 6).

Recent research suggested that the first event in RA pathogenesis is antigen-dependent activation of T lymphocytes. Regulatory T lymphocytes (Treg cells) have a critical role in maintaining peripheral immune tolerance and preventing chronic inflammation and autoimmune diseases (7, 8). Fewer Treg cells or Treg cells with defective function have been described in RA (9). The immunosuppressive function of $CD4^{+}CD25^{++}FoxP3^{+}$ Treg cells was proposed to depend on interactions between stimulatory and inhibitory signals, and Treg cells were also reported to stimulate dendritic cell activity via CD80/CD86, which would favour their tolerogenic activity (10, 11).

Abatacept (ABT) is a soluble fusion protein consisting of the extracellular domain of recombinant human cytotoxic T lymphocyte-associated antigen 4 and a fragment of the Fc domain human immunoglobulin IgG1 (CTLA-4Ig) (12). CTLA-4Ig has been proposed to reduce T lymphocyte responses by competing for CD80/CD86 to access CD28 and limit the CD28 signaling that T lymphocyte activation requires (13) and ABT may affect more on T lymphocytes (14). Whether intracellular signaling or other CTLA-4Ig-mediated effects contribute to a beneficial or adverse outcome, especially in the setting of the treatment of RA patients, is not entirely clear, and the exact role of CTLA-4Ig in biologic system, including patients with AA amyloidosis secondary to RA, is also unresolved. Only a lower cost-consequence for ABT during real-life treatment was reported (15).

Because the safety and efficacy of ABT in patients with AA amyloidosis secondary to RA have not been studied, we assessed the effectiveness of ABT in RA patients with AA amyloidosis.

Patients and methods

Demographic and clinical backgrounds of study patients and RA controls

Two patients with AA amyloidosis secondary to RA, who met both the American College of Rheumatology (ACR) revised classification criteria (16) and the new ACR/European League Against Rheumatism criteria (17), were eligible for ABT therapy and were followed-up in our outpatient clinic. They were treated with 500 mg of ABT at baseline, 2 weeks, and then every 4 weeks. As described below, they had a long RA disease history, with followed-up in our clinic. To determine a characterisation of a few serum cytokines and T lymphocytes, 18 RA patients without AA amyloidosis who met the RA classification criteria (16, 17) and untreated with ABT, were selected from our outpatients. We recorded age, sex, and duration of RA and of AA amyloidosis, as well as changes in laboratory indices; indicators of disease activity that we recorded included C-reactive protein (CRP), anti-circulated citrullinated peptide antibody (anti-CCP Ab), serum amyloid A protein (SAA), erythrocyte sedimentation rate (ESR), rheumatoid factor (RF), serum albumin, serum creatinine (Crea), 24-hour proteinuria, and estimated glomerular filtration rate (eGFR). We chose CRP as an indicator of clinical rheumatoid inflammation in the Disease Activity Score in 28 joints based on the CRP level (DAS28-CRP) (18), and serum albumin as an indicator of the severity of AA amyloidosis (19). We selected Crea and eGFR to assess the effectiveness of ABT treatment. We calculated eGFR values via the nomogram for modification of diet reported in a Japanese renal disease study. We also obtained information on patients' use of non-steroidal anti-inflammatory drugs (NSAIDs), disease-modifying antirheumatic drugs (DMARDs), immunosuppressants or prednisolone, and angiotensin-converting enzyme inhibitors and angiotensin II receptor block-

ers. We carefully checked for adverse effects, *i.e.* infection risks, myelosuppression, glucose intolerance, dyslipidemia, and carcinogenesis.

Determination of SAA1 genotype

To determine SAA1 gene polymorphism, we performed polymerase chain reaction-restriction fragment length polymorphism analysis with samples from patients with RA, as described elsewhere (20, 21).

