プロテオグリカン糖鎖合成酵素の遺伝子多型と多発性硬化症の関連

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研究要旨
プロテオグリカンはコア蛋白と長大な糖鎖からなる分子である．神経系の細胞外マトリックスの構成成分であり，その糖鎖の変化は，神経疾患の病態に影響すると考えられる．今回，プロテオグリカン糖鎖合成酵素の中の ChGn1 のゲノム解析を行った結果，著明な酵素活性の低下を伴う SNP（rs140161612）を見出した．MS のみでなく正常コントロールでも同程度の頻度で当該 SNP はみとめられたが，同 SNP をもつ男性 MS において MS の臨床的増悪が軽度であることを示唆するデータが得られた．近年，ChGn-1 のノックアウトマウスにおいて，脊髄損傷からの回復過程が早まることが示された．多発性硬化症の回復過程においてもプロテオグリカンの糖鎖が関与している可能性があり，今後さらに症例数を増やして検討する必要がある．

研究目的
プロテオグリカンはコア蛋白と長大な糖鎖（GAG）からなる分子である．神経系の細胞外マトリックスの構成成分であり，その糖鎖の変化は，神経疾患の病態に影響すると考えられるが，従来十分な検討はされていない．今回，GAG を形成するプロテオグリカン糖鎖合成酵素のひとつである β-1,4-N-acetylgalacto -saminyltransferase-1（ChGn-1）遺伝子に着目して，ゲノム解析を行い，見いだされた SNP の臨床的意義について検討した．

研究方法
147 名の多発性硬化症患者（MS）患者 (男性 45 名，女性 102 名) と 181 名(男性 54 名，女性 127 名)の正常コントロールについて ChGn-1 遺伝子のゲノム解析を行った．それにより見出された酵素活性の低下を伴う SNP と MS の臨床経過との関連をしらべた．

倫理面での配慮
ヒトゲノム・遺伝子解析研究に関する倫理指針に従い，近畿大学医学部倫理委員会で承認を受けた．個人情報には特段の配慮を行った．

研究結果
MS およびコントロールの一部において，ChGn-1 遺伝子の SNP（rs140161612）が認められた．この SNP のアミノ酸変異 p.S126L(377C/T genotypes) について *In vitro* における組換え型酵素による糖転移活性測定の結果，酵素活性の低下を認めた．この SNP の頻度を検討した所，CC 型 (p.S126S)は MS 群 147 名中 134 例であり，コントロール群は 181 例中 166 例であった．CT 型(p.S126L-hetero)は MS 群 147 名中 13 例，コントロール群 181 例中 14 例であった．TT 型（p.S126L-homo）は M S 群（n=147）中 0 人，コントロール群で 1 例を認めたのみであった．MS 患者の臨床症状との関係を検討すると，p.S126L ヘテロ MS 患者群の Progression index（年あたりの EDSS の悪化の程度）は，男性で 0.20±0.09，女性で 0.74±0.21　であり両群で有意差を認めた．また男性患者では，同 SNP を有する例は持たない例よりも Progression index が有意に低かった

考察
プロテオグリカン糖鎖合成酵素のうち，ChGn1 についてゲノム解析を行ったところ MS 患者の約 1 割弱に，酵素活性の著明な低下を伴う ChGn-1 の多型（rs140161612）を認めた．正常対照でも同程度の頻度で同部位の多型がみられ，疾患特異性は認めなかった．しかし MS の Progression index を比較すると，その SNP を有する男女間において有意差がみとめられた．また同 SNP をもつ男性例は，持たない男性例に比

較して Progression index が有意に低値を示した．
近年，ChGn-1 のノックアウトマウスにおいて，脊髄損傷をきたしたときに野生型に比べて有意に回復が早いことが示された．損傷ではなく炎症性疾患である多発性硬化症の回復過程においても ChGn-1 が作用している可能性がある．ChGn-1 の S126L を heterozygote で持った MS 患者は，酵素活性が半分に低下しており，そのことが疾患の増悪過程あるいは増悪からの回復過程において影響している可能性もある．

われわれは先に，同じくプロテオグリカンの糖鎖合成酵素である C6ST-1 の遺伝子改変マウスで MS のモデルとされる experimental autoimmune encephalomyelitis（EAE）の臨床経過が野生型と有意に異なることを報告した．今回のヒト MS のデータは，プロテオグリカンの糖鎖合成酵素遺伝子の多型が MS の臨床経過を修飾する可能性を示唆しており，今後さらに症例数を増やして検討する必要があると考えられる．

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健康危険情報

なし

知的財産権の出版・登録情報

特許：なし
実用新案登録：なし

二次性進行型多発性硬化症患者血清の抗BBB構成内皮細胞抗体が認識する標的抗原：プロテオーム解析を用いた検索

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研究要旨

今回われわれは，二次性進行型多発性硬化症（secondary progressive multiple sclerosis; SPMS）患者血中に存在する抗血液脳関門構成内皮細胞抗体が認識する標的抗原をプロテオーム解析を用いて検索した．プール血清を1次抗体に用いたウェスタン・ブロット法でSPMS患者血清のみにみられ，疾患コントロールでは反応がみられないスポットとしてgalectin-3が同定された．siRNA法で血液脳関門構成内皮細胞株（TY10）のgalectin-3をノックダウンすると，TY10のNFκBとICAM-1の蛋白とmRNA量が増加した．galectin-3は細胞接着や急性炎症に関与することが知られており，本研究の結果から，SPMS患者ではgalectin-3に対する自己抗体が内皮細胞のgalectin-3に結合することよりgalectin-3の機能を低下させ，NFκBを介してICAM-1の発現が増加することでBBBが破綻し，病状進行に関与する可能性が想定された．

