厚生労働科学研究研究費補助金

こころの健康科学研究事業

自己免疫疾患に伴う中枢神経障害に関連する抗神経抗体の検索と抗原機能の解析：病態の解明から治療法確立に向けて

平成18年度　総括研究報告書

主任研究者　木村暁夫

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Ⅰ　総括研究報告

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総括研究報告書

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研究要旨

全身性エリテマトーデスやシェーグレン症候群など全身性自己免疫疾患はしばしば中枢神経障害を合併する事が知られており、その臨床症状も精神症状、痙攣、認知障害、運動麻痺など多岐にわたり、いずれも患者の予後を左右する重要な病態である。これらの疾患には多種類の自己抗体が存在することが知られており診断項目の一つにもなっている。しかし一方でこれら自己抗体の特異性および病的意義に関しては不明な点が多い。本研究の目的は、中枢神経障害を合併した全身性自己免疫疾患患者の血清・髄液検体を用いて、疾患特異的抗神経抗体の検出ならびにその認識抗原を同定し、得られた抗原情報をもとに新たな診断および治療法の開発を行うことである。

【抗 Hsp60 抗体】本年度、我々は二次元免疫ブロット法および高感度ナノ LC-MS/MS システムを構築し CNS ループス患者、非ヘルペス性辺縁系脳炎患者、多発性硬化症(MS)患者、健常者の血清ならびに髄液検体を用い抗神経抗体の検出およびその認識抗原の同定を行った。その中に多発単ニューロパチーで発症し、その後特徴的な大脳・小脳・脳幹に広範な白質病変を呈した CNS ループス患者を経験し、その血清中にラット脳蛋白と免疫学的に反応する 5 つの抗神経抗体を検出した。その認識抗原は質量分析の結果、Beta-actin, Alpha-internexin, Heat-shock protein 60 (Hsp60), Hsp60(isoform), GFAP であった。このうちの抗 Hsp60 抗体の特異性につき検討した。ELISA キットを用い CNS ループス 11 例、神経症状を伴ったシェーグレン症候群 9 例、ギラン・バレー/フィッシャー症候群(GBS/MFS)10 例、多発性硬化症 10 例、髄膜炎 8 例、全身性自己免疫疾患を合併しない多発性脳梗塞 15 例, 健常者 17 例の血清中抗 Hsp60 抗体価を測定した。それぞれの群の抗体価の平均値は CNS ループス患者群およびシェーグレン症候群で高値をしめしたが健常者群との比較では統計学的有意差は認められなかった。しかし健常者群の抗体価の平均値＋2SD を cut off 値(41.99ng/mL)とした場合 12.5%(10/80)が陽性を示し、その疾患別内訳は CNS ループス 2 例、シェーグレン症候群 2 例、MS 3 例、多発性脳梗塞 2 例、MFS 1 例であった。陽性者の臨床的特徴を解析したところ 10 例中 9 例に頭部 MRI 上大脳白質病変を認め、抗体陽性の有無と同病変の合併に関して有意な相関関係を認めた(p=0.032、フィッシャー直接確率)。なお平均年齢 54.2 歳、男女比は 6:4 であり合併症として高血圧を 2 例に、糖尿病および高脂血症をそれぞれ 1 例ずつ認めた。

【抗 GluRε2 抗体】NMDA 型グルタミン酸受容体(GluR)ε2 サブユニットは前脳領域に発現し、記憶・学習に重要な役割を果たしていることが知られている。今回、2004 年より当科で経験した CNS ループス患者 6 名を含む計 23 名の急性脳炎・脳症患者の血清・髄液中に GluRε2 サブユニットに対する抗体が存在するか否かについてリコンビナント蛋白を用いたウエスタンブロット法により検索した。結果、CNS ループスの患者において IgG 型抗 GluRε2 抗体の陽性率が高値であった(血清: 感度 66.7%, 特異度 70.6%、髄液: 感度 60%, 特異度 82.4%)。臨床症状との関連では、痙攣重積の有無と髄液の IgM 型抗 GluRε2 抗体に関連がみられた(P<0.001、フィッシャー直接確立)。

A. 研究目的

中枢神経障害を合併した全身性自己免疫疾患患者に対し、疾患特異的抗神経抗体の検出ならびに認識抗原の同定を行い、得られた抗原情報を新たな診断および治療法の開発に反映し、同疾患による精神・身体障害を軽減することにより国民福祉に貢献する。

B. 研究方法

1) 患者サンプルの採取と抗神経組織抗体の検出

中枢神経障害を合併し、SLEの臨床診断基準(アメリカリウマチ協会、1997年)を満たす患者より血清および髄液を採取した。ラット大脳ホモジネートを抗原サンプルとして等電点電気泳動を行い、その後SDS-PAGEならびにブロッティングを施行した。ブロット後のPVDFメンブレンを用い上記患者血清を一次抗体とし、HRPで標識したヤギ抗ヒトIgG・A・M抗体を二次抗体として抗原・抗体反応を行い化学発光によりスポットを検出した。抗体の反応スポットと、同じサンプルを用いた二次元電気泳動のCBB染色したゲル上のスポットとマッチングを行った。

2) 認識抗原の同定

1)にて一致したスポットに対しゲル内トリプシン消化を行い nanoscale capillary liquid chromatography (LC) system (LV-VP, Shimadzu) および ion-trap を原理とした tandem mass spectrometer (LCQ Advantage max, Thermo electron)を組み合わせた高感度の質量分析システムを用い、データベース検索 [Mass data 収集ソフト X calibur TM (Thermo finnigan), Mass data 解析・蛋白同定ソフト MASCOT (Matrix Science)]により患者血清中の抗神経組織抗体の認識抗原の同定を試みた。

