TABLE II. Proteins Identified From 2-DE Gels of AD Hippocampus by MALDI-TOF Mass Spectrometry

Spot no.[a]	Protein	Probability	Sequence coverage (%)	NCBI accession Nos.	Theoretical value		Experimental value	
					pI	kDa	pI	kDa
A1	Vimentin	3.8E+02	68	CAA39600	5.1	53.7	5.1	49.2
A2	Vimentin	4.3E+02	71	CAA39600	5.1	53.7	5.1	49.1
B1	GFAP	3.1E+02	53	NP_002046	5.4	49.9	5.2	43.5
B2	GFAP	3.3E+02	57	NP_002046	5.4	49.9	5.2	44.8
B3	GFAP	3.2E+02	55	NP_002046	5.4	49.9	5.3	47.4
B4	GFAP	3.3E+02	60	NP_002046	5.4	49.9	5.3	49.9

[a]The numbering and lettering corresponding to the 2-DE gets image shown in Figure 2A.

performed by using Mascot Search (Matrix Science Ltd., London, United Kingdom).

Immunohistochemistry

The paraffin-embedded sections (6-μm-thick) of AD and control hippocampi were deparaffinized, rehydrated, and pretreated by heating in a microwave oven for 10 min in citrate buffer. Citrullinated proteins were detected as described previously (Ohsawa et al., 2001; Ishigami et al., 2002a). Briefly, sections were postfixed with 4% paraformaldehyde and 2.5% glutaraldehyde, and then they were incubated in the modification medium at 37°C for 3 hr to modify citrulline residues in situ. Immunostaining of citrullinated proteins was performed with antimodified citrulline IgG and the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA) using 3,3′-diaminobenzidine as a chromogenic substrate. Human PAD2 and GFAP were stained with rabbit anti-human PAD2 prepared as described previously (Ishigami et al., 2002b) and monoclonal antibodies to GFAP (Sigma-Aldrich), respectively. Sections were also subjected to hematoxylin staining for histological examinations. To evaluate the degree of citrullinated protein and PAD2 immunoreactivity, we used a scoring system, in which immunoreactivity was arbitrarily defined from grade 0 (no immunoreactivity detected) to grade 4 (the most intensive immunoreactivity detected). The scores obtained from 10 AD and nine control subjects were then averaged.

The citrullinated proteins, human PAD2 and GFAP, were detected with immunofluorescent labeling and confocal microscopy (LSM-510 laser scanning microscope; Carl Zeiss, Oberkochen, Germany). Primary antibodies were visualized with Alexa Fluor 488 goat anti-rabbit IgG and Alexa Fluor 594 goat anti-mouse IgG (Molecular Probes).

Statistical Analysis

The results are expressed as the mean ± SEM. The probability of statistical differences between experimental groups was determined by a Student's *t*-test, as indicated.

RESULTS

Identification and Characterization of Citrullinated Proteins in the Hippocampi of AD Patients and Normal Controls

Figure 1A shows total proteins in hippocampal extracts from brains of AD and normal individuals in which amido black staining delineated obviously different profiles. In particular, the protein bands between 30 and 50 kDa were notably smaller in the AD hippocampal extracts. Moreover, the citrullinated protein profile from AD hippocampal samples manifested species not detected in those from normal brains (Fig. 1B) as judged by the procedures described in Materials and Methods. In particular, a band migrating at approximately 20 kDa, which was previously shown to be myelin basic protein (MBP) in cultured oligodendrocytes (Akiyama et al., 1999), was present in relatively large quantities in the AD hippocampus but essentially undetectable in this region from normal brains. We confirmed the identity of this protein by immunoprecipitation using specific MBP antiserum (data not shown). Western blots of PAD2 revealed a single band migrating at approximately 75 kDa in hippocampal extracts from both normal and AD brains (Fig. 1C).

