(Fig. 2), and the patient's UCDAI score had declined to 2. After 3 months, all of the patient's colonic lesions had healed with scarring and her UCDAI score had reached 0, so the PSL was discontinued.

AM was first identified as a biologically active peptide with potent vasodilating action,[1] but is now known to exert a wide range of physiological effects, including cardiovascular protection,[2] neovascularization, and suppression of inflammation and apoptosis. We previously reported that AM therapy was effective in an animal colitis model,[3] and that AM's mechanism of action is likely attributable to its suppression of inflammatory cytokines and activation of regulatory cytokines in intestinal intraepithelial lymphocytes, as well as to its protection of intercellular junctions and its antibacterial activity.[4] In addition, AM reportedly suppresses cytokine production in trinitrobenzene sulfonic acid (TNBS)-induced colitis,[5] and exerts beneficial effects on microvascular function[6] and the reepithelialization[7] of ulcers in an experimental model of colitis.

Although AM has potent hypotensive activity, we observed only minor hemodynamic effects after administering a dose of 1.5 pmol/kg/min, which we considered safe based on human dose–response data in our possession.

Conventional treatment of active UC focuses on steroids, immunosuppressants, and biologics, but the use of these drugs is restricted in geriatric and immunocompromised patients.[8] AM, on the other hand, is a physiological peptide and is therefore anticipated to have excellent safety. Here we present the first reported case in which AM was used to treat a patient with intractable UC. AM treatment produced mucosal regeneration accompanied by marked neovascularization and vasodilation visible on endoscopic examination. These findings are suggestive of AM's potential to be a ground-breaking modality with a novel mechanism of action that differs from existing immunomodulation therapy.

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話　題

炎症性腸疾患に対するアドレノメデュリン療法*

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Key Words：adrenomedullin, inflammatory bowel disease, ulcerative colitis, bioactive peptide

はじめに

現在，炎症性腸疾患(IBD)に対する薬物療法として，アミノサリチル酸製剤，ステロイド，免疫調節剤，血球成分除去療法，生物学的製剤を用いた寛解導入および維持療法が行われている．活動期の治療として，従来のステロイド治療に加え，免疫調節剤療法，生物学的製剤療法の使用頻度が増加しており，特にステロイド抵抗性やステロイド依存性を示すいわゆる難治性潰瘍性大腸炎に対して，タクロリムス，シクロスポリン，アザチオプリンなどの免疫抑制剤やインフリキシマブといった生物学的製剤による治療が主流となってきている．しかし，これらの免疫調整治療は優れた治療効果が期待される一方，高齢者や糖尿病などの基礎疾患を有する患者における真菌症や結核，ニューモシスチス肺炎などの重篤な感染症の合併や，若年者における悪性リンパ腫の発生などの重篤な有害事象に関する懸念も否定できず，より安全性の高い治療薬の開発が望まれる．

アドレノメデュリン(AM)は強力な血管拡張作用を有する生理活性ペプチドであるが，心血管保護作用，血管新生作用や抗炎症作用など多彩な生理作用を有することが判明しており，近年，IBDモデル動物に対しても腸炎改善効果を有することが明らかとなった．AMは生理活性ペプチドであり，比較的安全性が高いと考えられ，IBD治療への応用が期待される．本項では，AMの基本事項や腸炎モデル動物に対する効果などの基礎研究に関する知見について概説し，さらにIBD患者に対する臨床研究に関しても言及する．

アドレノメデュリン(adrenomedullin；AM)とは

AMは1993年に褐色細胞腫抽出液より単離同定された，強力な血管拡張作用を有する生理活性ペプチドである．ヒトAMは52個のアミノ酸で構成され，カルシトニン遺伝子関連ペプチド(calcitonin gene-related peptide：CGRP)とアミノ酸配列の相動性を有するCGRPファミリーの一つである[1](図1)．循環調節性ペプチドとしての探索がはじまったが，その後の研究でAMが全身臓器で分布発現しており，血管新生作用，心血管系保護作用，腎保護作用，抗酸化作用，抗アポトーシス作用，インスリン抵抗性の改善などきわめて多彩な生理作用を有することが判明している[2]．また，AMの産生分泌は，心筋や血管壁伸展などの機械的刺激をはじめ，炎症性サイトカインなどの炎症惹起因子，アンジオテンシンII，酸化ストレス，虚血や低酸素刺激など種々の因子によって増加することが判明している．AMは多臓器にわたり多彩な生理作用を示すが，基本的解釈としては①循環動態，②心血管保護，

* Adrenomedullin therapy for inflammatory bowel disease.