3) リコンビナント蛋白を用いた検証

2)にて同定した抗原蛋白の全長に相当するリコンビナント蛋白を用い、再度患者血清とその他症例の血清を一次抗体としてウエスタンブロットを施行し、抗体の存在の再確認と特異性につき検討した。

4) 抗 Hsp60 抗体の ELISA 解析

ELISA kit (Stressgen, Ann Arbor, MI, USA)を用いCNSループス11例、神経症状を伴ったシェーグレン症候群9例、ギラン・バレー/フィッシャー症候群10例、多発性硬化症10例、髄膜炎8例、全身性自己免疫疾患を合併しない多発性脳梗塞15例, 健常者17例の血清中抗Hsp60抗体価を測定した。

5) 抗 GluR ε 2 抗体の検出

GluR ε 2分子全長を発現する培養細胞のホモジネートの上清中の抗原蛋白を用い western blotting を施行した。

(倫理面への配慮)

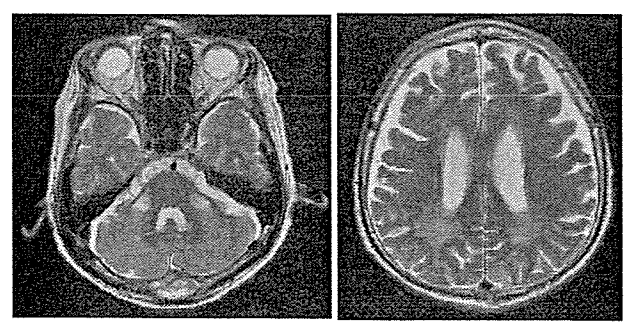
文書による対象患者もしくは家族の同意のもと血清および髄液を採取した。サンプルとして使用したラット脳の使用に関しては、岐阜大学大学院医学研究科動物実験委員会の承認を得た。

C. 研究結果

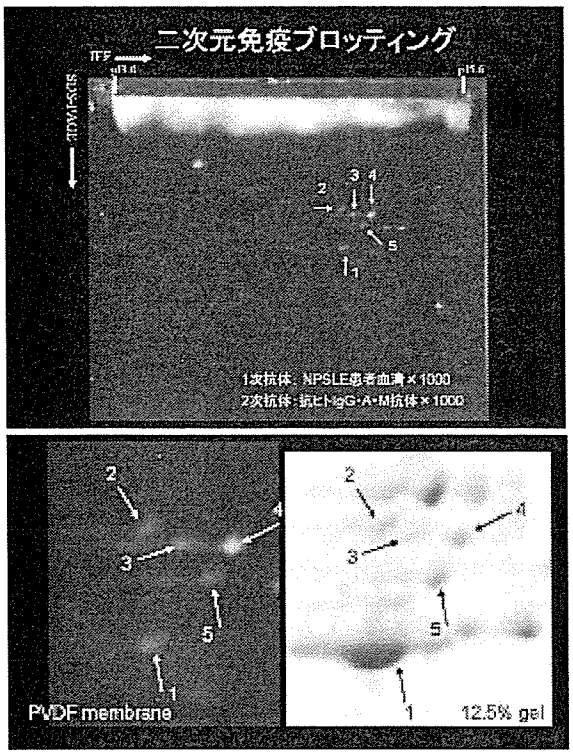
1) CNS lupus 患者血清における抗神経組織抗体の同定

今回検討したCNSループス患者の中に多発単ニューロパチーで発症し、その後特徴的な大脳・小脳・脳幹に拡がる広範な白質病変(図1)を呈したCNSループス男性患者を経験した。その血清中にラット脳ホモジネートと反応する5つのスポット(MW/PI:46KD/5.15, 63K/5.15, 57KD/5.25, 57KD/5.4, 53KD/5.3)を二次元免疫ブロット法にて確認した(図2)。これらスポットに対応する認識抗原は質量分析を行った結果、順に Beta-actin, Alpha-internexin, Heat-shock protein 60 (Hsp60), Hsp60(isoform), GFAP であった(表1)。

(図1)頭部 MRI T2WI



(図2) 二次元免疫ブロッティング



(表1) 質量分析の結果

Spot Numb.	protein name	score	peptides	Coverage %	M.W. (kDa)/pI	Theoretical M.W./pI
1	Beta-actin	421	16	41	46K/5.15	42K/5.29
2	Alpha-Inx	203	5	12	63K/5.15	56K/5.20
3	Hsp 60	112	3	6	57K/5.25	61K/5.91
4	Hsp 60	112	3	8	57K/5.4	61K/5.91
5	GFAP	113	2	2	53K/5.3	50K/5.35

(図3) Amino acid sequence (Hsp60)