AD hippocampal proteins were further characterized by 2-DE (Fig. 2A). Because citrullinated proteins were detected in abundance migrating over 35 kDa, as shown in Figure 1B, the gel region outlined in Figure 2A was cut out (Fig. 2B), and the citrullinated proteins were subjected to Western blotting for identification. Figure 2C shows several of the AD hippocampal citrullinated proteins detected and identification of these proteins by peptide mass fingerprinting and a subsequent database search. These methods identified vimentin as proteins A1 and A2 and GFAP as proteins B1, B2, B3, and B4 (Table II). Some citrullinated proteins were visible throughout the gel (Fig. 2A), even outside the region cut out (data not shown).

Immunohistochemical Localization of Citrullinated Proteins and PAD2 in Hippocampal Regions and Cell Type

Figure 3A shows the regional localization of citrullinated proteins in the hippocampus of an AD brain. Citrullinated proteins were detected throughout the hippocampus, especially in the conjugation region between molecular layers of hippocampus and dentate gyrus as well as polymorphic layers of dentate gyrus and stratum radiatum of CA1 and CA2 areas. No such proteins were found in the granular layer of the dentate gyrus. Moreover, no citrullinated proteins at all were detected in a normal hippocampus (Fig. 3B). A scoring system adopted to evaluate the degree of citrullinated protein immunoreactivity in the hippocampus then revealed significantly greater immunoreactivity in the AD hippocampus than in its normal counterpart (Fig. 4A). In contrast, PAD2 immunoreactivity was detect-