研究目的・背景

多発性硬化症（multiple sclerosis: MS）では血液脳関門（blood-brain barrier: BBB）の破綻は病状進行に関与する重要なイベントであると考えられている．我々はこれまでに，二次性進行型多発性硬化症（secondary progressive MS：SPMS）患者血清中に脳微小血管構成内皮細胞に対する自己抗体が存在し，その自己抗体がBBBを破綻させる可能性があることを報告してきた[1]．本研究では，SPMS患者血清中に存在するヒト脳微小血管内皮細胞に対する自己抗体の標的抗原を同定し，病態機序を解明することを目的とした．

研究方法

当教室で樹立したヒト脳微小血管内皮細胞株（TY10）より抽出した蛋白を2次元電気泳動で分離し，SPMS患者7例，正常コントロール20例，疾患コントロールとして再発寛解型MS（relapse-remitting MS）患者9例，視神経脊髄炎（neuromyelitis optica: NMO）患者14例，筋萎縮性側索硬化症（amyotrophic lateral sclerosis: ALS）患者9例，皮膚筋炎患者10例のプール血清を1次抗体としたウェスタン・ブロットで抗原に対する反応を比較した．SPMS患者血清に反応がみられ，正常コントロール血清には反応のない蛋白を同定するために，抗原抗体反応のあった蛋白質をゲル状から切り出し，ゲル内トリプシン消化で得られた抽出物を質量分析計で解析した（liquid chromatography-tandem mass spectrometry: LC-MS/MS）．

同定した蛋白質に対する自己抗体を確認するために，同定した蛋白質のリコンビナント蛋白質を用いて各プール血清を1次抗体としたウェスタン・ブロットを行った．また，同定した蛋白質のTY10での細胞内局在を免疫染色で検討した．

同定した蛋白質をsmall interfering RNA（siRNA）を用いてノックダウンし，TY10細胞での細胞接着分子（ICAM-1，MCAM，VCAM-1）とtight junction関連蛋白（claudin-5，occludin）の蛋白量/mRNA量の変化をウェスタン・ブロット/quantitative real-time PCRで検討した．

研究結果

SPMS患者血清でみられ，正常コントロール血清ではみられないスポットが7個みられた．そのうち，SPMS患者血清のみにみられ，疾患コントロールではみられないスポットが1個みられ，galectin-3が同定された．

galectin-3のリコンビナント蛋白を用いたウェスタン・ブロットでは，SPMS患者のプール血清にのみ，galectin-3の高さにバンドが検出された．

galectin-3のモノクローナル抗体を用いた免疫細胞化学的検討では，galectin-3はTY10の主に核内に存在したが，一部では細胞膜に局在していた．

SiRNAを用いてTY10のgalectin-3をノックダウンするとTY10のNFκBとICAM-1発現量が増

加したが，MCAM，VCAM-1，claudin-5，occludin には変化がなかった．

考察

糖尿病性腎症の研究で，mouse の galectin-3 をノックアウトすると，糖酸化生成物 を投与した場合に，腎皮質での NFκB p65 の活動性が高くなることが示されている[2]．また，腫瘍細胞を用いた研究では，galectin-3 は細胞と collagen type IV, fibronectin，laminin などの細胞外マトリックスの結合を濃度依存性に抑制することが報告されており[3]，galectin-3 は細胞接着を抑制する可能性がある．さらに，SLE 患者では抗 galectin-3 抗体が存在し，皮膚病変の改善とともに抗体価が低下すること，SLE 患者血清から精製した抗 galectin-3 抗体をラットの皮膚に投与すると血管炎を生じること，Rabbit-polyclonal anti galectin-3 antibody を経静脈的に投与すると皮膚血管炎が生じることから，抗 galectin-3 抗体は，SLE 患者の皮膚病変形成に関与するとされている[4]．以上からは，抗 galectin-3 抗体は病原性のある抗体であり，galectin-3 の機能を阻害することで細胞接着を促進する可能性が考えられる．SPMS 患者では，BBB 構成内皮細胞の galectin-3 と抗 galectin-3 抗体が結合することで BBB 構成内皮細胞の galectin-3 の機能が低下することで，NFκB 上昇を介して ICAM-1 の mRNA と蛋白の発現を増強し，SPMS の病状進行に関与している可能性がある．

結論

SPMS 血清中には BBB 構成内皮細胞に対する自己抗体が存在し，その標的抗原の一つが galectin-3 であることを同定した．抗 galectin-3 抗体は SPMS 患者の病状進行に関与している可能性があり，今後は，個々の患者血清を使用し，ELISA などで感度・特異度の検証を行う予定である．