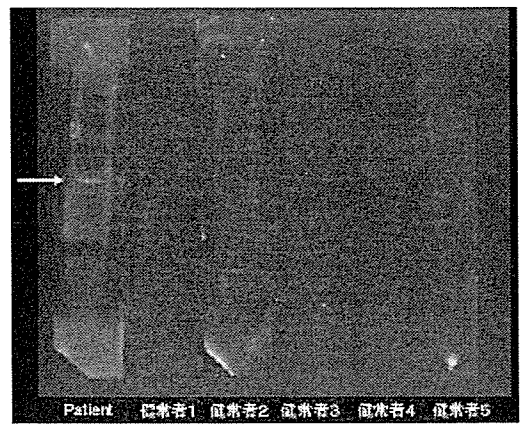
Matched peptides shown in underlined

1 MLRLPTVLRQ MRPVSRALAP HLTRAYAKDV KFGADARALM LQGVDLLADA
51 VAVTMGPKGR TVIIEQSWGS PKVTKDGVTV AKSIDLKDKY KNIGAKLVQD
101 VANNTNEEAG DGTTTATVLA RSIAKEGFEK ISKGANPVEI RRGVMLAVDA
151 VIAELKKQSK PVTTPEEIAQ VATISANGDK DIGNIISDAM KKVGRKGVIT
201 VKDGKTLNDE LEIIEGMKFD RGYISPYFIN TSKGQKCEFQ DAYVLLSEKK
251 ISSVQSIVPA LEIANAHRKP LVIIAEDVDG EALSTLVLNR LKVGLQVVAV
301 KAPGFGDNRK NQLKDMAIAT GGAVFGEEGL NLNLEDVQAH DLGKVGEVIV
351 TKDDAMLLKG KGDKAHIEKR IQEITEQLDI TTSEYEKEKL NERLAKLSDG
401 VAVLKVGGTS DVEVNEKKDR VTDALNATRA AVEEGIVLGG GCALLRCIPA
451 LDSLKPANED QKIGIEIIKR ALKIPAMTIA KNAGVEGSLI VEKILQSSSE
501 VGYDAMLGDF VNMVEKGIID PTKVVRTALL DAAGVASLLT TAEAVVTEIP
551 KEEKDPGMGA MGGMGGGMGG GMF

2) リコンビナント蛋白を用いた検証

Hsp60 および GFAP に関して、全長を発現したリコンビナント蛋白を用い再度患者血清とその他症例の血清を用い一次元免疫ブロットを施行した。両蛋白ともに患者血清と反応し、同定結果が正しいことが証明された。一方、その他症例での検討では GFAP は一部の健常者でもバンドが確認されたが、Hsp60 に関しては健常者では確認されなかった(図4)。

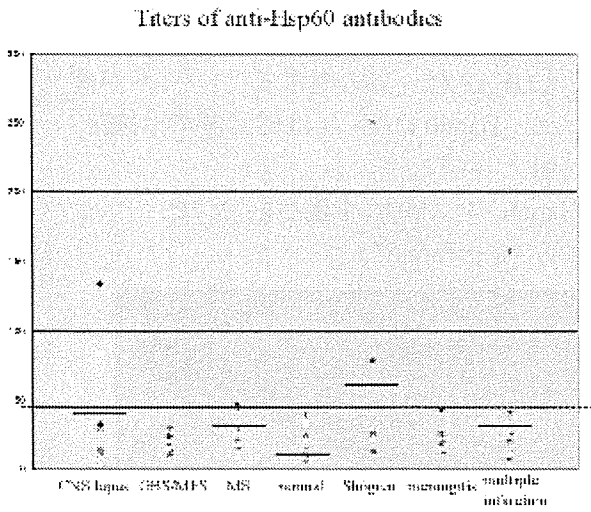
(図4) Hsp60 リコンビナント蛋白を用いた一次元免疫ブロッティング



3) 抗 Hsp60 抗体の ELISA 解析

各疾患および健常者の血清中抗 Hsp60 抗体価の結果を(図5)に示す。各群の平均値は CNS ループス 34.35, 神経症状を伴ったシェーグレン症候群 53.08, ギラン・バレー/フィッシャー症候群 24.63, 多発性硬化症 31.61, 髄膜炎 21.25, 全身性自己免疫疾患を合併しない多発性脳梗塞 31.51, 健常者 19.56 (単位は全て ng/mL)となり CNS ループス患者およびシェーグレン症候群で高値をしめしたが健常者群との比較で統計学的有意差は認められなかった。しかし健常者群の抗体価の平均値＋2SD を cut off 値 (41.99ng/mL)とした場合 12.5% (10/80)が陽性を示し、その疾患別内訳は CNS ループス 2 例、シェーグレン症候群 2 例、多発性硬化症 3 例、多発性脳梗塞 2 例、フィッシャー症候群 1 例であった(表2)。陽性者の臨床的特徴を解析したところ 10 例中 9 例に頭部 MRI 画像上大脳白質病変を認め、抗体の有無と同病変の合併に関して関連性を認めた(p=0.032、フィッシャー直接確率)(図6)。なお平均年齢 54.2 歳、男女比は 6:4 であり合併症として高血圧を 2 例に、糖尿病および高脂血症をそれぞれ 1 例ずつ認めた。

(図5)各疾患別血清中抗 Hsp60 抗体価



cut off value: mean (normal) ＋ 2SD (41.99ng/mL)

(表2) Cases with high titers of anti-Hsp60 antibodis

Disease Age/Sex	Complications	Titer (ng/mL)	WMHs [1]
MS, 69/M	Hyperlipidemia	44.5	＋
MS, 37/F	－	43.6	＋
MS, 31/F	－	56.7	＋
CNS lupus[2] 68/M	－	133.6	＋
CNS lupus 63/F	－	83.1	＋
Sjögren synd. 78/M	－	77.8	＋
Sjögren synd. 41/F	－	250.1	＋
Cereb. Inf.[3] 59/M	Hypertension	157.2	＋
Cereb. Inf. 59/M	Hypertension DM[4]	58.7	＋
Fisher synd. 39/M	－	88.1	－

[1] WMHs: cerebral white matter hyperintensities on brain MR T2WI/FLAIR image

[2] Bold italic: our case

[3] Cereb. Inf.: Multiple cerebral infarction without systemic collagen disease

[4] DM: Diabetes mellitus

(図6)頭部 MRI 大脳白質病変と関連因子

	With WMHs N=37	Without WMHs.N=25	P Value
Mean age, y	59.35±17.15 (55.86±16.07)	38.68±15.40	<0.001 (0.011)
Men:women	19:18 (5:4)	10:15	0.380 (0.338)
High titers of anti-Hsp60 Abs	9	1	0.032
Hypertension	11	0	0.002
DM	7	0	0.057