Case presentation

– Case 1

A 70-year-old Japanese woman, a housewife, had Steinbrocker Stage IV and Class 2 RA (21), had the SAA1.3/1.5 allele genotype, and was diagnosed as having AA amyloidosis 3 years before ABT therapy. She first noted tenderness in a joint of the right hand in 1986; since then, she had been treated with NSAIDs and/or DMARDs, but her disease activity progressed and then worsened around 1990. Therefore, she started taking immunosuppressive drugs including mizoribine and MTX. A right total knee arthroplasty was performed in 2000. She also had the complications of cervical spine bone involvement such as atlantoaxial subluxation and impaired glucose tolerance. In 2005, she began taking etanercept. In 2010, refractory diarrhea, weight loss, and repeated constipation emerged, and a routine upper gastrointestinal (GI) serial biopsy was performed. The presence of AA amyloid deposits was confirmed histologically via positive Congo red staining and green birefringence seen by polarisation microscopy after Congo red staining, as well as immunohistochemical analysis with anti-AA antibody and anti-immunoglobulin light-chain antibody to identify AL (immunoglobulin light-chain) amyloidosis. In December, 2011, she started ABT therapy, and we followed her clinical course since then. When ABT therapy began, the titers of RF and anti-CCP Ab in serum were 286 IU/ml and 526 U/ml, respectively.

– Case 2

A 65-year-old Japanese woman, a housewife, had Steinbrocker Stage IV and Class 2 RA (22). Her RA disease duration was 25 years; she had a biopsy-confirmed diagnosis of AA amyloidosis 17 years before ABT therapy, and she was homogygous for the SAA1.3 allele genotype. From 1988, her RA disease activity was waxed and waned. In 1995, renal dysfunction, *i.e.* proteinuria appeared, and a renal needle biopsy showed the presence of AA amyloid deposits. Despite the administration of tocilizumab in 2009, after etanercept in 2004, a reduction of rheumatoid inflammation to within normal limits could not be achieved. Chronic kidney disease gradually progressed, and in 2010 we switched to ABT treatment for both rheumatoid inflammation and AA amyloidosis. Although rheumatoid inflammation showed improvement, the DAS28-CRP level was still high. Proteinuria tended to decrease and eventually disappeared, but sometimes positive values were noted. Her renal function did not deteriorated and remained poor but stable. When she started ABT therapy, serum titers of RF and anti-CCP Ab were 826 IU/ml and 684 U/ml, respectively.

Assessment of the effectiveness of ABT therapy

After ABT treatment of the 2 patients with AA amyloidosis secondary to RA began, we monitored markers of rheumatoid disease activity, including DAS28-CRP, health assessment questionnaire (HAQ), and CRP. Also, we assessed values of SAA and eGFR and the degree of spot proteinuria in addition to performing histologic examinations of biopsy specimens obtained at 3 different times after ABT treatment.

Antibodies

Monoclonal antibodies (mAbs) targeting various molecules were used unlabelled or labelled with fluorescein isothiocyanate (FITC) or phycoerythrin (PE) (BD Biosciences, San Jose, CA, USA). The mAbs against CD4, CD8, and CD25 were obtained from Becton Dickinson, Franklin Lakes, NJ, USA. The mAbs against forkhead box P3 (FoxP3) were obtained from eBiosciences, San Diego, CA, USA. Anti-human CD68 antibody was from Dako North America, Carpinteria, CA, USA. Anti-formyl peptide receptor-like 1 (fPRL-1, the receptor involved in the production of reactive oxygen species, degranulation, and phagocyte migration) antibody was described elsewhere in detail (23).

Measurement of serum cytokine concentration

For suspension array analysis, serum samples were separated by centrifugation in the presence of ethylenediaminetetraacetic acid-2Na and were stored at -80°C until study. Serum interleukin (IL)-2 determinations were performed based on the one-step, solid-phase sandwich enzyme-linked immunosorbent assay (ELISA) with the microtiter tray, an anti-human IL-2 mAb that reacted with a horseradish peroxidase-marked IL-2 mAb. With this method, we measured absorptivity after adding the substrate liquid to nonreactant and washing it out from these immune complexes, so that we obtained IL-2 colour development with 450 nm absorptivity (Hitachi, Tokyo, Japan), and we calculated the value for each sample from a standard curve (24).

We measured tumour necrosis factor α (TNF-α) by the two-step sandwich ELISA. After reaction with anti-TNF-α mouse mAb and alkaline phosphatase (ALP)-labelled anti-TNF-α polyclonal antibody, the immune complexes of solid-phase TNF-α antibody and TNF-α-ALP-labelled TNF-α antibody formed. We also added a sensitiser to the complexes after adding the substrate liquid and washing out the liquid, after which we measured IL-2 (25).