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III. 学会発表等実績

委託業務題目「多発性硬化症生体試料バンクを活用したアジア人特有の遺伝環境因子探索による病態解明」
機関名　九州大学大学院医学研究院神経内科学

1. 学会等における口頭・ポスター発表

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多発性硬化症および視神経脊髄炎における T 細胞受容体遺伝子領域のコピー数多型の同定　（口演）	佐藤眞也，山本健，松下拓也，磯部紀子，河野祐治，吉村怜，飯沼今日子，渡邉充，米川智，眞﨑勝久，山﨑亮，吉良潤一.	第 55 回日本神経学会学術大会，福岡，	2014 年 5 月 21-24 日	国内
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多発性硬化症一卵性双生児不一致例でのゲノムの相違（ポスター）	河野祐治，松下拓也，眞﨑勝久，米川智，佐藤眞也，吉良潤一，土井晃一郎，吉村淳，森下真一.	第 55 回日本神経学会学術大会，福岡，	2014 年 5 月 21-24 日	国内
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多発性硬化症・視神経脊髄炎の全ゲノム関連解析の成績と今後の研究の方向性.	松下拓也，佐藤眞也，中村優理，磯部紀子，吉村怜，眞﨑勝久，河野祐治，吉良潤一.	厚生労働省難治性疾患（神経免疫疾患）政策および実用化研究班　平成 26 年度合同班会議，東京	2015 年 1 月 21-22 日	国内
MS 生体試料バンクを活用したアジア人特有の遺伝環境因子探索による病態解明研究班　概要説明：全国多発性硬化症臨床情報・生体試料バンクの樹立と研究の方向性. 多発性硬化症・視神経脊髄炎の全ゲノム関連解析の成績と今後の研究の方向性.	吉良潤一，佐藤眞也，中村優理，磯部紀子，吉村怜，眞﨑勝久，河野裕治，松下拓也，山﨑亮，新野正明，深浦彦彰，田中正美，越智博文，神田隆，横田隆徳，松井真，楠進，寺山靖夫，西澤正豊，大橋高志，西山和利，中辻裕司，越智一秀，松山晃文，樋野村亜希子，錫村明生，山本健，辻省次，下濱俊.	厚生労働省難治性疾患（神経免疫疾患）政策および実用化研究班　平成 26 年度合同班会議，東京	2015 年 1 月 21-22 日	国内
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2. 学会誌・雑誌等における論文掲載

掲載した論文（発表題目）	発表者氏名	発表した場所（学会誌・雑誌等名）	発表した時期	国内・外の別
Mechanism of multiple sclerosis based on the clinical trial results of molecular targeted therapy - Ochi - 2014 - Clinical and Experimental Neuroimmunology - Wiley Online Library.	Ochi H.	Clin Exp Neuroimmunol 2014	18-Dec-14	国外

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IV. 研究成果の刊行物・別刷

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REVIEW ARTICLE

Mechanism of multiple sclerosis based on the clinical trial results of molecular targeted therapy

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Keywords
alemtuzumab; daclizumab; infliximab; monoclonal antibody; secukinumab; ustekinumab

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Abstract

Multiple sclerosis (MS) is a complex immune mediated disease of the central nervous system. It is characterized by inflammatory and neurodegenerative processes that result in neuroaxonal damage. Its etiology is still unknown, and its pathogenesis is only partly understood. There have been major advances in the treatment of MS in the past two decades, and a wide range of immunomodulagtory and immunosuppressive therapies have been used for the management of MS. More recently, there has been a growing interest in immunotherapeutic strategies with selective actions that target biological molecules involved in MS pathogenesis. Thus, better understanding of the immunopathogenesis of MS is believed to result in the development of more efficacious treatment. However, in contrast to the successful introduced therapies, such as natalizumab and alemtuzumab, there have been a remarkable number of therapeutic failures as well. Despite the convincing immunological concepts and promising results from animal models of MS, some drugs showed no clinical efficacy or even worsened the disease. Clinical trial results of molecular targeted therapy that shed light on the improving understanding of the immunopathogenesis of MS are discussed in the present review. These trials include monoclonal antibodies against leukocyte differentiation molecules (anti-CD3 and anti-CD4 antibodies), tumor necrosis factor-α neutralization, targeting the interleukin (IL)-12/IL-23 pathways, immune cell-depleting ant-CD52 monoclonal antibody and targeting IL-2 receptor signaling.

Introduction

Multiple sclerosis (MS) is an autoimmune disease of the central nervous system (CNS).[1] Over the past two decades, immunomodulatory and immunosuppressive therapies have been widely used for the management of MS.[2] However, a growing understanding of the molecular pathogenetic cascade of MS coupled with the development of biotechnology has enabled remarkable progress in MS therapy. Recently, there has been a growing interest in immunotherapeutic strategies with selective actions that target biological molecules involved in MS pathogenesis.[3] This therapeutic strategy is known as a molecular targeted therapy, and includes monoclonal antibodies and small molecules.[4]

In contrast to successfully established and emerging molecular targeted therapies, such as natalizumab, alemtuzumab, daclizumab, rituximab and fingolimod, there have been several failed or interrupted clinical trials as well.[5] These failed drugs and strategies include monoclonal antibodies against leukocyte differentiation molecules (anti-CD3 and anti-CD4 antibodies), tumor necrosis factor (TNF)-α neutralization and interleukin (IL)-12/IL-23 neutralization. In the present review, clinical trial results of molecular targeted therapy that have added to our growing understanding of MS immunopathogenesis are discussed.