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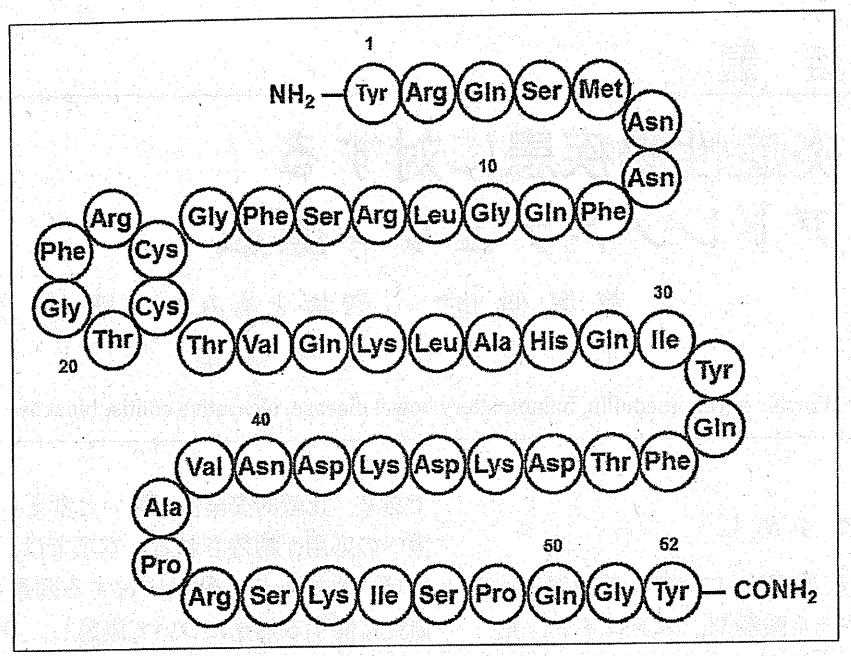

図1　ヒトアドレノメデュリンのアミノ酸配列

③抗炎症作用，④組織修復再生に寄与する働きを持つと説明できる(表1)．

AMの抗炎症作用

上記のようにAMは循環作動薬としての研究が進んできたが，AM発見後の早い時期から血管平滑筋培養細胞へのinterleukin(IL)-1やtumor necrosis factor(TNF)-α，lipopolysaccharide(LPS)などの炎症性サイトカインの刺激によりAMの遺伝子発現が著明に亢進することが判明していた．さらに，敗血症に伴う全身性炎症反応症候群(SIRS：systemic inflammatory response syndrome)患者において血中AM濃度が著明に上昇していることも明らかとなり[3]，炎症との関連が注目されている．動物実験レベルでは，LPSとD-ガラクトサミン投与による敗血症モデルマウスを用いた研究で，対照群では重度のうっ血肝を生じたことに比べ，AM過剰発現(トランスジェニック)マウスにおいてはうっ血肝がごく軽微となり，内因性AMがSIRSに対して臓器保護作用を有することが証明されている[4]．さらに，敗血症モデルマウスへAMを投与すると，血行動態の改善が認められるだけではなく，血漿中TNF-α，IL-1β，IL-6などの炎症性サイトカインが減少することや，in vitroにおいてもAMがマウスマクロファージ培養細胞からのTNF-αやIL-6の産生を抑制することが判明している[5]．このように，生体レベル，細胞レベルいずれにおいても，AMが炎症状態に対して抑制的に作用することが確認されており，今後，さまざまな炎症性疾患への応用が期待される．

表1　AMの代表的作用

血行動態改善	血管拡張 利尿・Na利尿 強心作用
心保護作用	酸化ストレス抑制 血管平滑筋増殖抑制 心肥大・線維化抑制 アルドステロン産制抑制
抗炎症作用	炎症性サイトカイン抑制 抗菌効果 SIRSへの臓器保護作用 胃腸炎改善
組織再生	血管新生・安定化 アポトーシス抑制 粘膜上皮細胞増生 骨髄細胞の末梢への動員

消化管におけるAM

消化管においてもAMは食道から大腸まで広く分布しており，特に胃と大腸での発現が強い[6]．胃においては胃内分泌細胞に発現しており，血流調節への関与が考えられている[7]．胃潰瘍患者

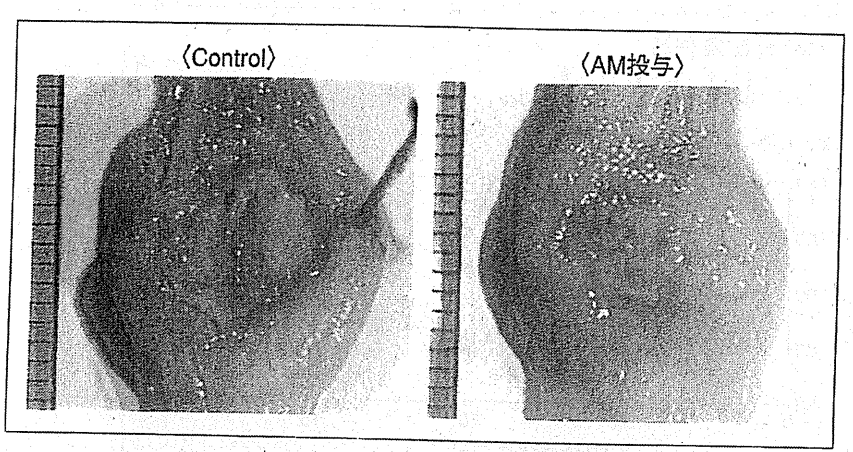

図2　酢酸誘発性大腸潰瘍に対するAM注腸の治療効果
左：対照群，右：AM投与群．大腸潰瘍作成後5日間AMを注腸投与したところ，対照群と比べ潰瘍の縮小と浮腫の軽減を認めた．
（文献[15]より許可を得て一部改変して転載）