On the basis of the chemiluminescence enzyme immunity method of measurement (a 2-step sandwich radioimmunoassay), we quantified IL-6 by using ferrite-coated particles, which were attached via a mouse anti-human IL-6 mAb to the surface for the solid phase. We used ALP as the marker enzyme. The enzyme-labelled antibody was the ALP-labelled antibody used with a mouse anti-human IL-6 mAb (26).

Phenotype analysis

We withdrew blood from the 2 patients with AA amyloidosis secondary to RA

and the 18 control patients with RA during routine laboratory testing. We isolated peripheral blood mononuclear cells (PBMCs) from heparinised blood by means of density gradient centrifugation. PBMCs were resuspended in phosphate-buffered saline/3% human IgG (Baxter International, Atlanta, GA, USA) to block Fc receptors and prevent nonspecific antibody binding, and then PBMCs were incubated for 15 minutes at 4°C in the dark with combinations of FITC-, PE-, CD4-, CD8-, CD25-, and FoxP3-conjugated mAb. Cells were then washed with phosphate-buffered saline/1% bovine serum albumin. We assessed background fluorescence by using appropriate isotype- and fluorochrome-matched control mAbs. After we stained cells with the indicated antibodies, we analysed them by flow cytometry (FACS Canto II; BD Biosciences). We calculated absolute numbers of cells from whole blood counts obtained during routine laboratory testing.

Two-colour flow cytometry analysis of Treg cells

Cells were immunostained for CD4, CD8, and CD25 by using specific mAbs coupled to FITC or PE for 20 minutes at 4°C. Cells were then washed, and intracellular staining was performed after fixation and permeabilitation with an anti-FoxP3-PCH101 clone (10 µl per 1 X 10^6 cells) or an anti-CTLA-4-PE mAb for 30 minutes at 4°C. Finally, the following PBMC subsets were analysed separately, gating first on lymphocytes identified by their forward and side scatter characteristics, in an FACS Calibur flow cytometer with CellQuest software: $CD4^+CD25^+$, $CD4^+FoxP3^+$, and $CD8^+FoxP3^+$. Data were expressed as the percent of double-positive cells or the mean fluorescence intensity of the indicated molecule (27).

Histologic examinations of biopsy specimens

Before and after ABT therapy started, biopsy samples of the upper GI tract were obtained yearly from the 2 patients with AA amyloidosis secondary to RA and were stained with hematoxylin-eosin, Congo red, and immunohistochemicals. Specimens obtained from Case 2 at the latest biopsy were stained with antibodies against fPRL1 and CD68.

Table I. Demographic and clinical characteristics of patients before starting abatacept therapy and RA control patients at baseline.

Characteristic	Case 1	Case 2	RA control (n=18)
Age (years old)	70	65	68.4 ± 3.7
Sex	Female	Female	Female
SAA1 allele polymorphism	1.3/1.5	1.3/1.3	1.3 homo (no.***) 1 1.3 hetero (no.) 8 Others (no.) 9
Months since RA onset	324	302	253.6 ± 75.6
Months since diagnosis of AA amyloidosis	36	204	N.A.**
Steinbrocker's classification*			
Stage	IV	IV	II/III/IV (no.) 2/10/6
Class	2	2	2/3/4 (no.) 12/6/0
MTX (mg/week)	8.0	12.0	7.2 ± 6.7; Yes (no.) 16
PSL dosage (mg/day)	1.5	2.5	4.2 ± 5.8; Yes (no.) 12
Previous biologics (Yes/No)	Yes	Yes	Yes (no.) 4
DAS28-CRP	6.4	5.6	2.72 ± 0.53
HAQ	15.8	10.8	2.34 ± 1.75
CRP (mg/dl)	7.8	5.4	0.28 ± 0.07
ESR (mm/hr)	92	84	20.6 ± 15.3
SAA (µg/ml)	364	289	12.6 ± 5.7
eGFR (ml/min/1.73m^2)	76.4	26.4	69.6 ± 38.4
Crea (mg/dl)	0.64	2.08	0.72 ± 0.10
24-Hour urinary protein (g)	0.06	1.20	0.09 ± 0.15
Alb (g/dl)	4.2	3.5	4.01 ± 0.82
RF (IU/ml)	286	826	146.2 ± 82.6
Anti-CCP Ab (U/ml)	526	684	63.9 ± 49.3