Rationale for targeting the adaptive immune system

MS is a complex chronic immune mediated disease of the CNS, arising from complex interactions between multiple genetic and environmental factors.[6] These interactions could, in part, be responsible for disease heterogeneity. MS is characterized by inflammatory and neurodegenerative processes.[7] Traditionally, two-stage disease pathogenesis has been widely accepted, in which the inflammation precedes neurodegeneration. However, this concept is increasingly being modified in light of neuropathological and magnetic resonance imaging (MRI) findings.[8] Even in the very early stage of the disease, innate immune cells are activated and together with adaptive immune cells, exert direct neurotoxic effects. These neurotoxic effects, together with non-immune mechanisms, such as glutamate toxicity, iron channelopathy, oxidative stress and mitochondrial dysfunction, might cause irreversible axonal and neuronal loss.[7] These are considered to be associated with most of the long-term disability.

Innate immune cells promote the differentiation of Th1 and Th17 cells, which drive acute inflammation. In addition, accumulation of evidence shows that the progressive phase of MS is also, in part, mediated by the innate immune system. However, the role of the innate immune system in the neurodegeneration of MS has been poorly understood.[9,10] Thus, there is no specific therapy to target the innate immune cells in MS. In contrast, far from complete, there is a growing understanding of the adaptive immune system in MS immunopathogenesis.[11] Detailed studies involving human MS immunology, animal models of MS, and clinical trials showed the crucial role of T cells in the pathogenesis of MS. More recent studies also showed the involvement of B cells and regulatory cells.[12] Taken together, the adaptive immune system is believed to play an important role in the inflammatory stage of disease, and thus so far, adaptive immune cells are the main targets for MS therapy.

Molecular targeted therapy

Based on the adaptive immune system in MS pathogenesis, several molecular targets have been translated into therapeutics. Current and emerging treatment options for MS are categorized as unspecific immunomodulators and immunosuppressants or specific molecular targeted treatment.[4] The latter are further classified as either monoclonal antibodies or small molecules. BAF-312,[13] fingolimod,[14] lamotrigine,[15] lipoic acid[16] and riluzole[17] are examples of small molecules that act as specific immunomodulators. Natalizumab,[18] alemtuzumab,[19] daclizumab,[20] ocrelizumab[21] and rituximab[22] are examples of monoclonal antibodies. Among them, natalizumab and fingolimod are landmarks for monoclonal antibodies and small molecules, respectively.

Emergence of therapeutic monoclonal antibodies

Structural characteristics of antibodies were described in the early 1960s, and early hybridoma technology allowed the development of fully murine monoclonal antibodies during the 1970s.[23,24] There are five antibody isotypes distinguished by differences in their heavy chains: immunoglobulin (Ig)M, IgD, IgA, IgE and IgG. Among these antibody isotypes, IgG is the most abundant antibody and typically acts in the secondary phase of an immune response. There are four IgG subclasses in humans; subclasses IgG1, IgG2, IgG3 and IgG4. Except for IgG3, the IgG subtypes have the longest elimination half-lives of approximately 20 days among antibody isotypes. Therefore, IgG is the most common isotype of immunoglobulin that is used for generation of therapeutic monoclonal antibody.

The first murine anti-CD3 monoclonal antibody, muromonab, was approved for the treatment of steroid-resistant acute allograft rejection in renal transplantation recipients.[25] However, its clinical use was hampered by serious side-effects linked to its immunogenicity and mitogenecity. So, recombinant DNA strategies were developed, and more humanized or fully humanized monoclonal antibodies were engineered. They are less immunogenic and mitogenic monoclonal antibodies. Therefore, most of the recently approved biologics are fully humanized monoclonal antibodies.

Failed clinical trials of molecular targeted therapy

Depleting anti-CD3 monoclonal antibody

MS is considered to be a mainly T cell-mediated autoimmune disease, and thus targeting of the T cell differentiation molecules (e.g. CD3 and CD4) with monoclonal antibodies might be a candidate for MS therapy. OKT3 is a mouse IgG2 subclass monoclonal antibody against human CD3. In 1991, Weinshenker et al. carried out the open-label trial of OKT3 in 16 patients with severely progressive MS.[26] In that trial, 73% of treated patients were stabilized clinically, and no new MRI lesions are detected in any of the

patients. However, the trial was hampered by serous side-effects linked to its immunogenesity and mitogenesity. Transient increases in interferon (IFN)-γ and TNF-α were observed, resulting in the cytokine syndrome. Subsequently, humanized Fc receptor-non-binding monoclonal antibody against human CD3, teplizumab, was engineered, and is now tested for type 1 diabetes.[27] This antibody depletes CD3 T cells and can induce CD25+ regulatory cells, and thus treatment with this antibody might be considered to have a good potential for MS therapy.