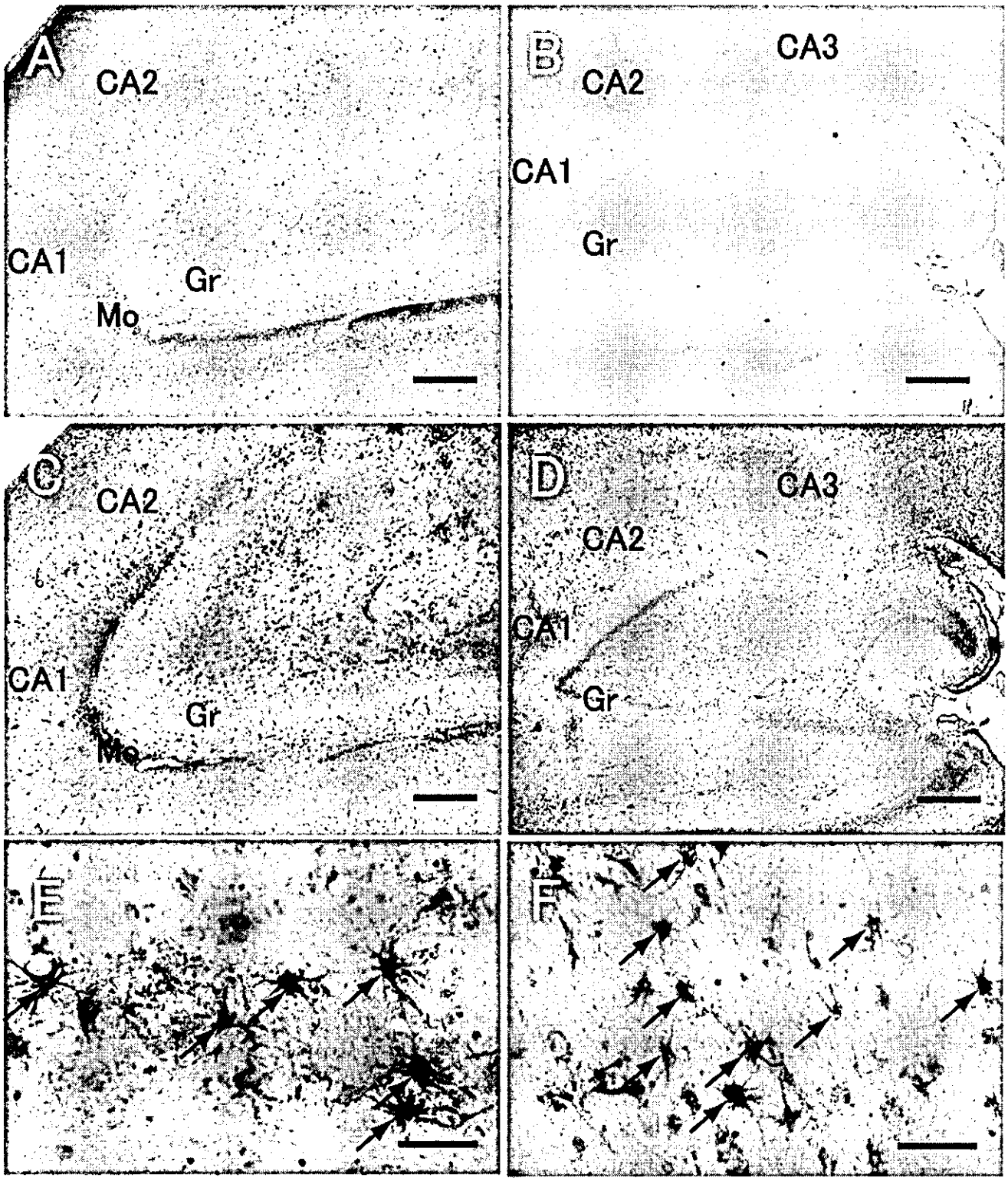

Fig. 3. Immunohistochemical staining of citrullinated proteins and PAD2. Hippocampal sections from AD (**A,C**) and control (**B,D**) brains were stained for citrullinated proteins (A,B) and PAD2 (C,D) as described in Materials and Methods. **E:** Higher magnification of A. Arrows indicate the citrullinated protein-positive cells. **F:** Higher magnification of C. Arrows indicate the PAD2-positive cells. Gr, granule cell layer; Mo, molecular cell layer. Scale bars = 500 μm in A–D, 50 μm in E,F.

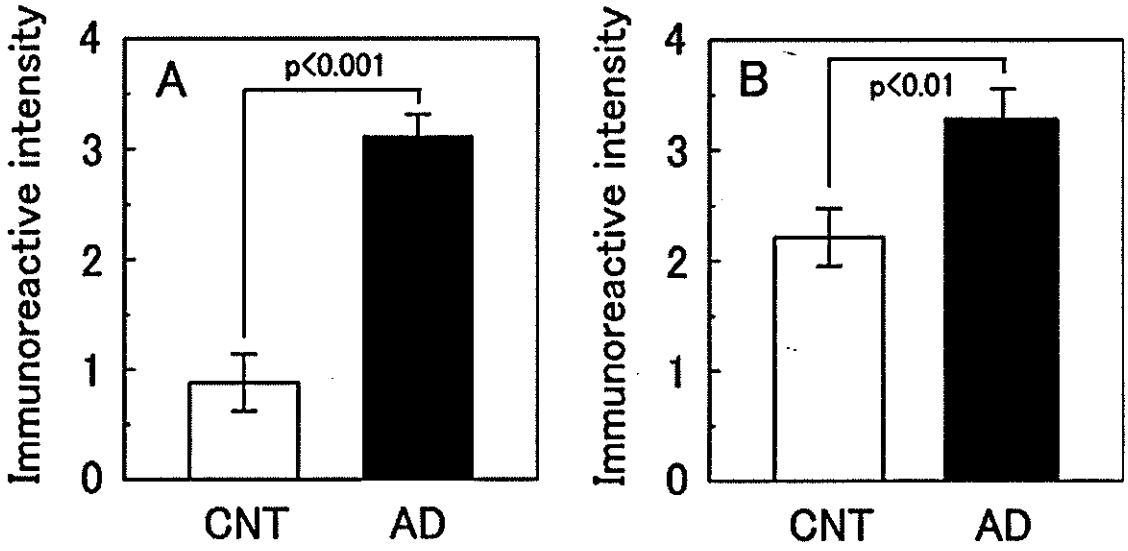

Fig. 4. Immunoreactive intensity of citrullinated proteins and PAD2 in the hippocampi of AD and control brains. The immunoreactivity of citrullinated proteins (A) and PAD2 (B) in the hippocampus was graded (grade 0 to 4) as described in Materials and Methods. Values are expressed as means ± SEM of 10 AD and nine control subjects. Data were compared by Student's *t*-test.