( )内は抗 Hsp60 抗体価高値症例

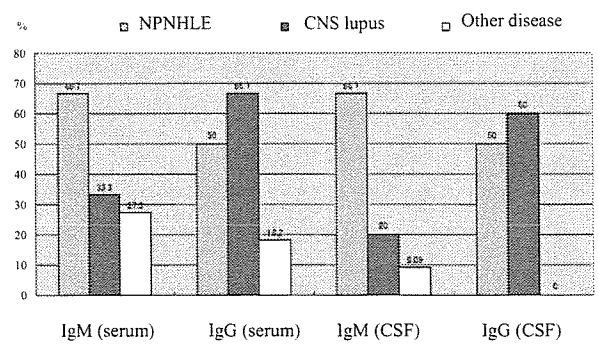
4) CNS lupus 患者における抗 GluR ε 2 抗体

2004 年より過去 3 年間に当科で経験した CNS ループス患者 6 名を含む計 23 名の急性脳炎および脳症患者の血清および髄液検体を用い抗 GluRε2 抗体の測定を行った。結果、IgM 型および IgG 型抗 GluRε2 抗体を血清中に 9 名、髄液中に 6 名ずつ検出した(表3)。疾患別では CNS ループスおよび非傍腫瘍性非ヘルペス性辺縁系脳炎(NPNHLE)において、これら2疾患を除くその他の脳炎・脳症と比較し抗体の陽性率が高かった。特に CNS ループスにおいては髄液 IgG 型抗 GluRε2 抗体の陽性率(n=6, 感度 60%, 特異度 82.4%)が、NPNHLE においては髄液 IgM 型抗 GluRε2 抗体の陽性率(n=6, 感度 66.7%, 特異度 87.5%)が高値であった(図7)。また、抗体と臨床症状(痙攣重積、記憶障害、精神症状・異常行動)および頭部 MRI 所見との関連性の検討では、痙攣重積と髄液中 IgM 型抗 GluRε2 抗体に関連性がみられた($p<0.001$, フィッシャー直接確立)(表4)。さらに急性期の血清で IgM 型抗 GluRε2 抗体が陽性であった NPNHLE 患者 4 例の血清を用いラット脳に対し免疫組織学的分析を行った結果、いずれの症例においても特異的に海馬および大脳皮質ニューロンの細胞質が染色性を示した(Kimura et al. Eur Neurol. in press)。

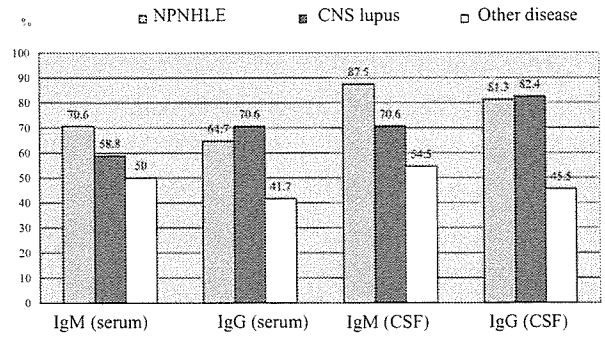
(表3) 急性脳炎・脳症と抗 GluR ε 2 抗体

age/sex	Clinical diagnosis	serum IgM/IgG	CSF IgM/IgG
27/M	ADEM	± / -	- / -
68/M	bacterial meningoencephalitis	- / -	- / -
22/M	CNS lupus	- / -	NE
31/F	CNS lupus	- / +	- / +
32/F	CNS lupus	- / +	- / +
61/F	CNS lupus	- / ±	- / -
55/F	CNS lupus	+ / -	± / -
68/M	CNS lupus	+ / +	- / +
57/F	cryptococcal meningoencephalitis	- / -	- / -
26/M	etiology-unkown meningoencephalitis	± / -	- / -
18/F	MELAS	- / -	- / -
57/M	Malignant lymphoma	- / -	- / -
59/F	Neuro-Sweet	- / -	- / -
22/F	NPNHLE	- / -	- / -
62/M	NPNHLE	- / -	+ / +
82/F	NPNHLE	+ / -	- / -
30/M	NPNHLE	+ / +	+ / -
45/F	NPNHLE	+ / +	+ / +
53/F	NPNHLE	+ / +	± / +
65/M	PLE (Hu positive)	- / +	- / -
43/F	Influenza encephalopathy	+ / -	- / -
20/M	Cerebral hemorrhage (cavernoma)	- / -	± / -
37/F	Brainstem encephalitis	- / +	- / -

(図7－1) 疾患別抗 GluRε2 抗体(感度)



(図7－2) 疾患別抗 GluRε2 抗体(特異度)



(表4) 臨床症状・画像所見と抗 GluRε2 抗体

Anti-GluRε2 Ab / Symptoms	IgM (Serum)	IgG (Serum)	IgM (CSF)	IgG (CSF)
Refractory status epilepticus	0.239	0.761	<0.001	0.946
Memory loss	0.306	0.306	0.229	0.229
Psychiatric synd. Abnormal behavior	0.247	0.247	0.318	0.074
Abnormal MR Findings (limbic system)	0.283	0.717	0.1	0.1

(p value: フィッシャー直接確立)