Depleting anti-CD4 monoclonal antibody

CD4 T cells are considered to play a major role in MS pathogenesis. An open trial showed that treatment of MS patients with the depleting chimeric anti-CD4 monoclonal antibody cM-T412, priliximab, induced a long-lasting reduction of CD4 T cells with a safe procedure.[28, 29] Based on these data, a controlled phase II trial of this antibody was carried out in 71 patients with active relapsing–remitting and secondary progressive MS.[30] However, that trial was essentially negative. In that trial, although there was a long-lasting reduction of peripheral CD4 T cells without causing major toxicity, this antibody did not show any significant effect on the number of active lesions on the monthly gadolinium-enhanced MRI over 9 months. In addition, secondary efficacy parameters, such as Expanded Disability Status Scale (EDSS) progression or the number of courses of methylprednisolone, were not influenced by anti-CD4 treatment. Immunological investigations showed the reduction in the number of peripheral T cells, which was preferentially observed in CD4 T cells.[31] This effect was more pronounced in naïve CD4 T cells, and prior activated CD4 T cells were relatively preserved. In addition, IFN-γ-producing cells were not affected, and IL-4-producing cells decreased. Thus, the significant increase of the Th1/Th2 ratio was observed. This might provide an explanation for the lack of clinical efficacy of depleting anti-CD4 monoclonal antibody. These observations show that the strategies targeting surface molecules exclusively expressed on activated T cells might have a more selective efficacy. There are several other mechanistic interpretations for the lack of clinical efficacy of depleting anti-CD4 monoclonal antibody.[31] In animal models of MS, anti-CD4 antibody treatment was successful when it was given during the period of disease induction. Development of the disease is considered to be mainly dependent on the recruitment of naïve T cells, and thus it is conceivable that treatment is effective. However, in animal models, treatment with depleting anti-CD4 monoclonal antibody did not have any effect on disease activity in the already established disease. This shows that anti-CD4 antibody doses not effectively influence the ongoing immune responses. Taken together, the timing of intervention by anti-CD4 antibody might be critical in ongoing immune responses, and future therapy has to be directed at the primed CD4 T cell subset. Another lesson from this trial is that CD8 T cells are also important effectors in MS. B cells and CD8 T cells are dispensable in many experimental autoimmune encephalomyelitis (EAE) models; however, MS lesions have similar or higher frequencies of CD8 T cells than that of CD4 T cells.[7]

TNF-α neutralization therapy

Several lines of evidence suggest that together with IFN-γ, TNF-α is an essential pathogenetic factor in MS. It has been detected in active MS plaques and *in vitro* study has also shows that TNF-α is cytotoxic for oligodendrocytes. In addition, TNF-α neutralization showed a positive effect on disease pathogenesis in various animal models of MS.[32] Thus, TNF-α neutralization is considered to be a potential therapy for MS. So far, two agents that antagonize TNF-α signaling were tested in MS. One is humanized mouse IgG1 chimeric monoclonal ant-TNF-α antibody CA2, infliximab. The other is recombinant TNF-receptor p55 immunoglobulin fusion protein sTNFR-IgG p55, lenercept.

In an open phase I trial, two patients with rapidly progressive MS were treated with infliximab.[33] Although there was no clinical deterioration, gadolinium-enhancing lesions increased transiently after the treatment in both patients, together with exacerbation of cerebrospinal fluid (CSF) findings, such as leukocytosis and elevated IgG index. This antibody is shown to be efficacious in Crohn's disease and rheumatoid arthritis. Acute demyelinating episodes are an infrequent, but feared complication of its treatment.[34] It is possible that infliximab might induce the intrathecal immune activation; however, there was no detectable antibody in the CSF of either patient.

The other agent, lenercept, is a TNF-α capture molecule, and was tested in a double-blind, randomized, placebo-controlled phase II trial in 168 patients with mainly relapsing–remitting MS.[35] There were no significant differences in terms of MRI parameters or EDSS scores. However, patients treated with

lenercept had a higher number of clinical exacerbations; more relapses, relapses lasted longer and tended to be more severe. The reason why there was a discrepancy between clinical exacerbations and MRI findings is not clear so far. Lanercept has the Fc portion of immunoglobulin, and it might activate Fc receptor-bearing immune cells.

There are several possible explanations for the lack of efficacy of TNF-α neutralization therapy. The first possibility is the potent immunosuppressive properties of TNF-α.[36] TNF-α is reported to induce IL-10 production, and thus blockage of IL-10 induction by TNF-α might, in part, account for the failure of beneficial impact. In the EAE model, TNF-α deficient mice showed prolonged auto-reactive T cell activity, leading to exacerbated disease. The second possibility is the existence of a window of therapeutic opportunity.[37] In animal studies, anti-TNF-α treatment was shown to be more efficacious when delivered before disease onset rather than during remission. In addition, TNF-α-deficient mice showed delayed onset of EAE with comparable disease severity.[38] This shows that an initial priming event might depend more on TNF-α signaling than relapse events.