の潰瘍周辺組織においてもAMが強く発現しており，さらに潰瘍治癒に伴いAMの発現が増強傾向を示すことから，AMが粘膜再生に関与する可能性が推測されている[8]．また，AMは胃粘膜傷害モデルラットに対して抗潰瘍作用を示すことが明らかとなっており，その機序としてAMが胃酸分泌を抑制すること[9]，胃動脈収縮を抑制し粘膜血流を維持すること[10]，血管新生を促進すること，粘膜上皮細胞の増殖を促進すること[11]などが推測されている．大腸におけるAMの検討としては，AMが粘膜上皮の細胞頂部と神経内分泌細胞に発現しており，defensinのように腸内細菌に対して抗菌作用を示すこと[12]，腸管血流を増加させること(Talero)などが報告されている．また，ヒト大腸癌および実験腸炎関連大腸癌において癌組織でAMの発現が増強しており，癌組織の発育へ関与する可能性が報告されている[13][14]．

腸炎モデルに対する粘膜障害改善効果

AMが炎症惹起状態において抗炎症作用を示すことに加え，胃粘膜障害モデルに対し粘膜保護・潰瘍改善効果を示すことから，下部消化管における炎症(IBD)に対しても抗炎症効果を有する可能性が考えられ，筆者らは酢酸誘発性大腸潰瘍モデルラットを用いてAMの抗潰瘍効果を検討した．ラット大腸の漿膜下に酢酸を注入することにより大腸人工潰瘍モデルを作成し，AMを経肛門的に5日間連日投与した．その結果，AM投与群では対照群と比較してAM用量依存的に潰瘍面積が縮小しており(図2)，組織学的検討でも潰瘍部への炎症細胞浸潤が軽度であった．また，AM投与群において潰瘍大腸組織におけるinterferon(IFN)-γやIL-6などの炎症性サイトカインが有意に低値であった[15]．次に実験的腸炎の汎用モデルであるデキストラン硫酸(DSS)腸炎モデルを用いて，抗炎症効果の再現性を確認するとともに，その機序を検討した．DSS腸炎モデルマウスに対しAMを7日間にわたって注腸投与したところ，AM投与群は対照群に比べ，体重減少，下痢，血便が著しく軽減し，病理組織学的所見でも好中球や炎症細胞の浸潤，粘膜びらんが顕著に軽減した(図3, 4)．また，大腸上皮間T細胞(intraepithelial T lymphocytes；IEL)からのサイトカイン産生の検討では，AM投与群において炎症性サイトカインであるINF-γ，TNF-α，IL-6の産生が有意に抑制され，さらに制御性サイトカインであるtransforming growth factor(TGF)-βの増加が認められた(図5)．大腸IELのFACS解析の結果，対照群では病態の増悪化に伴いT cell receptor(TCR)γδ IELの継時的減少が認められたが，AM投与群では維持されており，TCRγδの維持がTGF-β亢進に関与した可能性が考えられた．さらに，AM投与群では対照群に比べ腸炎に伴う大腸上皮細胞接合分子の損傷が軽度であったことや，大腸腸内細菌数の減少がみられたことも，腸炎改善効果の一因と考えられた[16]．

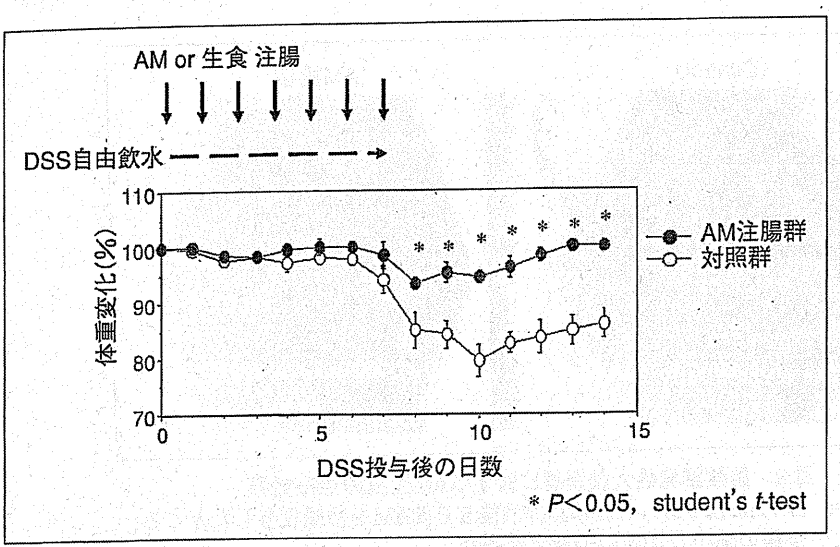

図3　デキストラン硫酸ナトリウム(DSS)腸炎モデルマウスへのAM療法
DSS投与下に，AM注腸(0.05μg/日)を7日間施行した．対照(生食)群と比較し，AM投与群ではDSS腸炎に伴う体重減少が軽微であった．* $P<0.05$
(文献[16]より許可を得て一部改変して転載)

