低下させる．また，C5aからアルギニンがとれた一次代謝物であるC5adesArgがその受容体であるC5L2(C5a-like receptor 2：C5a 様受容体)に結合すると，IL-12の産生を抑制するように働く(図3)．これらの報告から，アレルギー反応におけるC5aの制御はその症状を緩和するのに有効な方法の一つであると考えられる[10]．濱らはヒト肺組織を用いた喘息モデルにおいて，AcPepAが肥満細胞から放出されるメディエーターの一つCysLTsの産生を強力に抑制することを明らかにしており[11]，相補性ペプチドの抗喘息薬としての可能性も検討されている．

6．おわりに

現在，C5a阻害相補性ペプチドAcPepAはブタ新生児を用いたCLP(cecal ligation and purcture：腸管穿孔モデル)実験においても著明な延命効果やサイトカインの過剰放出抑制効果が認められている．その結果を基に，敗血症やSIRSなどの患者の救命効果を臨床治療実験で明らかにするトランスレーショナルリサーチへと進めることが可能であると考えている．また，相補性ペプチド創出技術を用いて，サイトカインストームの進行で形成されるTNF-αやHMGB1などほかの起炎性因子に対する相補性ペプチドも創生し，病態が進行した患者も救命できる治療薬としての開発を目指している．ペプチド剤は蛋白質分解酵素の作用を受けて速やかに分解されるため，蓄積毒性などの副作用リスクが少ないと考えられる．これらの特性を生かし，将来的には，相補性ペプチドが臨床病態における重要な因子を制御する戦略に幅広く応用されることになり，さらに多くの疾患における有効な治療薬の開発手段として発展していくことを期待している．

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Nagoya Med. J. (2009) 50, 101-105

第59回名古屋市立大学医学会総会
特 別 講 演 II

アンチセンスペプチド理論より創出した アナフィラトキシン阻害相補性ペプチド

岡 田 則 子
名古屋市立大学大学院医学研究科免疫学分野

Anaphylatoxin inhibitory peptide created by the application of the antisense peptide theory

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Department of Immunology
Nagoya City University Graduate School of Medical Sciences

要 約

自然界に存在するタンパク質の分子内にはセンスーアンチセンス関係にあるペプチドが集積した部分が存在し相互作用する事が明らかとなりその部分をアンチセンスホモロジーボックス（AHB）と称した．そこで標的アミノ酸配列に相互作用を示す相補性ペプチドをアンチセンスアミノ酸を指標に加えて人為的に創出するコンピュータプログラムMIMETICを作成し任意のタンパク質の標的ペプチド部分に結合してその生物活性を制御できる相補性ペプチドを創出して創薬の可能性を探っている．現在，補体系の活性化中間産物であるアナフィラトキシンC5a制御を目指して，C5a活性阻害を示す相補性ペプチドの研究を進め，敗血症などの補体過剰反応に起因する病態の解明や治療に向けての相補性ペプチドを用いた新たなアプローチを試みている．

は じ め に

Blalock & Smithによって1984年にアンチセンスアミノ酸の概念が始めて提唱されてより，多くの実験系でのセンスペプチドーアンチセンスペプチド相互作用が立証されてきた．その反応機構の詳細な解析が進められている現状でもあるが，我々はアンチセンスアミノ酸の存在意義を検証するために二つのタンパク質との間で相互にアンチセンス部分になっている部分を検索するコンピュータプログラムANTISを作成した．それを一つのタンパク質内で比較する手法にも応用して，同一分子内でのセンスーアンチセンスペプチド関係を検索した．その結果，自然界に存在するタンパク質の分子内にもセンスーアンチセンス関係にあるペプチドが集積して存在する事が明らかとなった．そこで，このアンチセンスアミノ酸理論をベースとして標的アミノ酸配列に相互作用を示すペプチドを人為的に創出するコンピュータプログラムMIMETICを作成し，任意のタンパク質の標的ペプチド部分に結合してその生物活性を制御できる候補ペプチドを創出した．その中から反応性の優れたペプチドを相補性ペプチドとして選出し，タンパク質機能を制御するリードペプチドとして創薬の可能性を探っている．現在，アナフィラトキシンC5aの活性阻害を示す相補性ペプチドの解析を進め，治療薬としての応用研究を進めている．

センスーアンチセンスペプチド相互作用

二本鎖 DNA のうち，negative strand DNA に相補的な RNA を鋳型として人工的に合成したペプチドが，相対する positive strand DNA に由来するペプチドと特異的に相互作用をするという考え方が，1984年に Blalock JE ら[1]によってアンチセンスアミノ酸の概念として提唱された（図１）．それ以降，それを示す知見が多数継続的に報告されている．アミノ酸をコードするコドンに相補的なコドンによってコードされるアミノ酸であるアンチセンスアミノ酸は疎水性あるいは親水性が各々逆になっていることが特徴として挙げられる．１つのアミノ酸をコードするコドンは複数存在する．従ってその複数のコドンを基にすると１個のアミノ酸に対して複数個のアンチセンスアミノ酸が存在する．センスーアンチセンスアミノ酸は３グループに分けられ，おのおののグループに属するアミノ酸は同じグループのアミノ酸とのみセンスーアンチセンス関係にある．その各グループは，αhelix，βturn，βsheet の２次構造をとりやすいアミノ酸がグループを形成している．そして，センスーアンチセンスアミノ酸間での hydropathic score は符号が逆であり絶対値があまり変わらない関係になっている[2]．さらにアンチセンス mRNA を３'－５'方向に翻訳してできるペプチドは５'－３'方向に翻訳してできるペプチドとではアミノ酸配列は全く異なるにも拘らず hydropathic score がほぼ同様となる．これはアンチパラレルアンチセンスペプチドと称されるが，センスペプチドと相互作用を示すことを検証している．これらアンチセンスペプチドの分子間認識の重要な要素に hydropathicity が関与することが検証されてきた．