TNF-α is also known as a pleotropic cytokine, and there are two signals through TNF receptor 1 (TNFR1) and TNF receptor 2 (TNFR2).[39,40] Stimulation of these receptors induces two opposing signaling events. In general, TNFR1 is a major mediator of TNFR-signaling, and this signaling results in apoptosis and inflammation. Both of which depend on the cellular environment, such as the state of activation and cell cycle. In contrast, TNFR2 expression is restricted to highly activated T cells in the immune system. In the central nervous system, this receptor is expressed on neuronal cells, oligodendroctes, microglia and astrocytes. TNFR2 signaling induces cell survival pathways that can result in cell proliferation. This signaling is also shown to be necessary for TNF-α-induced regeneration of myelin-forming oligodendrocyte precursor cells. Taken together, TNF-α might mediate an "on and off" signal in the MS pathogenetic cascade, and pleiotropic function might be a barrier toward translational development of TNF-α targeted therapy.

Targeting the IL-12/IL-23 pathways

Th1 and Th17 immune pathways play a crucial role in the pathogenesis of several immune-mediated disorders including MS. IL-12 induces Th1 cells, and IL-23 promotes the expansion and survival of Th17 cells. Therefore, targeting the IL-12/IL-23 pathways is an attractive approach in the treatment of MS.[41] IL-23 is a heterodimeric protein composed of p19 and p40 subunits. The p40 subunit is also a component of IL-12. Thus, neutralization of the p40 subunit is believed to be able to block both Th1 and Th17 inflammatory pathways. Ustekinumab is a fully humanized IgG1 monoclonal antibody that blocks the activity of the p40 subunit common to IL-12 and IL-23, and has been evaluated in 249 patients with relapsing–remitting MS.[42] Treatment with this antibody was generally safe and well tolerated. Ustekinumab treatment did not show a significant clinical improvement or a radiological improvement. The cumulative number of new gadolinium-enhancing T1-weighted lesions did not differ between ustekinumab and placebo groups. Overall, ustekinumab was not shown to be efficacious for the treatment of MS. There are several explanations for the lack of therapeutic efficacy of this antibody. The therapeutic efficacy of ustekinumab might depend on the disease stages. In the EAE model, this antibody was shown to be more effective in the prevention regimen than the therapeutic regimen for established disease.[43] Thus, IL-12/IL-23 might have greater involvement in the initiation and expansion of pathogenic T cells. Another possibility is the crucial role of B cells and CD8 T cells in the pathogenesis of MS. Additionally, the blood–brain barrier (BBB) might be insufficiently disrupted in MS as compared with EAE and ustekinumab, and with an approximate molecular weight of 150 kDa, it might not have crossed the BBB. The pP40 subunit needs to be neutralized in the CNS to achieve a significant therapeutic effect in MS.

Targeting the IL-17 pathway

IL-17 is a hallmark cytokine of Th17 cells, and is produced mainly by Th17 cells. Th17 activation and excessive production of IL-17 lead to autoimmunity and chronic inflammatory responses.[44] An increased number of Th17 cells has been shown in the lesions of MS, and dysregulation of Th17 cells is thought to play a critical role in MS immunopathogenesis.[45] Therefore, targeting the IL-17/Th17 pathway is an attractive approach in the treatment of MS. Although ustekinumab, which also targets IL-17 signaling indirectly through IL-23, failed to show clinical efficacy in MS, an early clinical trial of secukinumab, which is a fully humanized IgG1 monoclonal anti-IL-17A antibody and a highly selective inhibitor of IL-17A signaling, showed promising efficacy.[46] The first results of 73 patients showed a

significant reduction of combined unique active lesions during weeks 24–48 in the secukinumab group, and a trend towards reducing the annual relapse rate was also observed. This result supports the concept that the IL-17 pathway plays a crucial role in the MS pathogenetic cascade.

Novel and emerging molecular targeted therapy

Immune cell-depleting anti-CD52 monoclonal antibody (alemtuzumab)

Alemtuzumab is a humanized IgG1 monoclonal antibody against CD52.[47] CD52 is expressed on the surface of various immune cells, including B and T cells, natural killer cells, dendritic cells, and most monocytes and macrophages, the exact biological function of this molecule is not fully understood.[48] This antibody against CD52 was developed as an antibody that could selectively kill human lymphocytes, and was licensed as a treatment for fludarabine-resistant chronic B cell leukemia.[49] In addition, this antibody has been used off label in autoimmune hematological diseases, such as immune thrombocytopenic purpura, and in organ transplantation as an induction agent.

The first MS patients were treated with this antibody in 1991, and a total of 36 patients with progressive MS were treated by 1999.[50] A dramatic reduction in the number of gadolinium-enhanced lesions, maximally by over 90% and a 97% reduction in the relapse rate were observed for at least 18 months after a single pulse of treatment (given as a 20-mg daily intravenous infusion for 5 days).[51] Despite disruption of the inflammatory process, however, half of the patients experienced disease progression. In these patients, the accumulation of new lesions was suppressed; however, progressive cerebral atrophy on follow-up MRI was observed, and their disability worsened with time. Patients with progressive disability showed higher inflammatory activity before alemtuzumab therapy. This result raised the possibility that immunotherapy with alemtuzumab might be more beneficial if given in the early course of relapsing–remitting phase.