実験腸炎モデル動物に対するAMの有効性は，当教室以外にも複数の施設から報告されており，トリニトロベンゼンスルホン酸(TNBS)誘発腸炎モデル動物に対するAMの腹腔内投与でも腸炎の改善が認められる．その機序として，Gonzalez-Reyらは腸管サイトカインの詳細な検討からAMにより腸管免疫寛容が改善されることを報告し[17]，TaleroらはCOX-2やiNOS/NOに関連した腸管微小循環が改善されることを報告している[18]．また近年，HayashiらはDSS腸炎モデルマウスを用いての検討に加え，創傷治癒アッセイを用いたin vitroでの検討を行い，AMが損傷上皮細胞に対して上皮再生促進効果を有することを報告している[19]．

このようにAMは腸炎モデル動物に対し，抗炎症作用や腸上皮バリアの保護作用を示し，新たな機序を有する炎症性腸疾患治療に発展する可能性が示された．

AMの臨床応用

AMが血行動態改善，心血管保護，組織(血管)再生作用を有することから，循環器疾患患者に対する臨床研究がすでに開始されている．Katookaらは急性心筋梗塞患者に対して12時間のAM持続静注投与を行い，心筋梗塞発症3か月後の心機能評価でAM投与群では心臓壁運動や心筋虚血領域の軽減効果が認められた[20]．また，急性心筋梗塞や心不全治療以外にも，難治性肺高血圧症に対する血管拡張作用や，閉塞性動脈硬化症への血管新生効果に関する臨床応用も期待されている．これらの臨床研究および前述基礎研究での知見を背景に，われわれは炎症性腸疾患患者に対するAMの効果に関して臨床研究を開始した．これまでに数名の患者にAMを投与したところおおむね良好な治療効果が得られており，下記に代表的症例を提示する．

症　例

68歳女性．潰瘍性大腸炎(UC)歴3年，糖尿病治療中．前回再燃時はプレドニゾロン(PSL)強力静注療法，血球除去療法で臨床的寛解が得られたが粘膜修復が不十分であり，また，ステロイド依存性を示し，PSL継続とアザチオプリンにて加療していた．某年5月から急激に腹痛と血便(>10回/日)が増悪し，内視鏡所見上，大腸全域に深掘れ潰瘍とびらんが多発していた．再度PSL強力静注療法と血球除去療法にて加療したが寛解導入が得られなかった．免疫抑制剤や生物学的製剤の追加投与を検討したが，高齢者である上，耐糖能異常と陳旧性結核を有するため感染症併発の危険性が懸念された．手術適応も検討されたが，患者の希望によりAM療法を試みた．虚血性心疾患や脳血管障害，糖尿病性網膜症な