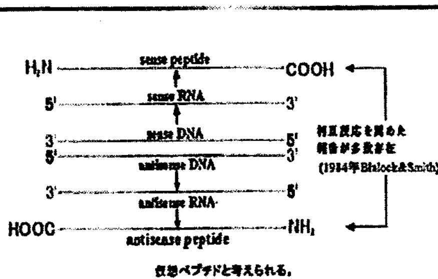

図１　アンチセンスペプチドとは．Blalock & Smith により1984年に提唱された Molecular Recognition Theory の概念図を示す

タンパク質が mRNA より翻訳されて相互作用をすることにより，すべての生命現象は統御されている．タンパク質が対応するタンパク質やペプチドを一組として認識できることが，生命の根源的な意味をなすものであり，タンパク質のセンスペプチドとアンチセンスペプチドが相互作用するという事象は極めて意味深いものがあると考えている．

アンチセンスホモロジーボックス（AHB）

アンチセンスペプチドの相互反応性に着目した研究が，リガンドに対応するレセプターの同定や，精製あるいは結合部位の同定にも応用されている[3]．この場合，特徴としてペプチドの anphiphilic structure が重要な要素である事も知られた．アミノ酸には複数個のアンチセンスアミノ酸が存在するので，このような関係をアンチセンスホモロジー（AH）と称するが，この AH を検索するコンピュタプログラム ANTIS を作成して，タンパク質における AH の存在様式を検索した結果，AH が集積して存在している部分が存在する事が見いだされ，AH ボックス（AHB）と称している[4]．この AHB の存在する部分はタンパク質の機能や高次構造に関連した部位あるいはその近傍に集積している事が明らかとなった（図２）．さらに関連するタンパク質間には共通する AHB が存在する事よりそれらは共通のタンパク質より進化し，遺伝子重複などにより派生したタンパク質にも AHB が受け継がれており，この AHB が保存されている事実はその部位が機能発現や高次構造に重要であることを示す[5]．このようにタンパク質の１次構造であるアミノ酸配列のなかに，ペプチド間相互作用部位が存在して高次構造を決定する要素が織り込まれていることは必定であると考えている．

C5aアナフィラトキシンの炎症拡大反応

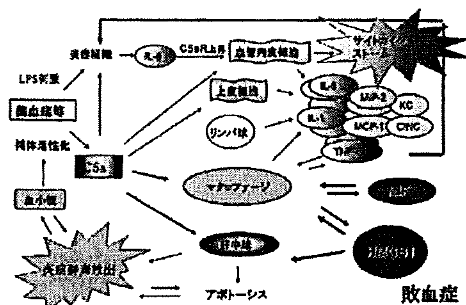

図2　C5aアナフィラトキシンの敗血症ショックにおける役割．C5aは細菌感染やLPSなどによる補体活性化にともない放出される．C5aRはLPSやC5aなどにより発現上昇が誘導される．血液細胞や血管内皮細胞が炎症反応に乗じてサイトカインストームを誘発する．反応拡大により炎症後期反応因子HMGB1，MIFなどが放出されて，致命的な敗血症へと誘導される．C5a阻害相補性ペプチドはHMGB1などの放出を阻止して敗血症ショックを回避させ救命できると推察される．

アナフィラトキシン阻害相補性ペプチドの創出

そこでANTIS/MIMETICを用いて候補ペプチドの設計を行い，タンパク質の作用を制御できる相補性ペプチドの解析を行った．例えば，HIV逆転写酵素（reverse transcriptase）の酵素活性阻害，TAFI（thrombin activatable fibrinolysis inhibitor）の活性阻害，thrombomodulinのthrombin補助作用の阻害，およびC5a anaphylatoxinの活性阻害などである．各々標的ペプチドに対して反応性を検討したところ試作相補性ペプチドの阻害活性検出率は約30％となっている（表1）．これらは我々の研究室内での解析結果であるが，現在，我々以外の研究施設に於いても他のタンパク質における解析が進められており，同等の解析結果も得られ始めている．

補体系は，我々が長年に渡り研究を進めている分野であるが，体液性の最たる生体防御常備軍であり，生体での異常を察知すると瞬時に活性化される．C5aは補体活性化反応の中間産物であり微量で白血球活性化などの生物活性を発

表1　相補性ペプチドのタンパク質機能制御．各標的タンパク質に於ける相補性ペプチド候補を試作して，その制御活性の検討を行った結果を示す．

相補性ペプチドの試作とその有効率

標的分子	作用	試作数	有効数	有効率（%）
HIV逆転写酵素（RT）	酵素活性阻害	10	3	30.0
ProCarboxypeptidase R (ProCPR)	活性化阻害	10	3	30.0
Thrombomodulin	Thrombin補助作用阻害	3	2	66.7
C5aアナフィラトキシン	活性阻害	19	7	36.8

MIMETICを用いて標的部分ペプチドに対する相補性ペプチド候補を創成し，in vitroにて各ペプチドの阻害活性を検討した。

揮する．過剰反応や持続活性化が起こると，C5aRの発現上昇が誘導されるとともに，炎症性サイトカインの産生も高まるので，過度の反応は敗血症や多臓器不全などの重篤な病態の要因になると考えられる．C5aのR37-E53部分がAHBとしてC5aRの活性化に関与することが確認できたので，そこを標的ペプチドとして相互作用を示すペプチドをMIMETICを用いて創成した[6,7]．その結果，候補ペプチド19個のうち7個でC5a活性に影響を与えるペプチドが検出され，特にC5a活性阻害効果の高い相補性ペプチドPepAについての検討を進めてきた．PepAは相補性ペプチドの特性としての両親媒性および標的ペプチドに対してhydropathicityの逆相関性を示すペプチドであり，C5a高次構造モデルを用いた3D docking手法においても高いフィットネスを示し，Biacore SPR解析に於いて，C5aに特異的に結合する事も確認された．さらにPepAはヒト好中球などのC5aR発現細胞のC5aによるCa-influxを阻害し，C5a機能を阻害することも検証された．ラットを用いてのエンドトキシン感作後に抗補体制御因子抗体の投与に誘発されるショック死モデル実験においても救命効果を発揮しており，PepAによるC5a阻害効果によるエンドトキシン病態の改善が期待された[8]．ペプチドは生体内に存在するペプチターゼなどによる分解を速