Except where indicated otherwise, values are the mean ± SD.
RA: rheumatoid arthritis; AA: amyloid A; DAS: disease activity score; HAQ: health assessment questionnaire; CRP: C-reactive protein; ESR: erythrocyte sedimentation rate; SAA: serum amyloid A protein; eGFR: estimated glomerular filtration rate; Crea: serum creatinine; Alb: serum albumin; RF: rheumatoid factor; Anti-CCP Ab: anti-circulated citrullinated peptide antibody; MTX: methotrexate; PSL: prednisolone.
*According to JAMA 1949;140:659-62 (Ref. No. 22). **N.A.: not applicable. ***no.: number.

Statistical analysis

All values are expressed as the arithmetic mean ± SD. One-way ANOVA and Student's *t*-test were performed for analysis of significance. Statistical significant means at $p<0.05$. We used SPSS Statistics 17.0, Base and Advanced, (SPSS Inc., Chicago, IL, USA) for statistical analysis.

Informed consent

Each patient gave written informed consent to participate in the study. Patient identities were not disclosed, data were anonymously used, and the work described in this paper was carried out according to the Helsinki Declaration. The Ethical Committee of Kumamoto Shinto General Hospital, Kumamoto, Japan approved the study design and protocols.

Results

Demographics and clinical characteristics of patients

The 2 patients with AA amyloidosis secondary to RA and 18 control patients with RA were all female, as Table I shows. Both study patients had a long RA disease duration, and the Case 2 patient had had AA amyloidosis for a long time, along with renal dysfunction. Both study patients carried the SAA1.3 allele and had a high titer of RF and anti-CCP Ab.

With regard to RA control patients, the mean (± SD) ages was 68.4±3.7 years, RA disease duration was 253.6±75.6 months, and CRP and ESR values were 0.28±0.07 mg/dl and 20.6±15.3 mm/hour, respectively. Whereas the 2 study patients had high rheumatoid inflammation measures, disease activity in RA control patients was controlled well. These control patients received MTX, 7.2±6.7 mg/week, and low to

intermediate doses of corticosteroids, 4.2±5.8 mg/day. Two-thirds of the RA control patients had received NSAIDs and DMARDs.

Effects of ABT in the 2 patients with AA amyloidosis secondary to RA

As seen in Fig. 1, during the more than 2 years of observation after the start of ABT therapy, both patients demonstrated reduced rheumatoid inflammatory disease activity and their diseases were well controlled, with Case 1 maintaining remission of RA. It was interesting that Case 1 and Case 2 had totally different scores in the HAQ, an indicator of functional daily quality of life activities. During the study, no serious adverse clinical and laboratory events and no severe infectious events occurred, except herpes zoster, which was completely cured in Case 1.

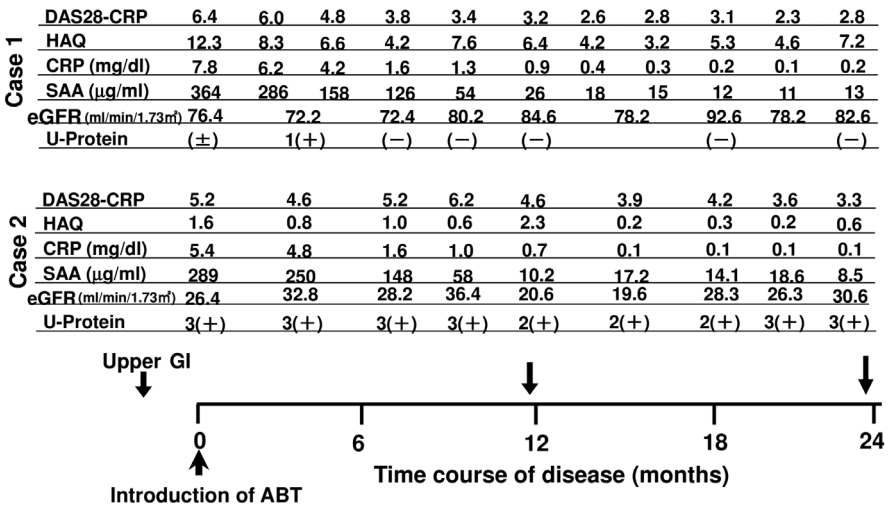

Fig. 1. Clinical course of Case 1 and Case 2. Clinical parameters related to rheumatoid inflammation and AA amyloidosis after abatacept treatment. DAS28-CRP: disease activity score in 28 joints based on the C-reactive protein (CRP) level; HAQ: Health Assessment Questionnaire; SAA: serum amyloid A protein; eGFR: estimated glomerular filtration rate; U-Protein: qualitative protein analysis of spot urine; Upper GI: serial upper gastrointestinal studies; ABT: abatacept. Arrows indicate upper GI biopsies.