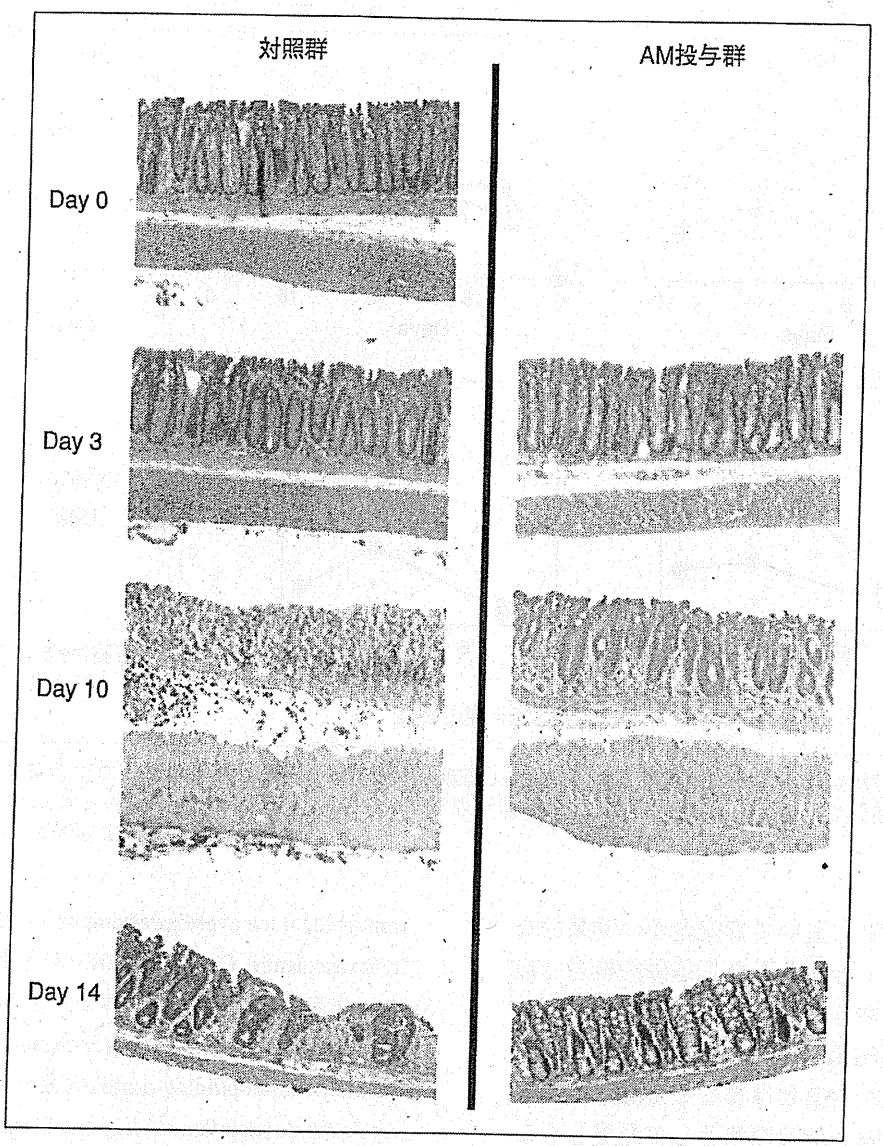

図4　DSS腸炎モデルマウスへのAM投与による腸炎改善効果
左列：生食投与群，右列：AM投与群(文献[16]より許可を得て一部改変して転載)

どの心血管系疾患や悪性疾患の存在を除外したのちに，AMの持続静脈投与を行った(1.5pmol/kg/min, 8時間/日，12日間)．治療開始後数日で腹痛や血便は改善傾向を示し，AM投与2週間後の内視鏡検査では潰瘍底および潰瘍辺縁に再生粘膜増生，瘢痕化が認められた．AM投与前後でUCDAIは7点から2点まで改善した．AM投与中に10mmHg程度の軽度血圧低下を認めたが，その他の明らかな有害事象は認めなかった．その後も寛解を維持し，AM投与3か月後の内視鏡評価ではすべての大腸病変が瘢痕治癒しており(UCDAI：0点)，PSL中止が可能となった．現在，1年以上が経過しているが寛解を維持している．

おわりに

AMの基本的特徴から消化器領域，特に炎症性腸疾患に関する臨床応用までを紹介した．AMが有する循環動態改善，抗炎症作用，抗酸化作用などといった臓器保護作用，血管新生，上皮細胞増殖といった組織修復作用は，循環器疾患だけだはなく他疾患領域での応用も期待され，消化管領域においてもさらなる基礎研究および臨床研究の発展が望まれる．また，AMはこれまで循環器領域を中心に研究が進んできた生理活性ペプチドであるが，腸炎モデル動物においても抗炎症効果や粘膜修復効果を示すことが判明し，

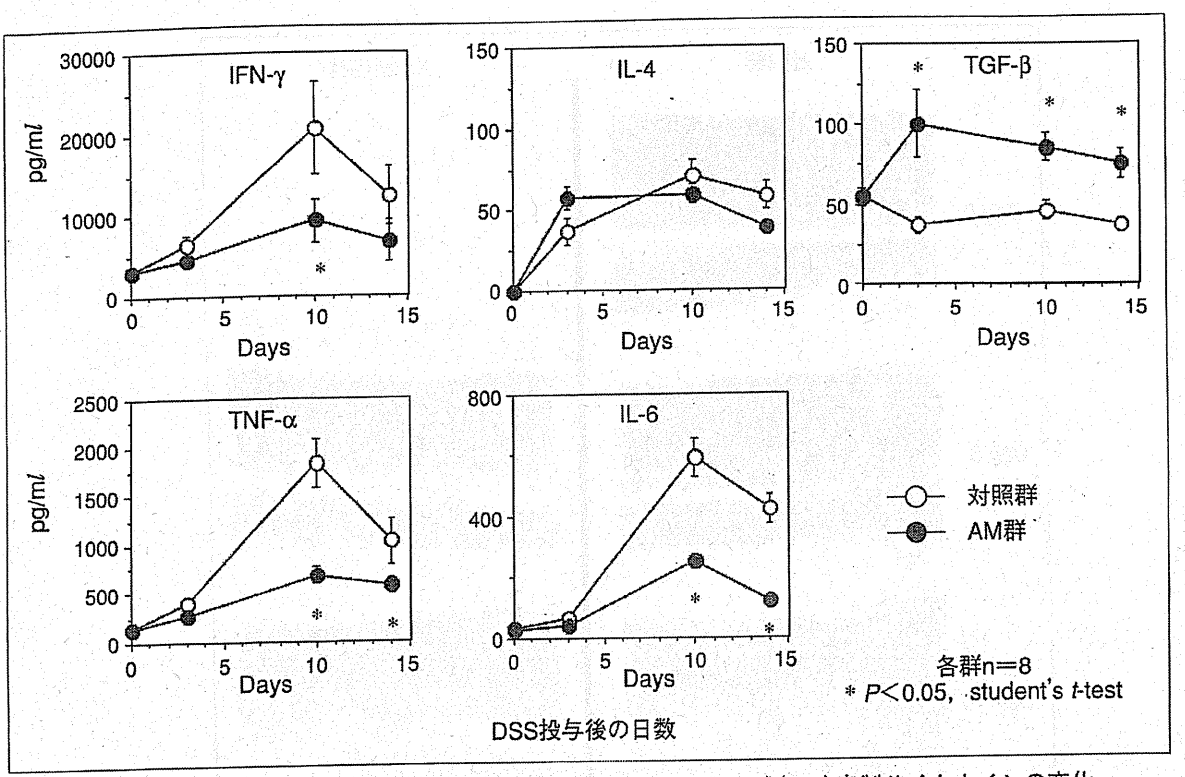

図5 DSS腸炎マウスへのAM投与による大腸上皮細胞間リンパ球(IEL)産制サイトカインの変化
AM投与群ではIFN-γ，TNF-α，IL-6産制の著明な抑制と，TGF-β産制の亢進が認められた．
(文献[16]より許可を得て転載)