やかに受けるために半減期が短いという難点がある．そこで生体内での安定性を高めるためにN末端をアセチル化したAcPepAを作成して検討した結果，期待通りに，PepAより優れた阻害効果が得られている[9]．

おわりに

C5aはアナフィラトキシンと呼称されるごとく，微量でショック病態を引き起こす極めてリスキーな体内産生分子として良く知られているが，そのレセプターに関しては，新たに第2のC5aRとしてC5LRが発見されるなど，C5aの機能発現に関する新たな進展が見られている．また，これらの欠損マウスなどの解析が始まり，新たなC5aの生理的機能として，Tリンパ球の発生分化に関与していることや，肝細胞再生に関与していることが知られた．さらに，病理的側面として，C5aが妊娠成立や維持，発育に影響を及ぼすことや，腫瘍形成浸潤の場におけるサプレッサー細胞の増殖を起こす等，病態に於ける危険因子としての報告も相次いでいる（表2）．

現在，我々はカニクイサルを用いてAcPepAの効果の検証を進めている．サルにLPSを投与してエンドトキシンショック死を誘発する実験系において，LPS投与後のAcPepA投与により，7例全例において，その救命に成功しており，敗血症ショックに於ける反応因子として注目されているHMGB1やMIFの血漿中への放出を阻止できる事が確認されている（図2）．重篤な敗血症患者の救命にC5a阻害ペプチドAcPepAが有用であると推察され，敗血症などの治療薬としての開発研究を進めている．相補性ペプチドは臨床病態に於ける重要な因子を制御する戦略に於いて幅広く応用されるようになり，さらに多くの疾患における有効な治療薬としての相補性ペプチドの研究が進む事を期待している．

表2　アナフィラトキシン研究の新展開．近年アナフィラトキシンレセプターの解析が進み，新たなレセプターの発見や機能発現の報告が相次いでいる．

アナフィラトキシン研究の新展開

新たな生理的意義	
T細胞分化	ナイーブCD4細胞の発生、分化を助ける。(Immunity 2008)
T細胞傷害	CTL活性を促進する。(Blood 2008)
肝再生	肝臓の再生を助ける。(J. Exp. Med. 2003)
新たな病理的意義	
腫瘍免疫	Suppressor細胞の増殖を助けて腫瘍組織へのCD8細胞浸潤を妨げる (Nat. Immunol. 2008)
生殖免疫	胎児の生育を妨げる。(Immunol. Invest. 2008)
移植免疫	同種異系β細胞移植を妨げる。(Mol. Immunol. 2008)
敗血症	アナフィラトキシンレセプターC5L2シグナルがHMGB1産生を助ける。(Nat. Med. 2008)
感染症	脳マラリアの感染病態を悪化させる。(J.Exp. Med. 2008)

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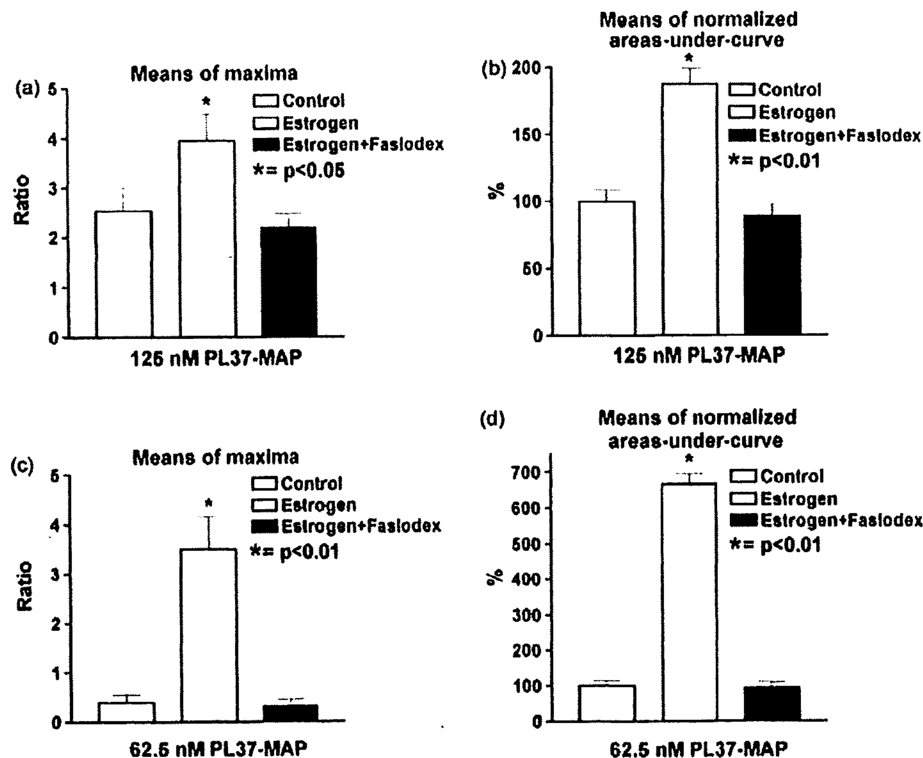