able all through the hippocampus, both in the AD and in normal brain, but not in the granular layer of the dentate gyrus (Fig. 3C,D). A scoring system adopted to evaluate the degree of PAD2 immunoreactivity in the hippocampus revealed significantly greater immunoreactivity in the AD hippocampus than in its normal counterpart (Fig. 4B). The PAD2-enriched region coincided well with the citrullinated protein-positive regions (Fig. 3A,C). At higher microscopic magnification in the polymorphic layer of dentate gyrus, figures of citrullinated protein-positive cells and PAD2-positive cells were apparent as astrocyte-like cells (Fig. 3E,F). To confirm whether these citrullinated protein- and PAD2-positive cells were astrocytes, we performed double-immunofluorescence staining for citrullinated protein or PAD2 and GFAP, which is an astrocyte-specific marker protein, followed by confocal microscopy (Fig. 5). GFAP-positive fluorescence signals clearly coincided with citrullinated protein-positive signals (Fig. 5A–C) as well as PAD2-positive signals (Fig. 5D–F). Almost all GFAP-positive cells showed immunoreactivity for PAD2, despite a few exceptions. Thus, PAD2 was distributed mainly in astrocytes.

DISCUSSION

We report here, for the first time, the abnormal accumulation of citrullinated proteins and abnormal activation of PAD2 in brain extracts from patients with AD. Citrullinated proteins were barely detectable in normal human brain extracts, although PAD2 was almost universally present.

Previously, we found similar results in the normal rat brain (Asaga and Ishigami, 2000, 2001). Additionally, physiologic insults such as hypoxia and kainic acid administration also resulted in the appearance of citrullinated proteins (Asaga and Ishigami, 2000, 2001; Asaga et al., 2002). PAD2 was present in the rat cerebrum and especially enriched in the dentate gyrus and stratum radiatum of hippocampus, the amygdala, the hypothalamus, and the cortex, but few or no citrullinated proteins were detected in those regions. Under hypoxic conditions (Asaga and Ishigami, 2000) and during kainic acid-evoked neurodegeneration (Asaga and Ishigami, 2001; Asaga et al., 2002), PAD2 became activated in regions undergoing neurodegeneration and functioned to citrullinate various cerebral proteins, indicating the involvement of protein citrullination in neurodegenerative processes. We are convinced that these past and present results confirm the involvement of protein citrullination in human neurodegenerative disease.

In the present study, Western blot analysis revealed numerous citrullinated proteins in the AD hippocampus. We identified citrullinated vimentin and GFAP, which were shown as several independent spots by 2-DE and proteomic analysis (Fig. 2C, Table II). Because protein citrullination results in a decrease in the net positive charge of proteins, each spot must be shown as several pI values with different degrees of citrullination resulting in neutralization of proteins. In the epidermis of mice, we previously identified citrullinated cytokeratins and filaggrin as several independent spots separable by 2-DE and detected by Western blotting (Senshu et al., 1995, 1999). Citrullination of cytokeratins and filaggrin occurs during the latest stages of epidermal differentiation and is thought to play a key role in the cornification process (Senshu et al., 1995). Although citrullination of vimentin and GFAP seems to be much more specific than that of other intracellular proteins, it is still unclear whether citrullination of vimentin and GFAP has physiologically