さらに実際の患者においても安全かつ効果的な効果が得られた．今後さらなる基礎的検討と症例蓄積が必要であるものの，炎症性腸疾患治療において，AM療法は生理活性ペプチドという既存の免疫調整性治療薬とは異なった機序を有する，新規かつ安全な治療戦略として発展しうる可能性が期待できる．

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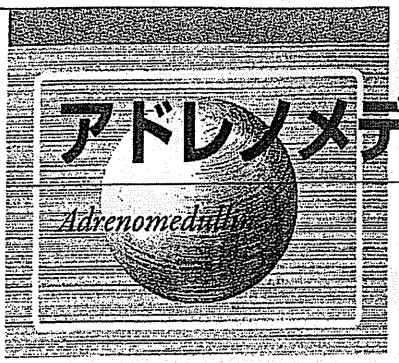


アドレノメデュリン

北村和雄

アドレノメデュリンとPAMPは，共通の前駆体より生合成される降圧作用を有した生理活性ペプチドで，心血管系をはじめとする幅広い組織で生合成されており，降圧因子として循環調節や心血管病の病態に関与している．

構造・分布

アドレノメデュリン(AM)はヒト褐色細胞腫組織抽出液から発見された強力な血管拡張性ペプチドであり，特徴として分子内に6個のアミノ酸よりなるリング構造とC末端のアミド構造をもっている．生理活性に必須であるこれらの構造はカルシトニン遺伝子関連ペプチド(calcitonin gene-related peptide；CGRP)やアミリンなどと一部相同性があり，1つのスーパーファミリーを形成している．さらに，AM前駆体からは**プロアドレノメデュリンN末端20ペプチド(proadrenomedullin N-terminal 20 peptide；PAMP)**が，降圧作用を有した別の生理活性ペプチドとして生合成されている(図)．AMは副腎髄質だけでなく全身の臓器で発現・分泌されており，肺，腎臓，心臓，血管など循環調節に重要な臓器での発現量も多い．

作用・作用機序・受容体

AMの主作用は強力で持続時間の長い血管拡張作用であるが，それ以外にアルドステロンの分泌抑制作用や利尿作用ももち，全体として血圧を低下させる方向に作用する．さらに，血管平滑筋や心筋の増殖抑制作用，血管内皮細胞のアポトーシス抑制作用を有し，臓器障害に対し保護的に作用している可能性も考えられる．なお，AMのトランスジェニックマウスが開発されており，血圧の低下が確認されている．また，AMの欠損マウスは胎生致死であり，その原因としては血管形成不全によると考えられている．

AMの血管拡張作用の機序としては，血管平滑筋細胞では，cAMPを濃度依存性に増加させ，血管拡張を起こすと考えられている．一方，血管内皮細胞に対してAMは，cAMPを増加させると同時にCa^{2+}やIP_3も増加させることが判明している．血管内皮細胞のCa^{2+}の増加はcNOS(constitutive nitric oxide synthase)を活性化しNO産生を増すことが知られており，種による差や血管床による違いはあるものの，AMの血管拡張作用はNO依存性血管拡張作用が関与していると考えられている．さらにAMの血管新生作用やアポトーシス抑制作用は，PI3K-Akt，ERK-MAPKおよび局所接着キナーゼ(p125 FAK)などを介することが示されている．