Fig. 4. Histograms of the data recorded in Fig. 3. E2 treatment significantly elevated the maximal amplitude of the recorded curves both at 125 nM (a, $p < 0.05$) and 62.5 nM PL37-MAP (c, $p < 0.01$). Faslodex blocked the elevation of the amplitude evoked by E2 (a and c). The areas-under-curve data show similar tendency. E2 pretreatment elevated the PL37-MAP elicited response of the cells significantly ($p < 0.01$) at both concentrations of PL37-MAP (b and d). All of the histograms were calculated using baseline correction and the areas-under-curve data were normalized by setting the control measurements as 100%. The mean ± S.E.M. values of the "means of maxima" were calculated by determining the maximum amplitude of calcium recording of each cell upon a certain treatment and then calculation of the mean and the S.E.M. was carried out.

reached a plateau (GT1-7 cells: 225 ± 54 pA; SON: 760 ± 172 pA; PVN: 1117 ± 214 pA). The extracellularly applied calcium channel blocker $CoCl_2$ (0.5 mM) abolished the current evoked by the PL37-MAP suggesting that the triggered inward pulses were due to calcium influx in the PVN (Fig. 5f). $CoCl_2$ also eliminated the evoked current in the GT1-7, SON and GnRH-GFP neurons (not shown). Pretreatment of the brain slice with C5a (5 μg/ml) in the extracellular solution significantly decreased the amplitude of the PL37-MAP-triggered inward current in the PVN (624 ± 79 pA, $n = 8$; $p < 0.05$; Fig. 5g) suggesting that the current was due to the activation of the C5aR. The PL37-MAP evoked current was diminished by the C5a-pretreatment in the GT1-7 cells (167 ± 45 pA; $n = 13$), the neurons of the SON (604 ± 110 pA; $n = 8$) and the GnRH-GFP cells (352 ± 57 pA; $n = 8$) as well ($p < 0.05$; not shown).

Before treatments with the PL37-MAP peptide, the cells were identified as neurons by applying +10 pA current with −10 pA prepulse in current clamp mode. The current injected has evoked action potential in the magnocellular cells of the SON and PVN (Fig. 6a and b). The protocol for the injected current is graphed in the inset of Fig. 6a. The action potentials presented no low threshold spike (LTS). In addition, a voltage gated transient outward rectifying (A-type) potassium ion current could be recorded in the neurons of the PVN (Fig. 6c). The voltage command protocol is in the inset: prepulse parameters were 20 ms and −90 mV whereas the pulses (30 ms) stepped from −80 mV up to +40 mV with steps of 10 mV. Lack of LTS in the SON and PVN and presence of the transient outward potassium current in the PVN suggested that these cells were neurosecretory magnocellular neurons (Hoffman et al., 1991; Tasker and Dudek, 1991). The GnRH-GFP cells also presented action potentials when current command was applied (Fig. 6d) demonstrating that the cells responding to the C5aR-agonist treatment were neurons. The injected current evoked action potentials in the cells of the AHA showing that these cells were neurons, too (Fig. 6e).

In order to demonstrate the action of estrogen in the neurons of the brain slice, GnRH-GFP neurons were treated with 2 μM PL37-MAP peptide in hypothalamic slices obtained from ovariectomized (OVX) and E2-substituted (OVX + E2) animals. PL37-MAP evoked inward pulses in the GnRH neurons of the OVX + E2 mice (610 ± 76 pA; $n = 8$) (Fig. 7a). The GnRH neurons of the OVX mice responded to the peptide with small inward current pulses (102 ± 45 pA; $n = 8$) (Fig. 7b) with significantly lower amplitude than in the neurons of the OVX + E2 mice ($p < 0.01$).

3.3. Real-time PCR experiments

To potentially explain the observed effect of E2 on the current evoked by the C5aR-agonist peptide, we examined