This result led to a change in therapeutic strategy, and an open-label pilot study of 58 patients with MS was carried out.[52] This trial consisted of 22 patients with early active relapsing–remitting MS and 36 patients with secondary progressive MS. In this trial, although significant effects on inflammatory activity were observed equally in both groups, the disability outcome was differently affected depending on the phase of the disease. In secondary progressive MS, sustained accumulation of disability and a reduction in cerebral volume were observed. In contrast, in early active relapsing–remitting MS, the mean EDSS score fell by 1.4 points and 16 of 22 (73%) patients improved in their disability by 1 year. This early trial of alemtuzumab clearly shows the existence of a "window of therapeutic opportunity" in the disease course, and supports the concept that MS is an immune-mediated disorder. Early rescue of neurons and axons from toxic inflammatory environment might result in the prevention of neuronal and axonal degeneration associated with long-term disability.

As a consequence, clinical development of alemtuzumab focused on patients with early active relapsing–remitting MS. In the phase II and III trials of alemtuzumab, patient selection was limited to those with short disease durations and limited disability measured by EDSS (Table 1).[53–55] Conclusions from these trails are that alemtuzumab treatment is more effective than subcutaneous IFNβ-1a with regard to relapse rate reduction and disability accumulation rate. In addition, there was a small improvement in the degree of disability during the phase II trial of early active relapsing–remitting MS.[53] Notably, the clinical effect of alemtuzumab is remarkably long-lasting, and appears to persist even 5 years after the last infusion. The 5-year follow up of the phase II trial showed that alemtuzumab lowered the relapse rate by 69% and the risk of sustained accumulation of disability by 72% compared with IFNβ-1a.[56]

The underlying mechanism is not yet fully understood. Alemtuzumab almost completely depletes the circulating T and B cells. The anti-inflammatory effect induced by lymphopenia could account for the early treatment efficacy; however, it is unlikely for the long-lasting efficacy. Long-term clinical efficacy of alemtuzumab can be attributed to qualitative changes in repopulating lymphocyte subset.[57] It is possible that these promote immune tolerance and suppression of the effector T cell repertories. An immunological mechanistic study showed that monocytes and B cells returned rapidly to the pretreatment value after the treatment of alemtuzumab. However, the recovery of T cells was much slower, with CD4 and CD8 T cells reaching 32.9% and 55.4% of pretreatment values, respectively, by 12 months. During immune repopulation, qualitative changes have been observed. For example, after the treatment of alemtuzumab, preferential expansion of CD25+ regulatory cells and significant expansion of Th2 cells were observed. In addition, B cell reconstitution was also observed. After their return to pretreatment value, B cells

Table 1 Phase II/III clinical trials of alemtuzumab focused on patients with early active relapsing–remitting multiple sclerosis

	CAMMS223[53]	CARE-MS I[55]	CARE-MS II[54]
Comparator	SC IFNβ-1a	SC IFNβ-1a	SC IFNβ-1a
Baseline demographics	Early active	Treatment naive	Relapse on DMT
Mean age (years)	32	33	35
Mean disease duration (years)	1.3 (≤3)	2.1 (≤5)	4.5 (≤10)
Mean EDSS	2.0 (≤3)	2.0 (≤5)	2.7 (≤5)
ARR	1.3	1.8	1.6
Clinical outcomes			
ARR at end of study (vs comparator)	0.1 (vs 0.36)	0.18 (vs 0.39)	0.26 (vs 0.52)
Relapse rate reduction	74%	55%	49%
Relapse-free patients (vs comparator)	80% (vs 52%)	78% (vs 59%)	65% (vs 47%)
Mean change of EDSS (vs comparator)	−0.39 (vs +0.38)	−0.14 (vs −0.14)	−0.17 (vs +0.24)
Sustained disability progression (vs comparator)	9% (vs 26%)	8% (vs 11%)	9% (vs 22%)

ARR, annualized relapse rate; CARE-MS, Comparison of Alemtuzumab and Rebif Efficacy in Multiple Sclerosis; DMT, disease modifying therapy; EDSS, Expanded Disability Status Scale; IFN, interferon; SC, subcutaneous.

exceed the pretreatment value by 124–165% at 12 months. In the B cell pool, mature naïve B cells became dominated, and memory B cells were suppressed by 25% of the pretreatment value even at 12 months. Furthermore, it is suggested that reconstituting lymphocytes might promote brain repair possibly through production of neurotrophic factors.[58] Alemtuzumab trials support the concept that MS is an immune-mediated disease, and dysregulation in the distribution of B and T cell subsets might underlie the disease activity.