5) CNS ループス患者の頭部画像所見の検討

CNS ループス患者 8 例に頭部 MRI および SPECT 検査を施行した。頭部 MRI では、8 例中 7 例にび漫性の大脳皮質萎縮性変化を認めた。SPECT では 8 例中 7 例に両側前頭葉の血流低下を 8 例中 4 例に頭頂葉(3 例が両側、1 例が右側のみ)の血流低下を認めた。

D. 考察

今回の研究において 1 人の CNS ループス患者より 5 つの神経組織に対する抗体と、それらが認識する 4 つの認識抗原蛋白を同定することができた。これまでの CNS ループス患者における自己抗体に関する報告では、抗リボゾーム P 抗体, 抗 microtuble-associated protein 2 抗体, 抗リン脂質抗体などとともに今回同定した抗 GFAP 抗体の報告もある(Sanna et al. Lupus, 2000)。同報告では SLE 患者において

抗 GFAP 抗体の陽性率と精神神経症状の関連性を指摘しているが、その一方で SLE 患者の血清中における同抗体の陽性率は 15.7%であり、精神神経症状の有無とは無関係であるとする報告もある(Valesini et al. Ann. N.Y.Acad. Sci. 2006)。今回我々の GFAP のリコンビナント蛋白を用いた一次元免疫ブロット解析では、CNS ループス患者と健常者を含めたその他の患者との間に陽性率の相違はみられなかった。

Hsp60 に関しては、通常ミトコンドリア内のシャペロン蛋白として機能するが、同時に細胞膜に発現することが知られている。血管内皮細胞にも同様に発現し抗血管内皮細胞抗体の重要なターゲットとなり、抗体が血管内皮細胞の apotosis を誘導し血管炎の病態形成に係わっているとする報告(Jamin, Arthritis Rheum, 2005)がある。さらに抗 Hsp60 抗体を有する SLE 患者では血栓症の合併頻度が高くなるとする報告(Dieude, Arthritis Rheum, 2004)もある。また同抗体と虚血性心疾患や動脈硬化症との関連性を指摘した報告(Bason, Lancet, 2003、Choy, J Mol Cell Cardiol, 2001)はあるが、脳血管障害との関連性を指摘した報告はない。我々の症例では血液検査にて凝固線溶系の亢進を示唆する所見もみられており、広範な白質病変をきたした背景に同抗体を介した微小血管レベルにおける血管内皮障害ならびに循環障害が存在する可能性を考えた。そこで我々は、抗 Hsp60 抗体と大脳白質病変との関連性に着目し検討を進めた。その結果、自己免疫疾患の患者群には抗 Hsp60 抗体価が高値を示す一群が存在し、高血圧や糖尿病の合併に関係なく大脳白質病変を伴いやすい可能性が示唆された。今後 prospective study を介し抗 Hsp60 抗体と大脳白質病変の関連性を明確にし、同抗体が大脳白質病変の拡大に伴う神経症状の悪化を予防する治療の指標となりうるか検討する必要があると考えられた。

これまでに CNS ループス患者に存在する抗二本鎖 DNA 抗体が NMDA 型抗グルタミン酸受容体の一部[NR2A (GluRε1)および NR2B (GluRε2)]と交叉反応性を示し、さらにはアポトーシス経路を介して神経細胞死をもたらすとする報告がある(DeGiorgio et al. Nature medicine 2001)。また最近、卵巣奇形腫を合併しその臨床症状および画像所見が辺縁系脳炎に類似した免疫療法反応性の脳炎患者で抗 GluRε2 抗体が検出され、抗体の病態への直接的な関与を指摘した報告がある(Dalmau, et al. Ann Neurolo 2007)。今回の我々の検討では、少数例の検討であるが、抗 GluRε2 抗体は CNS ループス患者ならびに非傍腫瘍性非ヘルペス性辺縁系脳炎(NPNHLE)で陽性率が高値であった。この事は、抗 GluRε2 抗体が陽性となる脳炎・脳症の中に、傍腫瘍性辺縁系脳炎のみならず NPNHLE ならびに CNS ループスが含まれ、いずれも抗体が辺縁系を中心とした前脳領域に分布する GluRε2 サブユニットに作用し共通した臨床症状(精神症状・記憶障害・痙攣発作など)をもたらしている可能性が推測された。一方、NPNHLE と CNS ループス患者における相違として前者では髄液中 IgM 型抗 GluRε2 抗体が、後者では髄液中 IgG 型抗 GluR ε 2 抗体の特異度が高く、いずれも疾患の診断マーカーと成りうる可能性が示唆されるとともに、両者間の症状や経過の相違に関連している可能性が考えられた。

E. 結論

1、全身性の自己免疫疾患を合併する脳炎・脳症患者群の中に抗 Hsp60 抗体価が高値を示す一群が存在し、高血圧や糖尿病の合併に関係なく大脳白質病変を伴いやすい可能性が示唆された。

2、抗 GluRε2 抗体は、一部の NPNHLE 患者や CNS ループス患者で陽性となり、前者では髄液中 IgM 型抗 GluRε2 抗体が、後者では髄液中 IgG 型抗 GluR ε 2 抗体の特異度が高く、いずれも疾患の診断マーカーと成りうる可能性が示唆された。