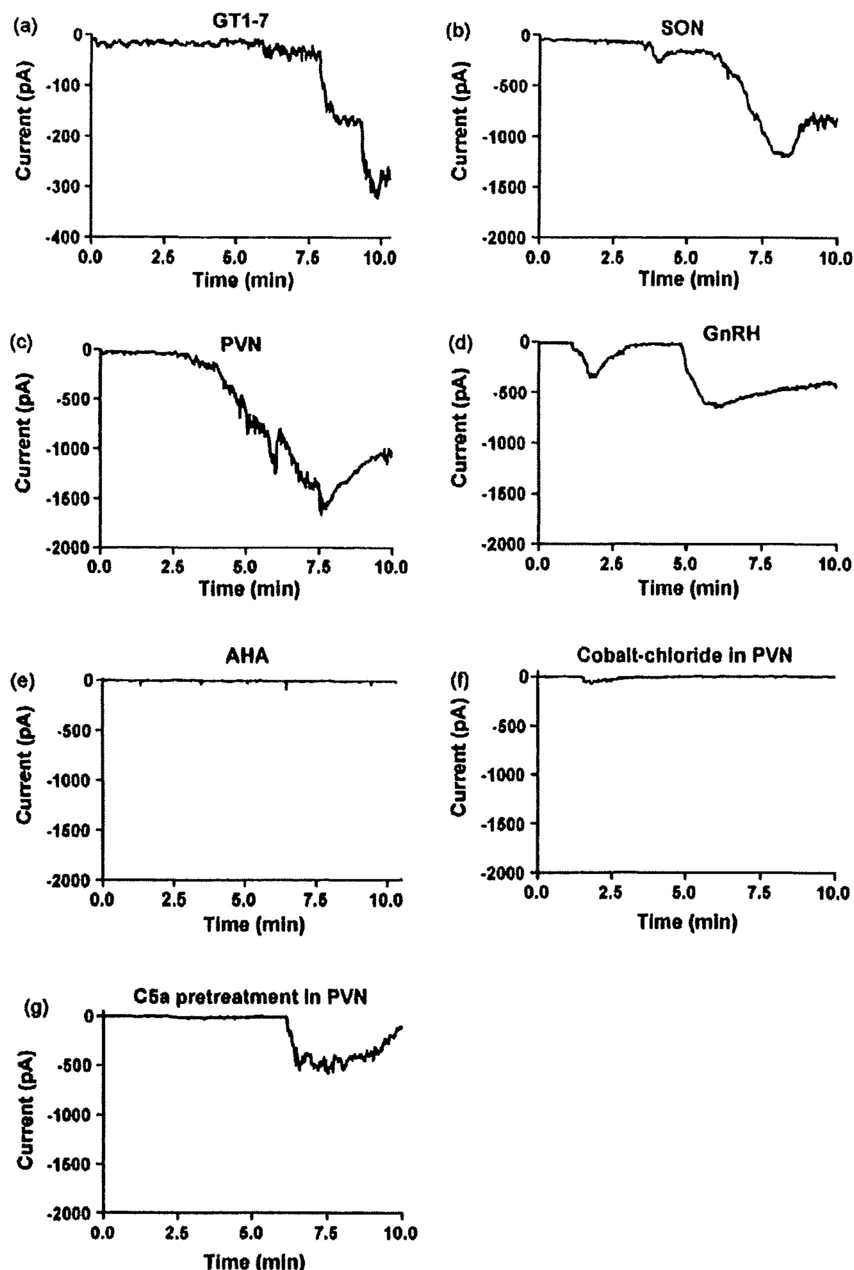

Fig. 5. The C5a-agonist peptide evoked inward current pulses, as determined by whole cell clamp electrophysiology in various hypothalamic cells. GT1-7 cells (a), magnocellular cells of the supraoptic (SON, b) and paraventricular nuclei (PVN, c) and GnRH cells (d) responded to PL37-MAP peptide with inward ion current. Cells of the anterior hypothalamic area (AHA) did not respond to the peptide administration (e). Calcium channel blocker $CoCl_2$ eliminated the evoked current in the PVN cells (f). Pretreatment of the brain slice with C5a diminished the amplitude of the inward current triggered by PL37-MAP in the PVN cells (g).

the expression of the C5aR in GT1-7 cells with real-time PCR. The expression ratio of C5aR and hypoxanthine-guanine phosphoribosil transferase (HPRT) genes in each sample (normalized data) was determined and compared (Fig. 8). In non-treated, control cells only low level of expression of the C5aR was detectable. Treatment of the cells with 20 nM E2 for 30 min, 2 h and 8 h resulted in mRNA signal intensities that did not significantly differ from those of the control. Nevertheless, treatment for 24 h caused a marked elevation in the C5aR mRNA level—reaching a significant 57-fold level (56.9 ± 32.44) compared to the signal intensities detected in non-treated cultures ($p < 0.01$). This showed that E2 induced C5aR expression and this process required E2 treatment for longer than 8 h. An additional day of E2 treatment (48 h) resulted in a decrease of the gene product. This indicated that the E2-dependent induction was transient in spite of the continuous presence of E2.

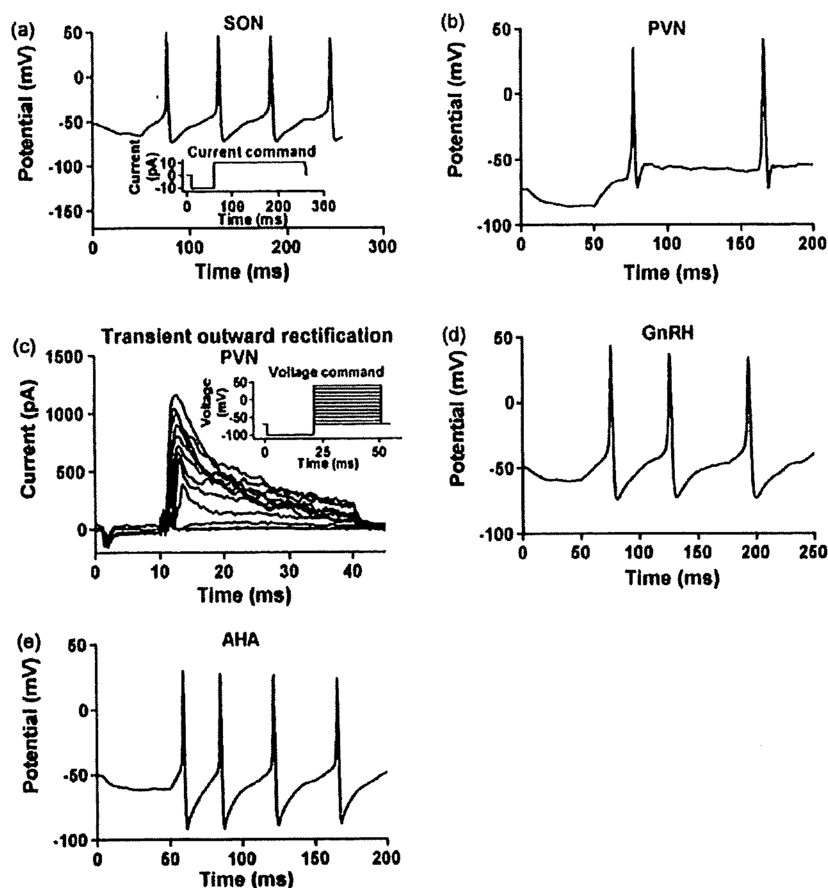

Fig. 6. Identifying the neuronal phenotype of the recorded cells shown in Fig. 5. The recorded cells of the SON, PVN and AHA in Fig. 5 were identified as neurons by their characteristic location and morphology and by the evoked action potentials using current clamp method (SON, a; PVN, b; AHA, e). Current clamp protocol details are given in the inset of (a). In addition, cells of the PVN were identified as magnocellular neurosecretory neurons by the presence of the transient outward rectification current (c) using voltage command seen in the inset. GnRH neurons were identified by their GFP fluorescence and the evoked action potentials (d).