Targeting IL-2 receptor signaling: Anti-CD25 monoclonal antibody (daclizumab)

IL-2 plays a pivotal role in the differentiation and homeostasis of T cells.[59] IL-2 has been called the "T cell growth factor," because IL-2 signaling mediates clonal expansion of activated T cells and promotes their effector functions. The high affinity of IL-2 receptor (IL-2R) is upregulated on activated or abnormal T cells, such as those in autoimmune disease. These observations reinforced the idea that IL-2 promotes T cell immunity. As a consequence, it was believed that blockage of IL-2 signaling would be a candidate for the treatment of MS.[60]

IL-2R is composed of three distinct subunits: α chain, β chain and γ chain.[61] To achieve high-affinity binding to IL-2, all three chains are required; the α chain confers high-affinity binging to IL-2, the β and γ chains compose the intermediate-affinity receptor, and α-chain alone represents the low-affinity receptor. Daclizumab is a humanized IgG1 monoclonal antibody against the α chain of the IL-2R (CD25) that blocks the interaction of CD25 with IL-2 by binding specifically to the Tac epitope of CD25.[62] Consequently, daclizumab blocks low- and high-affinity IL-2R, whereas there is no effect on IL-2 signaling through the intermediate affinity IL-2R. The high affinity IL-2R is expressed on not only activated T cells, but also CD4+ CD25+ Foxp3+ regulatory cells (Treg). Treg play an important immunoregulatory role in MS by suppressing effector autoreactive T cells, and are dependent on IL-2 for their survival and immunoregulatory function.[63] Blockage of CD25 on these cells results in the reduction in their number *in vivo*, and their suppressive function *in vitro*.[64] In addition, blockage of CD25 by daclizumab results in the inhibition of apoptosis of effector T cells.[65] Furthermore, humans with a genetic deletion of CD25 also showed both lymphoproliferative disorders and severe immunodeficiency.[66] Taken together, it can be speculated that daclizumab therapy should activate T cell immunity. However, positive treatment effects and the favorable safety profile of daclizumab were observed in other chronic human inflammatory conditions, such as human T lymphotropic virus 1-associated myelopathy/tropical spastic paraparesis and inflammatory uveitis.[67] As a consequence, based on the assumption that daclizumab would block the activation and expansion of autoreactive T cells that play a central role in the immunopathogenesis of MS, four small open-label trials were carried out using daclizumab in active relapsing–remitting MS and secondary progressive MS (Table 2).[20,68–70] These phase IIa trials showed a profound inhibition of inflammatory disease activity, and were followed by two large phase IIb trials in relapsing–remitting MS.[71,72] At present, there are two ongoing phase III trials.

Table 2 Phase II/III clinical trials of daclizumab

Trial	Phase	Design	Population	Size	Duration (months)	Results
Bielekova et al.[68]	IIa	Open label	RRMS/SPMS	10	6	78% ↓ new Gd 70% ↓ total Gd
Rose et al.[70]	IIa	Open label	RRMS/SPMS	19	5–25	Significant ↓ in ARR, EDSS
Rose et al.[20]	IIa	Open label	RRMS/SPMS	9	28	Significant ↓ in Gd, ARR, EDSS
Bielekova et al.[69]	IIa	Open label	RRMS/SPMS	15	16	72% ↓ new Gd 77% ↓ total Gd
CHOICE[71]	IIb	Add-on to IFNβ	RRMS/SPMS	230	6	72% ↓ Gd 68% ↓ new T2WI
SELECT[72]	IIb	vs Placebo	RRMS	600	12	ARR ↓ (54%, 50%) New Gd ↓ (69%, 78%) Disability ↓ (57%, 43%)
DECIDE	III	vs IFNβ	RRMS	1800	24–36	Ongoing
OBSERVE	III	Open label	RRMS	150	11	Ongoing

ARR, annualized relapse rate; EDSS, Expanded Disability Status Scale; Gd, gadolinium; IFN, interferon; RRMS, relapsing–remitting multiple sclerosis; SPMS, secondary progressive multiple sclerosis.

Mechanistic studies could not confirm the inhibitory effect of daclizumab on activated T cells.[73] Furthermore, the number of Treg, their *in vivo* proliferation and *in vitro* suppressive functions are all significantly inhibited. This apparent discrepancy between the clinical and immunological effect of daclizumab in patients with MS suggests the existence of the additional effects of daclizumab on the human immune system. Subsequent immunological mechanistic studies showed the seven- to eightfold increased $CD56^{bright}$ Natural Killer (NK) cell number in the peripheral blood and the CSF.[64,69,74,75] In addition, an increased number of $CD56^{bright}$ NK cells correlated negatively with inflammatory activity on MRI.[74] This NK cell subset is known as a regulatory NK cell, and can kill autologous activated T cells by the granzyme pathway.[76] Blockage of autocrine IL-2 stimulation by daclizumab leads to an increase of local IL-2 availability. Therefore, more IL-2 becomes available for cells expressing intermediate affinity IL-2R. This results in the expansion of immunoregulatory $CD56^{bright}$ NK cells that express intermediate affinity IL-2R. In addition, daclizumab blocks IL-2 transpresentation of mature dendritic cells to primed T cells expressing intermediate affinity IL-2R, leading to the targeted inhibition of dendritic cell-activated antigen-specific T cells.[77] These pathways are considered to result in the reduction of inflammatory activity in MS.

Conclusion

Successful trials with theoretically promising agents can provide the profound understanding of the immunopathological mechanism of MS. The extremely promising clinical efficacy of alemtuzumab supported the concept that MS is an immune-mediated disorder with an abnormally modified immune cell repertoire. Mechanistic studies of daclizumab provided a novel insight into the biology of IL-2 and IL-2R interactions in the human immune system. However, failed trials are more important for critical revision of the assumed immunopathogenetic cascade of MS.

Disclosure

Hirofumi Ochi is a consultant for Biogen Idec Japan.

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