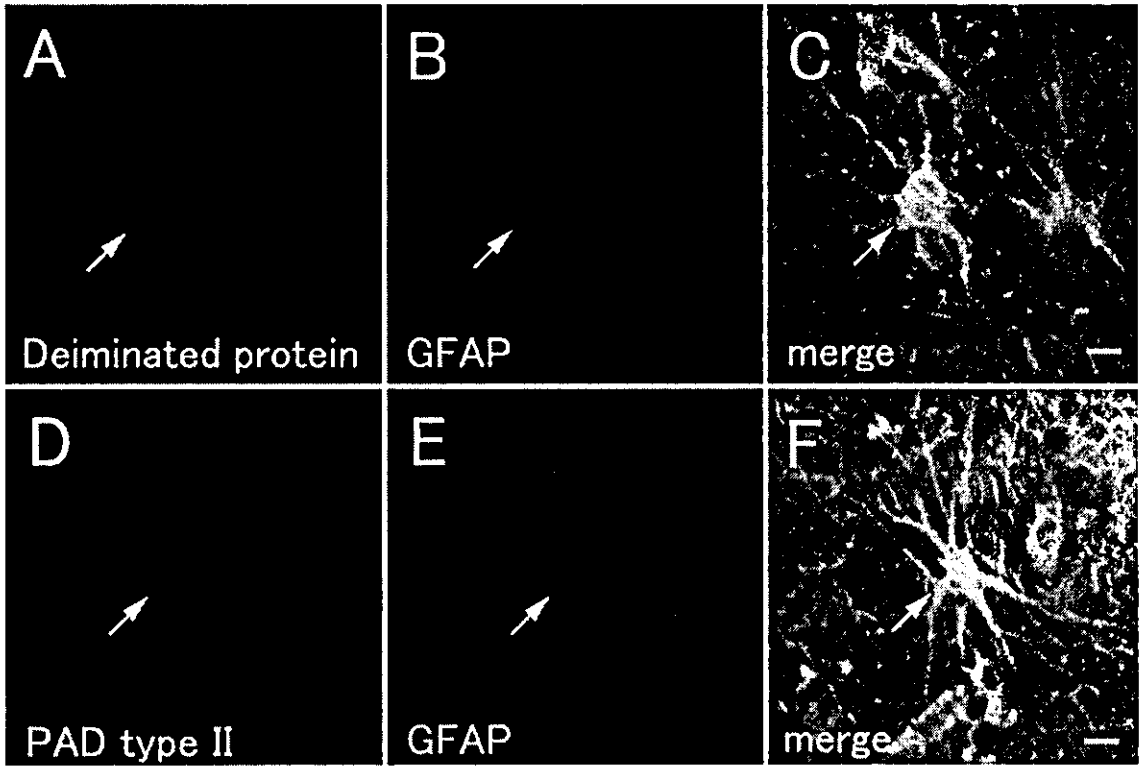

Fig. 5. Identification of citrullinated protein-positive and PAD2-positive cells by double immunofluorescence staining. Sections of AD hippocampus were doubly immunostained with monoclonal anti-GFAP antibody and with polyclonal antimodified citrulline IgG or polyclonal anti-human PAD2 antibody. The primary antibodies were visualized with anti-rabbit Alexa Fluor 488 (green) and anti-mouse Alexa Fluor 594 (red). **A,D:** Alexa 488 (green) for citrullinated protein (A) or PAD2 (D). **B,E:** Alexa 568 (red) for GFAP. **C,F:** Merged views for A/B and D/E, respectively. Arrows indicate coincident position. Scale bars = 5 μm.

important functions in the brains of AD patients. Inagaki et al. (1989) reported that vimentin and GFAP were highly susceptible to the attack of PAD2 in vitro; for example, citrullination of vimentin induced disassembly of intermediate filaments.

Here, we also identified citrullinated MBP, which is an authentic marker of oligodendrocyte, in the AD-afflicted hippocampus. Recently, we found PAD2 localized in a stage-specific, immature oligodendrocyte from a rat's cerebral hemisphere in vitro (Akiyama et al., 1999). Gould et al. (2000) reported that PAD2 cDNA was highly expressed in myelin sheath assembly sites with a combination of subcellular fractionation and suppression subtractive hybridization. Moreover, Moscarello et al. (1994, 2002) reported that PAD enzyme and citrullinated MBP are relatively enriched in immature myelin and that MBP citrullination has an important role in myelin development and in the human demyelinating disease multiple sclerosis. Recently, many investigators have suggested that myelin breakdown may be a contributing factor in the pathology of AD (Bartzokis, 2004; Tian et al., 2004). MBP citrullination might also contribute to the myelin breakdown.