4. Discussion

Few reports have suggested interactions between the complement system and the hypothalamus, particularly in the regulation of fever (Sehic et al., 1998; Blatteis and Sehic, 1998). A direct evidence for the central actions of C5aR was obtained following examination of the binding specificity of C5a injected into the hypothalamus, which suggested a presynaptic action for this anaphylatoxin (Schupf et al., 1989). The view that hypothalamic neurons express C5aR is also supported by the finding that direct injection of C5a into the perifornical region of the lateral hypothalamus of the rat changed eating and drinking behavior. C5a injection into this area stimulated eating in the satiated rats

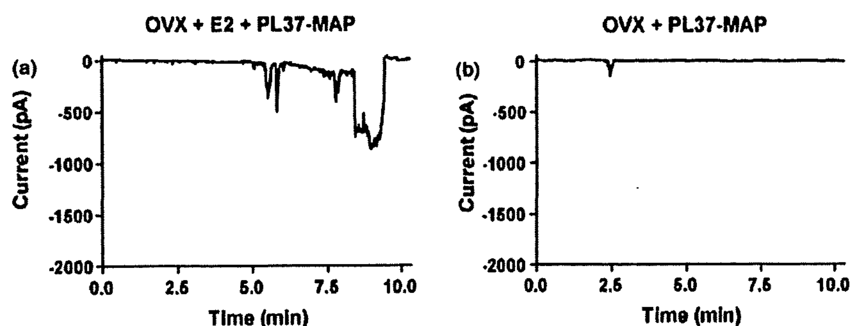

Fig. 7. The PL37-MAP peptide evoked inward current pulses in the GnRH-GFP neurons of the brain slice obtained from ovariectomized and E2 substituted (OVX + E2, a) and in ovariectomized (OVX, b) female mice. Amplitude of the triggered pulses in OVX brain slices was lower than in OVX + E2 slices showing that E2 increased the response elicited by PL37-MAP.

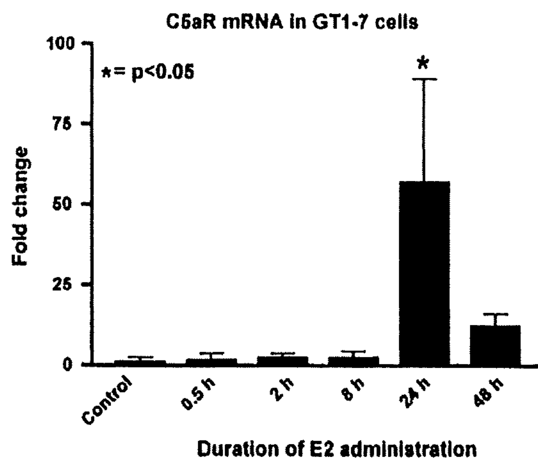

Fig. 8. Real-time PCR measurement of the E2-dependency of the expression of C5aR in GT1-7 cells. Treatment of the cells with E2 for 24 h elevated expression of C5aR followed by a decrease after 48 h.

and decreased carbamyl-choline-induced drinking (Williams et al., 1985).

Hippocampal and cortical pyramidal cells, cerebellar Purkinje cells and certain ventral and dorsal horn neurons of the spinal cord were earlier shown to bear C5aR (Farkas et al., 2003; Stahel et al., 1997a,b; VanBeek et al., 2000; O'Barr et al., 2001). Our experiments have now revealed that several neurosecretory cell types in the rat and mouse hypothalami – a brain area which has not been thoroughly investigated for expression of neuronal C5aR so far – also expressed complement C5aR. Magnocellular neurons of the supraoptic and paraventricular nuclei and GnRH-producing neurons responded to administration of the C5aR-agonist peptide demonstrating the presence of the functional C5aR in their membrane. However, neurons tested from the anterior hypothalamic area, which are not neurosecretory cells, were not activated by PL37-MAP suggesting that not all hypothalamic neurons bear C5aR. This result is supported by other works reporting that C5aR was only present in specific groups of neurons in the cortex and the hippocampus (Farkas et al., 2003; O'Barr et al., 2001).

Expression of the C5aR in the neurosecretory cells of the SON and PVN indicates that the inflammatory signals triggered inside the CNS might modulate the hormonal responses by these neurons. Although inflammatory signals generated in the periphery could also alter the hormonal response, the access of such signals to neurons is confined to brain regions lacking the blood–brain barrier. These include the organum vasculosum laminae terminalis (OVLT) and the median eminence. In addition, modulation of neuronal functions by the peripheral C5a is also limited by the short half-life of the circulating C5a. Nevertheless, modulation of the hormonal responses by the inflammatory signals is supported by the lipopolisaccharide (LPS)-evoked increase in c-fos immunoreactivity in neurons of the SON and PVN, followed by elevated plasma levels of vasopressin and oxytocin (de Carvalho Borges et al., 2006). The effect of LPS might be mediated via the production of cytokines such as tumor necrosis factor α (TNF-α), interleukins IL-1 and IL-6 (Haddad et al., 2002) which induce nuclear c-fos expression in the magnocellular neurons of the PVN and SON (Xia and Krukoff, 2003). However, TNF-α, the first of the cytokines to appear is not detectable until 30 min or even longer after the LPS challenge (Perlik et al., 2005), whereas vasopressin has already reached its highest level by this time (Giusti-Paiva et al., 2002). Nevertheless, a faster signal can be triggered via activation of the complement system. LPS in blood or tissue activates the alternative pathway of the C almost immediately. This results in the appearance of the complement cascade elements, including C5a, in 2–3 min (Giusti-Paiva et al., 2002; Blatteis, 2006). Activation of C5aR can elevate calcium content and c-fos expression via a G-protein-dependent pathway in neurons (Farkas et al., 1998a,b). In addition, C5a activation can elicit cytokine release from various cell types (Riedemann et al., 2004). In case of inflammation, C5a can be generated either locally in the brain (Terai et al., 1997; Gasque et al., 2000; Strohmeyer et al., 2000) or could enter the hypothalamus via structures lacking the blood–brain barrier (OVLT and median eminence, in case of the hypothalamus). Therefore, C5a bound to the C5aR may be considered as a putative early phase mediator in the hormonal response evoked by an inflammatory signal in the magnocellular neurons of the SON and PVN leading to increased vasopressin and oxytocin contents in the plasma. However, the validation of this hypothesis requires further experiments.