F. 健康危険情報

なし

G. 研究発表

1. 論文発表

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H. 知的財産権の出願・登録状況

1. 特許取得

なし

2. 実用新案登録

なし

3. その他

なし

II　研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Kimura A, Sakurai T, Suzuki Y, Hayashi Y, Hozumi I, Watanabe O, Arimura K, Takahashi Y, Inuzuka T	Autoantibodies against glutamate receptor ε2 subunit detected in a subgroup of patients with reversible autoimmune limbic encephalitis	Eur Neurol.	In press		
Kimura A, Tan CF, Wakida K, Saio M, Hozumi I, Inuzuka T, Takahashi H	Venous Congestive Myelopathy of the Cervical Spinal Cord: An Autopsy Case Showing a Rapidly Progressive Clinical Course	Neuropathology	In press		
Nozaki H, Ikeuchi T, Kawakami A, Kimura A, Koide R, Tsuchiya M, Nakamura Y, Mutoh T, Yamamoto H, Nakao N, Sahashi K, Nishizawa M, Onodera O	Clinical and genetic characterizations of 16q-linked autosomal dominant spinocerebellar ataxia (AD-SCA) and frequency analysis of AD-SCA in the Japanese population	Mov Disord.	Epub ahead of print		2007
Yoshino H, Kimura A	Investigation of the therapeutic effects of edaravone, a free radical scavenger, on amyotrophic lateral sclerosis (Phase II study)	Amyotroph Lateral Scler.	7	241-245	2006
木村暁夫、櫻井岳郎、香村彰宏、鈴木欣宏、田中優司、保住功、犬塚 貴	多発ニューロパチーにて発症し、頭部 MRI 上対称性びまん性病巣を呈した CNS ループスの 1 例	Neuroimmunology	14	169-174	2006
櫻井岳郎、木村暁夫、田中優司、保住 功、犬塚 貴	急性小脳失調で発症した HIV 感染症を伴う神経梅毒の 1 例	神経内科	64	609-613	2006

Ⅲ　研究成果の刊行物・別刷

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Dear Dr. Kimura,

Thank you for submitting a revised version of your manuscript to European Neurology. We are pleased to inform you that it has now been accepted for publication and passed on to our production department. The galley proofs will be sent to you as soon as they are available.

We hope you will continue to submit work from your group to European Neurology in the future.

With kind regards,
Prof. Julien Bogousslavsky
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Autoantibodies against glutamate receptor ε2 subunit detected in a subgroup of patients with reversible autoimmune limbic encephalis

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Key words: autoantibody, ε2 subunit, immunotherapy, limbic encephalitis, NMDA glutamate receptor, paraneoplastic syndrome
Running title: autoantibodies against GluRε2 subunit

Abstract: We investigated the presence of autoantibodies against glutamate receptor (GluR) ε2 in serum and cerebrospinal fluid (CSF) samples from 12 consecutive patients with acute encephalitis/encephalopathy by immunoblotting using recombinant GluRε2 as antigen. In four patients (Patients 1-4), IgM autoantibodies against GluRε2 were detected in CSF in the early phase of the disease but were not detectable after several months. Seizures and psychiatric symptoms were noted during the acute phase of the disease in these four patients, who showed various degrees of residual amnesia. Immunotherapy was performed on three patients (Patients 1, 3, and 4), and they showed marked improvements. Immunohistochemistry using these patients' sera showed that immunoreactivity is specifically detected in the cytoplasm of rat hippocampal and cortical neurons. The clinical features and neuroimaging findings of patients with IgM autoantibodies against GluRε2 in CSF resemble those of patients with reversible autoimmune limbic encephalitis.

Introduction

There are some reports that indicate the production of autoantibodies in patients with encephalitis [1-3] and encephalopathy [4]. Recently, autoantibodies against the NMDA-type glutamate receptor (GluR) ε2 have been detected in patients with epilepsia partialis conitinua (EPC) causally related to Rasmussen syndrome [5], non-paraneoplastic limbic encephalitis [6], and acute encephalitis [5, 7]. The NMDA receptor, which is one of the three major ionotropic GluRs, is a heterodimer composed of ε and ζ subunit families [8]. There are four members in the ε subunit family (ε1-ε4) [9]. The NMDA receptor channel is unique in terms of its functional properties [10, 11]. After birth, the expression of GluRε2 subunit mRNA becomes restricted to the forebrain, which includes the cerebral cortex and limbic system [12]. GluRε2 is associated with memory and learning [10, 11]. Therefore, we investigated autoantibodies against GluRε2 in serum and CSF samples from patients with acute encephalitis/encephalopathy to clarify its clinical features and immunological aspects.

Materials and Methods

We obtained serum and CSF samples from 12 consecutive patients with acute encephalitis/encephalopathy in our department from August 2003 to January 2005 [n=12; male:female=6:6; age range, 18-68; mean age, 47; idiopathic limbic encephalitis, 5; etiology-unknown meningoencephalitis, 1; anti-Hu antibody-positive paraneoplastic limbic encephalitis, 1; bacterial meningoencephalitis, 1; mitochondrial encephalopathy with lactic acidosis and strokelike episodes (MELAS), 1; cryptococcal meningoencephalitis, 1; brainstem encephalitis, 1; and Neuro-Sweet disease, 1].

Detection of autoantibodies against GluRε2

The method used was previously reported (Takahashi et al., 2003). The supernatants of cell extracts from stable NIH3T3-transformant cell lines expressing full-length GluRε2 were subjected to SDS-PAGE, and the separated proteins on the gels were transferred to nitrocellulose membranes. The membranes were reacted with diluted sera or CSF and stained with alkaline phosphatase-labeled secondary antibodies (IgG or IgM; Jackson ImmunoResearch, West Grove, PA). Anti-GluRε2 autoantibodies were detected as a band corresponding to approximately 180 kDa.