Changes in serum concentrations of cytokines

The baselines of serum values in IL-6, TNF-α, and IL-2 were 11.2±1.29 pg/ml, 1.04±0.36 pg/ml, and <0.8 U/ml, respectively, according to the analysis of 18 RA patients with well controlled RA disease activity and without AA amyloidosis, who were not treated with ABT, matched age, sex, and RA disease duration with the present two cases. After administration of ABT, the serum concentrations of IL-6 and TNF-α gradually decreased. In Case 2, levels of these cytokines declined to baseline levels after 12 months, with an abrupt decrease after the start of ABT treatment (Fig. 2). In contrast, both study patients demonstrated no change in serum IL-2 concentrations during 1 year of ABT treatment (Fig. 2). Further, IL-2 levels did not change during treatment but they were normal in both cases before ABT was started.

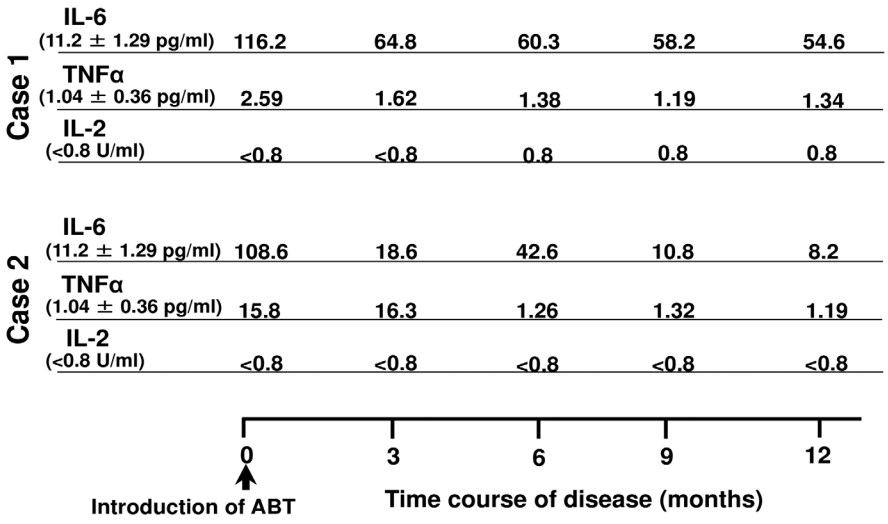

Fig. 2. Changes in serum levels of the inflammatory cytokines IL-6, TNF-α, and IL-2 for Case 1 and Case 2. Values in parentheses are baseline values for the 18 control RA patients whose rheumatoid disease activities were controlled. IL-6 : interleukin-6; TNF-α: tumour necrosis α; IL-2: interleukin-2; ABT: abatacept.

Changes in the Treg cell population distribution

The baseline values in the population of $CD4^+CD25^{++}FoxP3^+$ Treg cells gated on total T lymphocytes and $CD4^+$ T lymphocytes were 1.60±0.85% and 4.79±1.61%, respectively, judging from characteristics on 18 RA patients. With ABT treatment, the population of $CD4^+CD25^{++}FoxP3^+$ Treg cells gated on total T lymphocytes and $CD4^+$ T lymphocytes decreased over time in both study patients. The baseline level was reached 3 months from the start of ABT administration, which emerged was relatively earlier than the times for achievement of baseline rheumatoid inflammation and AA amyloidosis disease activity. However, these dynamic changes in Treg cells were similar to changes in rheumatoid inflammation and inflammatory cytokines (Fig. 3).

Histologic evaluation of AA amyloidosis

Before administration of ABT, Case 1 had Congo red-positive deposits in the submucosa or glandular tissues; however, these Congo red-positive deposits were reduced in biopsy specimens after ABT therapy, which reflected the lower intensity of rheumatoid inflammation (Fig. 4). Histologic observation at 2 years after the start of ABT treatment, however, revealed that AA amyloid deposits remained in Case 2 (Fig. 4).