The C5aR was found in GnRH-producing neurons, too, indicating that C5a/C5aR might contribute to the inflammation-related pathology of the GnRH system. These neurons are located in the preoptic area around the OVLT suggesting that not only local stimuli but also peripherally induced C5a might reach these cells. Indeed, the reproductive axis is heavily suppressed under inflammation involving inhibition of GnRH release (He et al., 2003). Inflammatory stress can even disrupt the ovarian cyclicity (Karsch et al., 2002). These results support the idea that C5aR may interfere with reproduction via altering the function of GnRH neurons.

Our present data show that the response of the GnRH neurons to the activation of C5aR can be modified by estrogen. The calcium influx evoked by administration of the C5aR-agonist peptide was amplified upon E2 treatment. In addition, the effect of this hormone might be mediated via the estrogen receptor because the estrogen receptor antagonist Faslodex inhibited the amplification. Electrophysiological recordings revealed that E2 elevated the amplitude of the inward ion current recorded upon applying PL37-MAP to GnRH neurons. These results suggest that elements of the C and estrogen could interact in the GnRH neurons. Similar interactions between estrogen and certain elements of the C have already been reported in the uterus where complement C3 is considered as one of the most sensitive marker of estrogenic effects (Christoffel et al., 2006). The significance of amplification of the C5aR-mediated response by estrogen in GnRH neurons has not been elucidated yet. Recent results, however, indicate that estrogen is required for a proper immune response to bacterial and viral pathogens in the brain of female mice (Soucy et al., 2005).

In addition to its function in mediating the immune response, C5aR is also considered to play a role in neurodegeneration (Farkas et al., 2003; Woodruff et al., 2006). It is known that persistent high calcium concentration could trigger detrimental effects in neurons. Our earlier results demonstrated that non-physiological activation of the C5aR related with high calcium influx could evoke apoptotic signals (Farkas et al., 1998a,b). Although according to our present knowledge there is no reason to suppose that activation of the C5aR by C5a under physiological condition could elicit harmful outcome, the earlier data mentioned above suggest that the persistent high calcium influx evoked by PL37-MAP in the hypothalamic neurons could finally result in a degenerative process. This also raises the possibility that chronic release of C5a or presence of its fragments under pathological conditions – which could be modeled by presence of the PL37-MAP – could elicit detrimental effects in the neurosecretory neurons of the hypothalamus. In addition, the C5aR is associated with multiple signal transduction pathways including the ones that trigger apoptosis in neurons and thymocytes (Riedemann et al., 2002; Farkas et al., 1998a,b). Furthermore, apoptotic signal is related to an elevated expression of the C5aR (Riedemann et al., 2002). Therefore, overamplification of the C5aR signal could be detrimental. The real-time PCR measurements presented here have shown that estrogen increased the expression of the C5aR in the GnRH-producing GT1-7 neurons. These data are in harmony and strengthen those of our patch clamp and calcium imaging studies showing that estrogen is capable of amplifying the C5aR-mediated signal. Therefore, from our results we predict that under chronic inflammatory conditions estrogen could be involved in a positive feedback loop amplifying the signals – including calcium influx – evoked by activation of the C5aR. Deleterious effects of estrogen have already been shown in GT1-7 cells where it enhanced glutamate-induced neurotoxicity (Yang et al., 2003). Other authors also claimed that the impact of estrogen highly depends on the circumstances (type and concentration of estrogen, exposure time, gender, age, etc.) under which it acts (Nordell et al., 2003; Sohrabji, 2005; Chen et al., 2006). Further experiments are required to investigate such a role of estrogen in the future.

In conclusion, we have shown that magnocellular neurons of the supraoptic and paraventricular nuclei and GnRH-producing neurons of the hypothalamus express functional receptor for the C5a. In addition, we have revealed that estrogen treatment modulates the C5aR-mediated signal and the action of estrogen could be related to the estrogen receptor. Furthermore, we have provided evidence for the up-regulation of the C5aR transcript by estrogen. The significance of these findings relates to the better understanding of the inflammatory and neurodegeneratory diseases of the hypothalamus and the related neuroendocrine and autonomic compensatory responses.

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An inhibitory peptide of c5a anaphylatoxin rescues monkeys from lethal endotoxin shock by suppressing HMGB1 release[1]

Running Title: **RESCUE OF ENDOTOXIN SHOCK MONKEYS BY ANTI-C5a PEPTIDE**

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Abstract:

A 17 amino acid peptide (ASGAPAPGPAGPLRPMF) named PepA binds to C5a and prevents complement-mediated lethal shock in rats (1). PepA harboring an acetyl group at the N-terminal alanine showed increased inhibitory activity against C5a and was named AcPepA (2). Cynomolgus monkeys intravenously infused with a lethal dose of bacterial endotoxin (4mg/kg) were rescued by intravenous administration of 2 mg/kg/h of AcPepA for 3h starting 30 min after the lethal endotoxin injection. In these monkeys, high mobility group box 1 (HMGB1) (ref.3,4) did not increase whereas TNFα and other cytokines increased. Inhibition of C5a by AcPepA should have interfered with its ability to stimulate C5L2 (ref. 5) which is responsible for HMGB1 release (6).