Immunohistochemistry using patient's serum

Under ether anesthesia, adult Sprague-Dawley rats were sacrificed. The cerebrums were immediately removed and frozen in dry-ice powder. Frozen sections (8 μm thick) of the cerebrums were fixed in 4% paraformaldehyde. The sections were incubated with serially diluted serum from a patient or with an anti-NMDAε2 antibody (1:500) [Santa Cruz Biotechnology, USA]. Then, the sections were incubated with a goat biotinylated anti-human IgM (μ-chain specific) antibody [Vector, USA] or a rabbit biotinylated anti-goat IgG (H+L) antibody [Chemicon, USA]. After washing, the sections were

reacted with a streptoavidin-peroxidase complex [Nichirei, Japan]. The reactions were finally developed with 3,3'-diaminobenzidine tetrahydrochloride [Wako, Japan] and 0.01% H_2O_2 in PBS. For adsorption tests, frozen sections after blocking were immunostained with a patient's serum (1:2000) or with the anti-NMDAε2 antibody (1:500) that was incubated for 24 hours with extracts from transformant cells expressing full-length GluRε2.

Results

Detection of autoantibodies against GluRε2 (Table 1)

The IgM autoantibody against GluRε2 in CSF was detected in four patients (Patients 1-4) out of 12 consecutive patients in the early phase of the disease but was not detectable after several months. The IgG autoantibody against GluRε2 in CSF was detected in three of these four patients (Patients 1-3). No autoantibodies against GluRε2 were detected in the CSF of the other patients. In the early phase of the disease, Patients 1 and 4 had IgM and IgG autoantibodies against GluRε2 in their serum. Patient 3 had only the IgM autoantibody, but became positive for the IgG autoantibody 2 months later.

Clinical features (Table 2)

All the patients who had the IgM autoantibody against GluRε2 in CSF presented with seizures (i.e., partial seizures evolving to secondary generalized seizures) and psychiatric symptoms (i.e., hallucination, behavioral changes, and agitation) in the early phase of the disease and developed prolonged consciousness disturbances with status epilepticus. None of these patients presented with paralysis or disturbances in sensation in the chronic stage. However, all of them presented with various degrees of recent memory disturbance and amnesia. Patient 3 showed residual psychiatric symptoms after treatment. Three patients (Patients 1, 3, and 4) received intravenous methylprednisolone pulse therapy and showed improvement in their seizures and consciousness levels.

Case presentation

Here, we describe a representative patient with IgM autoantibodies against GluRε2 in CSF. Patient 1 was described previously (Hayashi et al., 2005).

Patient 4

Patient 4 was a 30-year-old male initially complaining of headaches with fever. Throughout 1 week, he showed no improvement in these symptoms and presented with agitation and behavioral disturbance. He was admitted to a nearby hospital because he suddenly suffered a generalized tonic seizure. After admission to the hospital, he was treated with antiepleptic drugs and intravenous acyclovir. However, he developed consciousness disturbance and status epileptics. Then, he was transferred to our hospital. He had an unremarkable medical history, a temperature of 37.7°C, a pulse of 74/min, and a blood pressure of 105/68 mm Hg. On neurological examination, he exhibited somnolence. There was lateral gaze-evoked nystagmus. The deep tendon reflexes of all four limbs were slightly hypoactive. There were no pathological reflexes. No meningeal signs were observed. After his consciousness level improved, muscle strength, sensation, and coordination became normal. Laboratory tests revealed leukocytosis and elevated levels of myogenic enzymes: WBC count, $13.8\times10^3/\mu l$; CK level, 3285 IU/l; AST level, 80 IU/l; and LDH level, 394 IU/l. The serum CRP level was slightly elevated to 1.89 mg/dl. The serum anti-nuclear antibody was absent. IgM and IgG autoantibodies against GluRε2 were present in serum on the day of admission. Analysis of CSF showed 34 cells/mm^3 (mononuclear cells only), 36 mg/dl total protein, and 68 mg/dl glucose. He had a mildly elevated IgG index (0.73). IgM and IgG antibodies against herpes simplex virus, cytomegalovirus, and varicella zoster virus were absent in paired sera and CSF tested at 2- week intervals. PCR analysis showed negativity for herpes simplex virus, cytomegalovirus, and human herpes virus-6/7 DNA in CSF. The IgM autoantibody against GluRε2 was present, but the IgG

autoantibody was absent in CSF on the day of admission. EEG revealed diffuse θ waves with small spikes in the left temporal lobe. Brain MRI revealed no abnormal intensity changes, but revealed diffuse cortical edema. ^{99m}Tc-ECD SPECT performed on the 14th day of hospitalization revealed hypoperfusion in the diffuse cerebral cortex and bilateral mesial temporal lobes. After admission, he was treated with phenytoin. However, the seizures were difficult to control and required treatment with anesthetic agents (pentobarbital sodium and midazolam) under respiratory management. He was treated with an intravenous infusion of 1 g of methylprednisolone for three consecutive days. He showed improvement in consciousness level and the frequency of the convulsions decreased following the treatment. However, approximately two weeks after steroid therapy, his seizures increased in frequency again and intravenous immunoglobulin (IVIg; 400 mg/kg·day for five consecutive days) was administered. His condition slowly improved. On the 36th day of hospitalization, he required no respiratory management. Afterwards, we carried out the administration of IVIg and steroid pulse therapy, and his seizures disappeared completely. However, he showed disorientation and severe amnesia. HDS-R and MMSE scores determined approximately 3 months after admission were 10/30 and 13/30, respectively. His WAIS-R full-scale IQ was less than 40, and his verbal and performance subscale scores were 51 and less than 45, respectively. All WMS-R indexes showed significantly low scores (general memory<50; verbal memory=54; delayed memory<50; visual memory<50; attention and concentration<50). It was difficult for him to immediately recall things related to logical memory after hearing them. He showed no higher functional impairments such as aphasia, apraxia and agnosia. His memory and cognitive state slowly improved, and he was discharged four months after admission. Eight months after the disease onset, his memory and cognitive scale score improved significantly [HDS-R score was 25/30, WAIS-R IQ (total IQ, 70; verbal IQ, 63; performance IQ, 87), WMS-R scores (general memory, 53; verbal memory, 57; delayed memory, 60; visual memory, 70; attention and concentration, 61)]. Brain MRI revealed no intensity changes; however, mild cortical atrophy was observed six months after the disease onset.