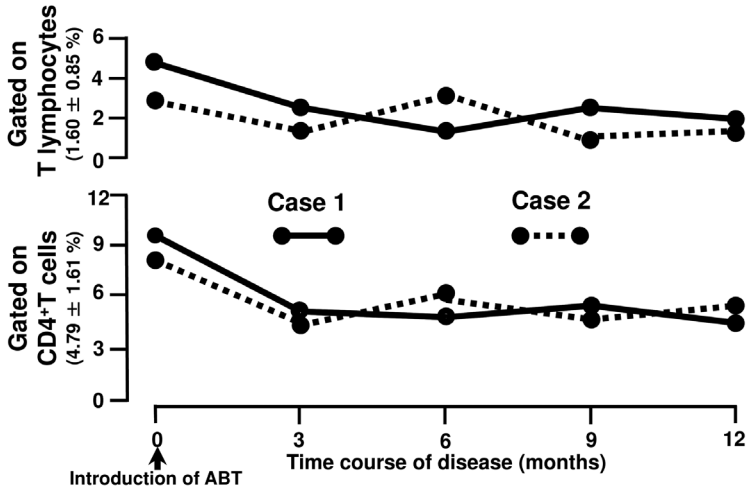

Fig. 3. Changes in serum Treg cell population distribution for Case 1 and Case 2. $CD4^{+}CD25^{++}FoxP3^{+}$ T lymphocytes were analysed by means of two-colour flow cytometry. Values in parentheses are baseline values for the 18 control RA patients whose rheumatoid disease activities were controlled. ABT: abatacept.

Participation of phagocytes in the regression of AA amyloid deposits

For Case 2, we used anti-fPRL1 and anti-CD68 antibodies in immunohistochemical studies of biopsy specimens from the upper GI tract after 2 years of ABT treatment. These antibodies showed positive staining of infiltrating cells in the submucosa where AA amyloid deposits were found (Fig. 5). We assumed that these cells were neutrophils and macrophages, because the antibodies used for staining were specific to molecules expressed in these cell types. In fact, we found that macrophages stained positively with anti-CD68 antibody, surrounding the AA amyloid deposits. Macrophages were often observed close to and being interacted with AA amyloid deposits (Fig. 5).

Discussion

T lymphocyte costimulation is critical for regulation of immune tolerance, immune response, and autoimmunity. Full stimulation of T lymphocytes requires a second signal; activation does not occur with the simple engagement of T cell receptors. Among the various molecules those act as costimulators, CD28/CD80 has an important role in modulating T lymphocyte responses. A physiologic antagonist of CD28 is CTLA4, whose synthetic analog, ABT, was recently approved to treat RA (28). Both study patients –70- and 65-year-old Japanese women– had had RA for 28 years and 25 years and had biopsy-confirmed AA amyloidosis with the SAA1.3/1.5 and SAA1.3/1.3 allele genotypes, respectively. We previously reported that the SAA1.3 allele is a risk factor for the association of AA amyloidosis and a prognostic factor indicating poor life expectancy in Japanese RA patients (29). ABT treatment of the study patients started in January 2011; Fig. 1 shows their clinical courses. We realise that the present study is limited to only 2 cases, but we believe that ABT was effective and safe for these patients with AA amyloidosis secondary to RA. Histopathologic findings from the upper GI biopsy studies before and after ABT therapy revealed that AA amyloid fibril deposits disappeared or decreased (Fig. 4). The changes in the cytokines values and in the Treg cell population distribution suggest an immunologic action of ABT in these cases (Figs. 2 and 3). Although additional elucidation is required, these results indicate the effectiveness of ABT for both RA and AA amyloidosis.