Introduction:

Sepsis is inflammatory response syndrome (SIRS) that causes disseminated intravascular coagulation (DIC) and multiple organ failure (MOF) which are usually fatal. C5a anaphylatoxin, a 74-amino acid peptide released from the fifth component of complement (C5) by C5 convertase generated during complement activation (7,8), has been suggested to play a role in the septic shock process (9,10). Antibodies to C5a have been demonstrated to be effective in treating experimental septic primate models (10, 11) indicating that C5a inhibitors should be useful for treatment of patients suffering from hyper-inflammation as in sepsis and multiple organ failure (11). However, attempts to restrict the effect of C5a with C5a receptor (C5aR) antagonists have not been successful because C5a is also capable of reacting with another C5a receptor, C5L2 (ref. 5), causing release of HMGB1 (ref. 6).

Although antibody to C5a was effective in treatment of experimental septic primates (10, 11), antibodies in the body which persist for several weeks may interfere with the inflammatory process necessary to prevent another subsequent infection. Therefore, an unstable agent that directly inactivates C5a and persists for a limited time is desirable. We have recently generated an inhibitory complementary peptide to a region in C5a (1). Amino acids 37 to 53 of C5a (RAARISLGPRCIKAFTE) define an antisense peptide to

Antisense Homology Box (AHB) peptides (12) of the C5aR, and this peptide has been designated PL37 (ref. 13). This region of C5a is presumed to be a potential site for C5aR stimulation (14). Using the computer program; MIMETIC (15-17), we generated a complementary peptide to PL37 which interferes with C5a function. This peptide with the amino acid sequence, ASGAPAPGPAGPLRPMF, has been termed PepA (1). To improve stability, we modified PepA by acetylation of its N-terminal alanine generating acetylated PepA (AcPepA) which was more stable in animal experiments (2). In preliminary experiments with human lung tissues, AcPepA successfully suppressed the allergic response ex-vivo (18). Therefore, in lieu of using human subjects, we performed experiments in cynomolgus monkeys that were in a septic shock state following a lethal intravenous dose injection of LPS (4mg/kg body weight).

Materials and Methods

PepA (ASGAPAPGPAGPLRPMF) whose N-terminal alanine is acetylated (AcPepA) was synthesized and purified (over 95% purity) by Biologica Co. Ltd. (Nagoya, Japan). The peptide was dissolved in saline at a concentration of 2mg/ml and passed through a 0.22 μm Millipore filter prior to intravenous administration with an automated injection

pump.

Cynomolgus monkeys were obtained from a breeding colony maintained by the Corporation for Production and Research of Laboratory Primates (CPRLP), Tsukuba, Japan. The Institutional Animal Ethical Committee of the Choju Medical Institute, Fukushimura Hospital, and the Institutional Animal Care Use Committees of the Tsukuba Primate Research Center, National Institute of Biomedical Innovation approved the study protocol. Animals weighed 4 to 5.5 kg, had hematocrits exceeding 36% and were free of infection including tuberculosis. Animals were held for one month at CPRLP prior to injection of LPS (E. coli 0111:B4, Sigma) at 4mg/kg body weight.

TNFα in monkey plasma was determined using an ELISA kit purchased from Quantikine Immunoassay (Minneapolis, MN). For determination of high mobility group box 1 (HMGB1), an ELISA kit from Shino-Test Co. (Sagamihara-shi, Kanagawa, Japan) was used.

Results and Discussion

Although C5a generated in vivo is regulated by carboxypeptidase N and more efficiently by carboxypeptidase R (CPR) (19, 20), excessive generation of C5a appears

to exceed the capacity of CPR, since administration to rats of a lethal dose of LPS exhausted their CPR capacity before death (21). Therefore, septic patients would lose carboxypeptidase activity responsible for inactivaing anaphylatoxins by removing carboxyterminal arginine. For inactivation of C5a anaphylatoxin, AcPepA has proven to be highly effective (2).

As shown in Fig. 1 and Table 1, AcPepA treatment rescued monkeys suffering from endotoxin shock. The AcPepA- treated monkeys might have escaped a cytokine storm induced by a feedback inflammatory circuit progression at a late stage of the endotoxin shock syndrome. Since HMGB1 is the endogenous stimulator of TLR4 and TLR2 (ref. 22, 23), inhibition of HMGB1 induction presumably prevented further progression in the septic state (6, 24, 25). Therefore, suppressed HMGB1 induction in monkeys treated with AcPepA (Figure 2) could explain the therapeutic effect of AcPepA in endotoxin shock monkeys. C5a continuously generated by LPS reacts with C5L2 on activated leukocytyes, promoting release of HMGB1 which stimulates TLR4 on recruited leukocytes causing a cytokine storm that results in a lethal effect on the host (Fig. 3). Suppression of HMGB1 induction by inactivation of C5a could be directly correlated with the survival observed following AcPepA treatment of monkeys injected with a lethal dose of LPS.

Conclusion

Stimulation of C5L2 by C5a on activated leukocytes induces release of HMGB1 which then reacts with TLR-4 on other leukocytes, as does LPS, resulting in further recruitment of activated leukocytes that express C5L2. Inhibition of C5a by AcPepA should have interfered with the ability to stimulate C5L2 which is responsible for HMGB1 release effective on TLR4 for amplification of inflammation.

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