The mechanism(s) by which citrullinated proteins occur in the hippocampus during AD remains unclear. Possibly PAD2 becomes activated, abundant, and functional only in the presence of AD, insofar as the amount of PAD2 increased notably in hippocampi of the AD patients we assessed compared with that in normal subjects. Although PAD2 was also present in hippocampal extracts from normal subjects, that enzyme remained in a steady state during which no enzyme activation occurred. For enzyme activation, the intracellular calcium concentration must become elevated. No other factors can regulate PAD activity in vivo or in vitro, to the best of our knowledge. A loss of neuronal calcium homeostasis leading to increases in the intracellular calcium concentration has been proposed to play a major role in hypoxic and ischemic brain injury (Choi, 1988; Hossmann, 1999). Haun et al. (1992) suggested that an influx of extracellular calcium contributes to astroglial injury during ischemia on the basis of their experimental results with simulated ischemia in a primary culture of astrocytes. Our previous report showed that PAD2 activated and citrullinated various cerebral proteins under hypoxic conditions (Asaga and Ishigami, 2000) and during kainic acid-evoked neurodegeneration (Asaga and Ishigami, 2001; Asaga et al., 2002). Abnormal PAD activation resulted in random protein citrullination, which could then trigger the onset of neurodegenerative disease.

In conclusion, the present data indicate that patients with AD bear an abnormal accumulation of citrullinated

proteins and abnormal activation of PAD2 in the hippocampus. Therefore, citrullinated proteins may be a useful marker for neurodegenerative disease. Moreover, the development of an inhibitory drug specific for PAD2 could conceivably prevent the onset and progression of neurodegeneration.

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免疫学

シトルリン化蛋白質と関節リウマチ

Citrullinated protein and rheumatoid arthritis

関節リウマチ(rheumatoid arthritis：RA)は，全身の関節が腫れて痛み，関節の破壊をもたらす自己免疫疾患のひとつである．RAは30～50歳代に多く発症し，女性の罹患率は男性のそれの4～5倍であり，加齢とともに罹患率が上昇する．RAの原因として以前より，遺伝要因・環境要因の両方が関与していると考えられていた．最近，1遺伝子多型(single nucleotide polymorphism：SNP)を用いたホールゲノム疾患関連遺伝子解析により，RA発症の遺伝要因の一端が明らかになってきた．

シトルリン化フィラグリン抗体の発見

従来より，RA患者の血清中に含まれる自己抗体としてrheumatoid factor(RF)，anti-keratin(AKA)，anti-perinuclear factor(APF)，anti-Sa抗体や軟骨，または関節膜蛋白に対する抗体の存在が知られている．これらのなかで，RFはもっとも一般的な血清マーカーとして使用されているが，特異性が低く，感染症や健常な高齢者などでも陽性に出てしまうことがある．RFに比べてAKAは90%以上の高い診断特異性を示す．AKAは1979年にラット上皮組織を螢光染色した結果，表皮細胞に存在するケラチンとして同定された．その後のエピトープ解析により，AKAはケラチンではなく，ケラチン線維を束ねる作用をもつフィラグリンであることがわかった．表皮角化の最終段階においてフィラグリンは，高分子量前駆体，プロフィラグリンとして合成され，細胞質中のケラトヒアリン顆粒に蓄えられる．角化の間，プロフィラグリンは，リン酸化，脱リン酸化，プロテアーゼ消化により，分子量37,000の機能的塩基性フィラグリンユニットとして遊離される．さらに，これら塩基性フィラグリンユニットは，二次的修飾として蛋白質中のアルギニンがシトルリンに変換されることにより塩基性が失われ，中性フィラグリンとなる．この反応は，ペプチジルアルギニンデイミナーゼ(PAD)という酵素により仲介される[1](図1)．

1998年，1999年にオランダとフランスの研究グループがあいついで，AKA抗体がシトルリン化フィラグリンを特異的に認識していると報告した[2,3]．さらに，いままで別の抗原と考えられていたAPFとAKAは，実は同じシトルリン化フィラグリンであることも判明した．