In experimental mouse models of AA amyloidosis, blocking T lymphocytes function by using the calcineurin inhibitor tacrolimus showed that tacrolimus inhibited AA amyloid fibril deposits in a dose-dependent manner and $CD4^{+}$ T lymphocytes localised identical to AA amyloid fibril deposits in the spleen, which suggests a role of T lymphocytes in the pathogenesis of AA amyloidosis (30). In addition to having an effect on T lymphocytes, ABT was suggested to affect other cell types, in particular, antigen-presenting cells. Several studies showed that ABT induced reverse signaling in these cells, although somewhat conflicting results have been reported (31, 32). In our study here, ABT, as a modulator of T lymphocyte function, affected the kinetics of inflammatory cytokines over time and reduced the levels of these cytokines to control values, but ABT had no effect on IL-2 kinetics (Fig. 3). In addition, the population of Treg cells decreased when inflammation indicators improved, which suggested a complex cross-talk between T lymphocytes and cytokines. These inflammatory cytokines recruit activated lymphocytes into synovial cavities and induce production of proteases, reactive oxygen intermediates, and prostaglandins, which damage the cartilage matrix of joints and cause pain (33). However, T lymphocytes obtained from joints of RA patients showed reduced production of inflammatory cytokines, and Treg cell accumulation in inflamed joints of RA patients was reported (34). The exact role of Treg cells in RA remains controversial, because investigations showed that elimination of Treg cells may prevent adjuvant-induced arthritis (35).

The two study patients, with more than 20 years of RA history, carried the SAA1.3 allele genotype, which is a risk factor for AA amyloidosis in Japanese RA patients. ABT therapy gradually improved their RA disease activities, proteinuria, and various GI symptoms and was clinically effective in these patients – to some degree in one case and completely in the other – for both rheumatoid inflammation and AA amyloidosis. The growing understanding of the molecular and cellular pathophysiologic mechanisms underlying RA has stimulated an avalanche of new and highly effective biologic therapies targeting inflammatory cytokines or their receptors. Both etanercept and tocilizumab were reported as being useful for patients with AA

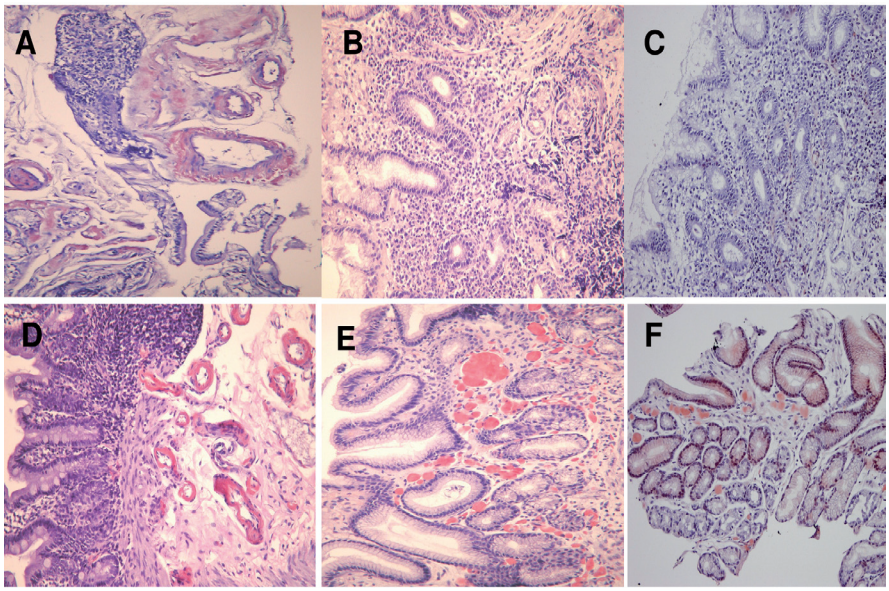

Fig. 4. Congo red-positive staining in a biopsy specimen obtained from serial upper GI studies of Case 1. Biopsies were performed in December 2010 (**A**), November 2011 (**B**), and December 2012 (**C**). With regard to Case 2, biopsies were performed in November 2010 (**D**), December 2011 (**E**), and December 2012 (**F**). Original magnification x100.