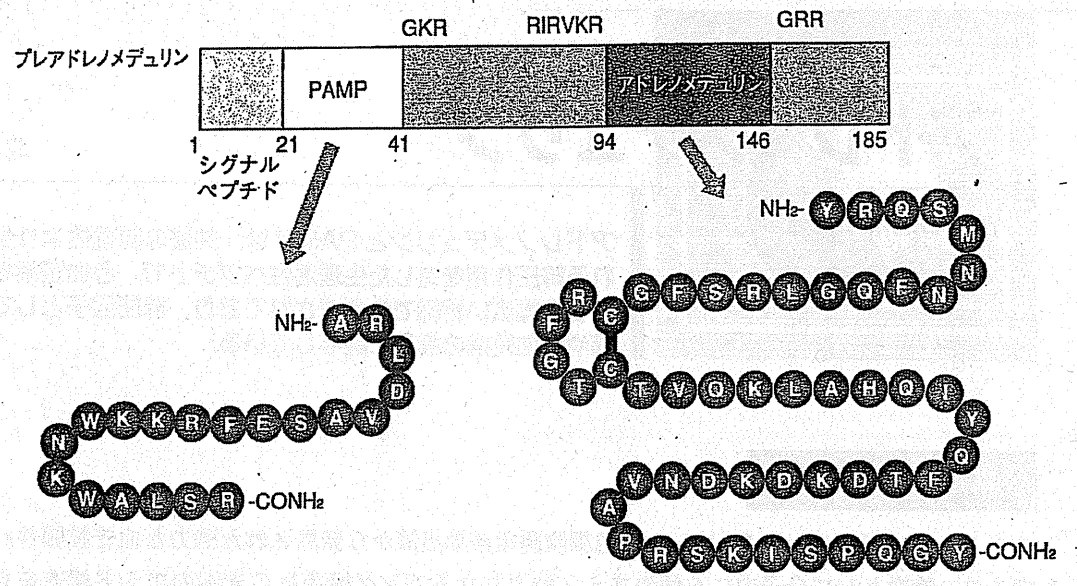
AM受容体に関しては，AMとCGRPが受容体としてCRLR(calcitonin-receptor-like receptor)という7回膜貫通型G蛋白共役型受容体を共有する．CRLRがAMとCGRPの受容体として機能を発揮するためには，1回膜貫通型の受容体活性調節蛋白(RAMP)と呼ばれる膜蛋白が必須である．RAMPがなければCRLR単独では膜表面に移行せず活性も示さず，RAMP単独でも受容体として機能しない．さらに，RAMPはAMとCGRPに対する特異性も決定する．RAMPにはRAMP1～3の相互に相同性を有する3種類の蛋白が存在し，CRLR＋RAMP1でCGRPの，CRLR＋RAMP2またはCRLR＋RAMP3でAMの受容体を形成する．CRLR＋RAMP2はAMへの親和性が高く，かつRAMP2欠

用語解説――プロアドレノメデュリンN末端20ペプチド(PAMP)

AM前駆体から生合成されるもう1つの降圧活性ペプチドで，降圧機序はAMと異なり，協調して循環調節に関与していると考えられる．

略語

AM：adrenomedullin
CRLR：calcitonin-receptor-like receptor
PAMP：proadrenomedullin N-terminal 20 peptide
cAMP：cyclic adenosine monophosphate
IP_3：inositol (1,4,5-) triphosphate
RAMP：receptor activity modifying protein



プロアドレノメデュリンN末端20ペプチド(PAMP)
降圧，交感神経抑制，アルドステロン抑制

アドレノメデュリン
降圧，利尿，抗炎症，細胞増殖の制御ほか

図　ヒトアドレノメデュリン(AM)前駆体の構造と生合成機構およびAMとPAMPの構造

AM前駆体からはAM以外に，PAMPが別の生理活性ペプチドとして生合成される．両ペプチドとも降圧作用を示すが，それらの作用機序は異なっている．

損動物ではAM欠損動物と同じく胎生致死となることから，AMの受容体としてのCRLR＋RAMP2が重要であると考えられている．

病態との関連

AMおよびPAMPの血漿中濃度は，本態性高血圧患者をはじめ腎不全や心不全患者で重症度に従い増加している．また，敗血症性ショックの患者では，AMの血中濃度が重症心不全患者の10倍以上にも増加しており，AMの敗血症性ショックでの病態生理学的意義が注目されている．AMは血管において多量に生合成分泌されており，血管内皮や平滑筋細胞からのAMの生合成・分泌はtumor necrosis factor(TNF)-αをはじめとする多くのサイトカインや血管作動物質等により調節されている可能性が示されている．血管で産生されたAMが血管に対して，エンドセリン-1やアンジオテンシンⅡに拮抗するようなかたちで，血管トーヌスの調節や動脈硬化症の病態などに深く関与していると考えられる．

トピックス：臨床応用の可能性

AMの作用の多くが心血管保護的であり，血管新生作用も有することから，心血管病治療への臨床応用に関する研究が展開されてきた．高血圧症や動脈硬化症への治療応用が期待されているが，AMペプチドそのものの単なる経口投与は不可能であり，新たな投与手段の開発が求められる．急性心筋梗塞，脳梗塞，肺高血圧症に対しては，比較的短時間の静脈内投与あるいは吸入投与にて治療効果が得られる可能性が示唆されており，今後の発展が期待されている．AMの抗炎症，抗線維化作用も強力であり，心血管病以外にも，炎症性腸疾患や各種線維症の治療薬としても検討されている．

Recommended Readings

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関連事項

アンジオテンシンⅡ ▶▶ 76頁
アルドステロン ▶▶ 88頁
NO ▶▶ 92頁
閉塞性動脈硬化症 ▶▶ 162頁

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Adrenomedullin production is increased in colorectal adenocarcinomas; its relation to matrix metalloproteinase-9