RA関連遺伝子の同定

2003年に理化学研究所，東京大学，三共株式会社の共同研究グループは1遺伝子多型を用いたホールゲノム疾患関連遺伝子解析により，RA発症の原因遺伝子のひとつがペプチジルアルギニンデイミナーゼタイプ4(PADI4)であることを報告した[4]．これはシトルリン化フィラグリンがRAの自己抗原である先の結果を強く支持する．生体内で生じるシトルリン化蛋白質は免疫系から異物として認識され，それに対する自己抗体が産生される．Schellekensら[2]は，フィラグリンの配列からシトルリンを含むペプチドを合成し，RA患者血清との反応性を調べた．その結果，合成ペプチドに対する抗体はRA患者血清の76%に存在し，96%の特異度を示した．さらに，この合成ペプチドを環状にした抗原は直線状よりも感度が高いこともわかった．近年，ヨーロッパでは環状合成シトルリンペプチド(CCP)を抗原とした関節リウマチの血清診断法が臨床応用され，感度70～80%，特異度95～99%と優れた検出成績をあげている．

今後の展望

ペプチジルアルギニンデイミナーゼは，はじめに発見された表皮を含む幅広い組織で発現が認められる．また，滑膜ではタイプ2とタイプ4の発現が報告されている．一方，フィラグリンは表皮特異的に発現し，滑膜には存在しない．したがって，シトルリン化フィラグリンがRAの真の自己抗原である可能性は低いと考えられる．シトルリン化フィラグリンとよく似たエピトープをもつ別のシトルリン化抗原の存在が示唆される．Masson-Bessiereら[5]は，血中

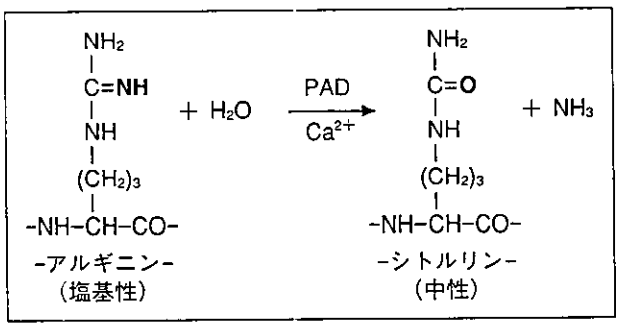

図1 ペプチジルアルギニンデイミナーゼ(PAD)によるアルギニンからシトルリンへの変換
酵素活性発現にカルシウムイオンを必要とする．ヒトでは遺伝子の異なる5種類(タイプ1，2，3，4，6)の存在が知られている．

のシトルリン化フィブリンα鎖，β鎖が自己抗原であると報告している．しかし，RAに対する特異度はシトルリン化フィラグリンに比べてかなり低い．

今後，真に病因と関連するシトルリン化抗原が明らかになれば，RA発症の詳細なメカニズムが解明され，診断や治療に大きく貢献するであろう．

1) Ishigami, A. et al.：Human peptidylarginine deiminase type II：molecular cloning, gene organization, and expression in human skin. *Arch. Biochem. Biophys.*, **407**：25-31, 2002.
2) Schellekens, G. A. et al.：Citrulline is an essential constituent of antigenic determinants recognized by rheumatoid arthritis-specific autoantibodies. *J. Clin. Invest.*, **101**：273-281, 1998.
3) Girbal-Neuhauser, E. et al.：The epitopes targeted by the rheumatoid arthritis-associated antifilaggrin autoantibodies are posttranslationally generated on various sites of (pro) filaggrin by deimination of arginine residues. *J. Immunol.*, **162**：585-594, 1999.
4) Suzuki, A. et al.：Functional haplotypes of PADI4, encoding citrullinating enzyme peptidylarginine deiminase 4, are associated with rheumatoid arthritis. *Nat. Genet.*, **34**：395-402, 2003.
5) Masson-Bessiere, C. et al.：The major synovial targets of the rheumatoid arthritis-specific antifilaggrin autoantibodies are deiminated forms of the α-and β-chains of fibrin. *J. Immunol.*, **166**：4177-4184, 2001.