Laboratory findings (Table 3)
Virological examinations showed negativity for IgM and IgG antibodies against the herpes simplex virus, cytomegalovirus, and varicella zoster virus in paired sera and CSF tested at 2- week intervals. PCR analysis showed negativity for the herpes simplex virus and human herpes virus-6/7 DNA in CSF. Patient 3 had the anti-nuclear antibody (ELISA: 34.4, normal<20.0) in her serum. Patient 1 showed an elevated level of the anti-thyroperoxidase (TPO) antibody in the serum, which was not detected in CSF. We measured the titers of voltage-gated potassium channel (VGKC) antibodies by radioimmunoassay using whole rabbit-brain homogenate as described previously [13]. The titers of VGKC antibodies showed normal levels in these four patients (Patient 1: 0 pM, Patient 2: 21pM, Patient 3: 0 pM, Patient 4: 18 pM, normal range<100 pM).

Neuroimaging and physiological examination
Brain MRI at disease onset showed signal abnormalities in the bilateral mesial temporal lobes in Patients 1 and 3 and on one side of the mesial temporal lobe in Patient 2. In Patient 4, brain MRI showed no signal abnormalities. However, several months (range, 6 months-1 year) after the disease onset, cerebral atrophy of various degrees, including atrophy in the mesial temporal lobe, was common (Figure 1). SPECT was performed in the acute or subacute phase. All of the patients except Patient 1 showed hypoperfusion in the mesial temporal lobe. In Patient 1, ^{99m}Tc-HMPAO SPECT performed the day after admission revealed hyperperfusion in the bilateral mesial temporal lobes; however, hypoperfusion was observed 17 months later. All the patients showed irregular diffuse cortical hypoperfusion. In three patients, EEG revealed small focal spikes. In all the patients, a mixture of diffuse θ waves was observed. No tumors were detected in any of the patients using various methods [chest, abdomen, and pelvic CTs (all patients), whole-body FDG-PET (Patient 1), and gallium scintigraphy (Patient 2)].

Immunohistochemical findings

The diluted serum samples from Patients 1, 3, and 4 reacted with the cytoplasm of rat hippocampal and cerebral cortical neurons (Figure 2). The most appropriate dilution of serum for immunohistochemical staining was 1:2000~4000. The sections incubated with the anti-NMDAε2 antibody showed the same pattern of immunoreactivity as those incubated with the patients' sera. The serum of the healthy control did not significantly immunoreact with the sections. The immunoreactivity of sera of the three above-mentioned patients to the anti-GluRε2 antibody was markedly decreased by prior incubation with the supernatants of extracts from stable transformant cells expressing GluRε2. Patients 1, 2 and 3 were negative for serum paraneoplastic anti-Yo, anti-Hu, anti-Ri, anti-CV2, anti-Tr, anti-Ma-2, and anti-amphiphysin antibodies.

Discussion

We presented four patients with acute encephalitis who had IgM autoantibodies against GluRε2 in CSF in the early phase of the disease; however, these antibodies were not detectable after several months. Other acute encephalitis/encephalopathy patients (Patients 5-12) had neither the IgG nor IgM autoantibody against GluRε2 in CSF. Four antibody-positive patients had the characteristic clinical features of reversible autoimmune limbic encephalitis such as intractable convulsion, psychiatric symptoms, recent memory disturbance and sufficient responsiveness to immunotherapy. Concerning the cause of limbic encephalitis, there are many reports in which limbic encephalitis is associated with cancer, most commonly a small cell carcinoma of the lung [14-20]. The four antibody-positive patients had no findings of viral infection or cancer. Several reports have been published concerning the immunotherapy response form of non-paraneoplastic limbic encephalitis [21-26]. Recently, a VGKC antibody has been detected in patients with reversible autoimmune limbic encephalitis [27-32]. However, the titers of VGKC antibodies were normal in our four antibody-positive patients' sera. We speculate that reversible autoimmune limbic encephalitis is heterogeneous. Some forms of this disease may be mediated by autoantibodies against antigens such as ion channels or ionotropic receptors in the limbic system.

In our immunohistochemical analysis of the sera of patients with the IgM autoantibody against GluRε2, immunoreactivity was detected in the cytoplasm of neurons in the hippocampus and cerebral cortex. The immunoreactivity was specifically demonstrated using an immunoadsorption test. The NMDA receptor is one of the ionotropic glutamate receptors essential for excitatory neurotransmission and synaptic plasticity, which underlie memory and learning [10, 11]. An antibody-mediated disturbance of NMDA-type GluR function might influence synaptic plasticity in the hippocampus and cortical neuronal excitability. The clinical response to immunotherapy and the results of immunoblotting and immunohistochemistry suggest that IgM autoantibodies may be related to pathogenesis in a subgroup of patients with reversible autoimmune limbic encephalitis.

In a previous study, autoantibodies against GluRε2 were detected in patients with chronic EPC causally related to Rasmussen syndrome [7]. There are some reports that these antibodies have also been detected in patients with encephalitides other than chronic EPC [6, 7]. In this study, we detected autoantibodies against GluRε2 in patients with reversible autoimmune limbic encephalitis. We suggest that autoantibodies against GluRε2 contribute to the onset of localized encephalitides such as EPC and reversible autoimmune limbic encephalitis [7].

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