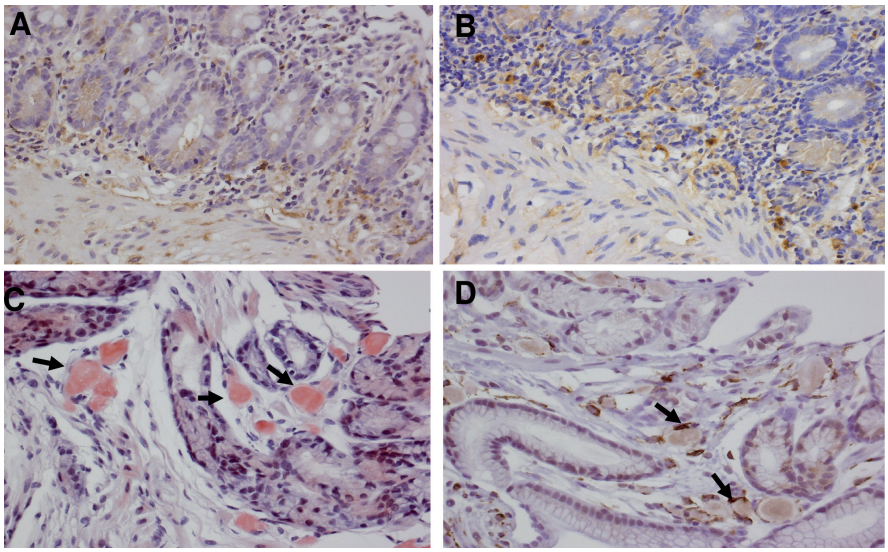

Fig. 5. Immunohistochemical analysis of the biopsy specimen from Case 2 obtained in December 2012 (see Fig. 4F), stained with anti-fPRL-1 antibody (**A**) and anti-CD68 antibody (**B**). Original magnification X200. Histology of specimen from Case 2 obtained in December 2012 (see Fig. 4F). Congo red-positive staining indicated AA amyloid deposits (Arrows) (**C**). Immunohistochemical analysis of the same specimen with anti-CD68 antibody (**D**). Anti-CD68 antibody-positive macrophages surrounded AA amyloid deposits (Arrows). Original magnification x400.

amyloidosis secondary to RA (36, 37). Results from our present study also suggest that ABT targeting costimulatory molecules may be effective for treating patients with AA amyloidosis secondary to RA and that ABT shows potential as an alternative to anticytokine therapies for AA amyloidosis complicating RA. Modulating or inhibiting T lymphocyte function may affect the pathogenesis of AA amyloidosis, and this finding may help elucidate the pathogenesis of AA amyloidosis complicating RA from an immunological point of view.

In patients with AA amyloidosis, degraded SAA products appear to aggregate into fibrils. It is unknown whether cleavage of SAA occurs before and/or after aggregation of monomers during fibrillogenesis (38). On the basis of our histologic studies, we now believe that phagocytic cells such as neutrophils and macrophages may play an important role in the regression of AA amyloid deposits during ABT treatment (Fig. 5). This suggests that T lymphocytes may influence the formation or metabolism of AA amyloid fibrils. These cells were co-localised within AA amyloid deposits, which indicates that phagocytes may function in the metabolism or turnover of AA amyloid deposits. In fact, we assumed the critical function of phagocytes in regression of AA amyloid fibrils. That macrophages are involved in AA amyloid reduction has been proposed, and this hypothesis is supported by observations that macrophages-derived proteases completely degradate AA amyloid (39). Resolution of AA amyloid deposits appears to begin when inflammation subsides and SAA levels normalise. Additional information about the natural clearance of AA amyloid is vital, both for a better understanding of the dynamics of amyloidogenesis and for the development of effective treatment methods for patients with AA amyloidosis secondary to RA.

Our study had several limitations. First, the number of patients assessed was quite small; however, the results obtained here were consistent and striking in the RA study patients as well as in the RA control patients. Because AA amyloidosis secondary to RA is so rare and the sharp-line is unable to be drawn within these two diseases, we could not clearly differentiate the effects of ABT on RA inflammation from those on AA amyloidosis. Also, the relation between ABT and Treg cells in AA amyloidosis secondary to RA has not been previously reported, despite many attempts to characterise additional pathways of the mode of action of ABT. One reason for this situation may be that previous studies on ABT-mediated effects focused mainly on T lymphocyte subsets those manifested no substantial shifts in the distribution (40). Finally, we performed our analysis within the first 3 years of ABT therapy and therefore did not relate them to clinical outcomes. However, whether the effect on phagocytes is accompanied by a clinical effect is not critically important, because

clinical responses are quite variable and many patients respond differently to various agents despite the clear-cut pharmacologic effect, which highlights the heterogeneity of the pathways leading to RA.

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