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消化器内科学

HCVセリンプロテアーゼ阻害剤

――新しいHCV治療薬

HCV serine protease inhibitor as a new anti-HCV agent

C型慢性肝炎患者に対する抗ウイルス療法としては，これまでインターフェロン療法単独やリバビリンとの併用療法が行われてきた．また，平成16年(2004)の年末からはPEG化インターフェロンとリバビリンの48週間の併用療法が保険収載される予定で，治療奏効率の上昇が期待される．しかし，それでもなおgenotype 1b HCV高ウイルス量感染者では50%以上がインターフェロン療法抵抗性であり，新しい抗HCV治療薬の開発が待たれている．

HCVはエンベロープを有する一本鎖の(+)鎖RNAウイルスである．感染後肝細胞内で脱殻し，HCV RNAが翻訳されて1本の長いポリプロテインを生成する．そのポリプロテインは，宿主由来およびHCV由来のプロテアーゼによりC, E1, E2, NS2, NS3, NS4A, NS4B, NS5A, NS5Bの10種の蛋白にプロセスされる．このなかで，NS3セリンプロテアーゼとヘリケース，NS5B RNA依存性RNAポリメラーゼはHCV RNAとともに，HCV複製複合体を形成し，HCV増殖のkey enzymeとされている．NS3(正確にはNS3-4A)セリンプロテアーゼはNS4A, NS4B, NS5A, NS5Bをプロセッシングし，そのNS4A領域の54残基のアミノ酸の中心領域(21～30アミノ酸)がNS3セリンプロテアーゼ活性増強に必要かつ十分な部位であることが明らかにされている．このNS4Aの中心領域はNS3蛋白と強固なヘテロダイマーを形成していることも，1996年にX線結晶化解析から明らかとなっている[1]．

以上の基礎情報をもとにHCVセリンプロテアーゼ阻害剤の開発が行われ，Boehringer Ingelheim社から報告されたBILN 2061と，Vertex社のVX-950が最近注目されている(図1)[2,3]．BILN 2061は世界で最初のHCVセリンプロテアーゼ阻害剤で，*in vitro*でgenotype 1bのHCVセリンプロテアーゼを強力かつ特異的に阻害する．また，低分子量であるため経口投与が可能である．

昨年(2003)の『*Nature*』誌に報告されたphase Iの臨床試験の結果では，genotype 1bのHCV感染患者(8名，対照者2名)に2日間，1日2回経口投与を行って2～3 logのHCV RNAの低下を認めている[3]．投与2日間ではBILN 2061投与による致死的副作用は認めず，投与終了後1～2週間で投与前のHCV RNAレベルに戻ったと報告されているが，長期投与試験に移る段階で副作用が出現し，現在の化合物は何らかの副作用を軽減するような修飾が必要であるとの情報が得られている．

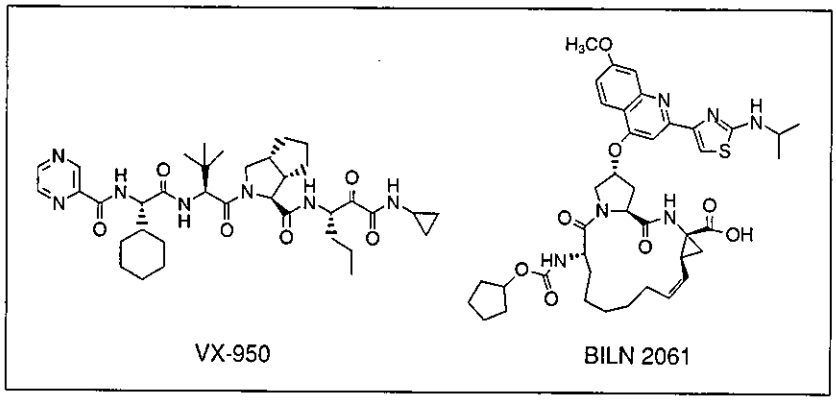

図1　HCV NS3プロテアーゼ阻害剤，VX-950とBILN 2061の化学構造[2]