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ABSTRACT

Adrenomedullin (AM) is highly expressed in various cancer cell lines, suggesting a possible association with cancer growth. In the present study, we examined the expression and/or concentration of AM, its related peptide, adrenomedullin2/intermedin (AM2/IMD) and their receptors in human colorectal cancer and the surrounding normal tissue. In addition, we assessed the correlation between the expression of AM and AM2/IMD with that of vascular endothelial growth factor (VEGF)-A and matrix metalloproteinase (MMP)-9. Using a specific immunoradiometric assay, we found that AM concentrations were 2–11-fold higher in colorectal cancer tissues than in the surrounding normal tissues. Moreover, real-time quantitative RT-PCR showed that the expression levels of preproAM (+548%), preproAM2/IMD (+2674%), calcitonin receptor-like receptor (CLR) (+518%), receptor activity modifying protein (RAMP)2 (+281%), RAMP3 (+178%), VEGF-A (+277%) and MMP-9 (+864%) mRNAs were significantly higher in cancer tissues than in the surrounding normal tissues, and there was a positive correlation between the gene expressions of MMP-9 and preproAM ($r=0.352$; $p=0.005$), but not with preproAM2/IMD ($r=0.041$, $p=0.406$). Both AM and AM2/IMD immunoreactivity were detected mainly within cancer cells, whereas MMP-9 immunoreactivity was mostly seen in the surrounding stroma. These findings suggest that AM produced in colorectal tumors acts in concert with MMP-9 in the stroma to contribute to the pathogenesis of colorectal cancer.

1. Introduction

Colorectal cancer is the fourth most common cause of cancer death, worldwide [10]. It proliferates locally and metastasizes to liver, lung, bone and/or brain, and often recurs after surgical resection. Invasion and metastasis are multistep processes that require sequential interactions between the cancer cells and stromal components, such as the extracellular matrix [12] and micro-neovessels [35]. Matrix metalloproteinases (MMPs) and vascular endothelial growth factor (VEGF) are key factors that facilitate invasion and metastasis, respectively by degrading extracellular matrix [35] and promoting angiogenesis [8,15] for solid tumor growth.

Adrenomedullin (AM) is a pluripotent bioactive peptide initially isolated from human pheochromocytoma [21]. Since its isolation, AM has been shown to be widely distributed among various organs and tissues, including the human digestive system [22,26], and to be involved a variety of physiological functions, such as vasodilatation, hormone secretion, neurotransmission, embryogenesis, wound healing and immunoregulation [2]. Adrenomedullin2/intermedin (AM2/IMD) was identified as an AM-related peptide by genomic searching [34,36]. AM2/IMD possesses not only a structural similarity to AM such as a ring structure and an amidated C terminus, but also a functional analogy to exert vasodilation and anti-oxidative stress [14]. The functions of AM are mediated through specific receptors comprising calcitonin receptor-like receptor (CLR) and a receptor activity-modifying protein (RAMP); when co-expressed with RAMP2 or RAMP3, CLR functions as a specific AM receptor [23]. Meanwhile, AM2/IMD binds non-selectively to three types of CLR/RAMP complexes: CLR/RAMP1, CLR/RAMP2 and CLR/RAMP3 [23]. AM and its receptors are highly expressed in some tumors and cancer cell lines, which suggests that AM may participate in tumor progression through its potent mitogenic, angiogenic and anti-apoptotic properties [1,13,29]. However, its site-specific expression profile and its concentration in human colorectal cancers and the surrounding normal tissue have not been fully explored. Therefore, our aims in the present study were to measure the expression and/or concentration of AM and its receptors in both cancerous and normal colorectal tissue in humans, and to correlate AM levels

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Table 1
Sequence of the primers and probes.

Gene	Primer/probe sequences	Product size (bp)
AM	S: 5′-TGCCCAGACCCTTATTCGG-3′ AS: 5′-CCGGAGGCCCTGGAAGT-3′ Probe: 5′-AGTCAAGCGCTACCGCCAGAGCA-3′	130
AM2/IMD	Applied Biosystems Gene Expression Assays (ID;Hs00363866_m1)	
CLR	S: 5′-CTGTACATGAAAGCTGTGAGAGCTACT-3′ AS: 5′-TGGAAGTGCATAAGGATGTGCATGATG-3′ Probe: 5′-TCCATGGCGACCTGAAGGAAAGATTGCAGA-3′	140
RAMP2	S: 5′-GCAGAGAGGATCATCTTTGAGACTC-3′ AS: 5′-CCTCCATACTACAAGAGTGATGAGGAAG-3′ Probe: 5′-TGCTCCCTGGTGCAGCCCACCTCTTCTGAC-3′	156
RAMP3	S: 5′-CCGAGTTCATCGTGTACTATGAGAG-3′ AS: 5′-CTGTGGATGCCGGTGATGAAGC-3′ Probe: 5′-AGGCCAATGTCGTGGGCTACTGGCCCA-3′	115
VEGF-A	S: 5′-GTGTGAGTGGTTGACCTTCCTC-3′ AS: 5′-CCGTATATAAAACACTTTCTCTTTTCTCTG-3′ Probe: 5′-CCTGGTCCTTCCCTTCCCTTCCCGA-3′	125
MMP-9	S: 5′-CCCTGGAGACCTGAGAACCA-3′ AS: 5′-AACCATAGCGGTACAGGTATTCCT-3′ Probe: 5′-TCTCACCGACAGGCAGCTGGCA-3′	78
β-actin	S: 5′-AGCCTCGCCTTTGCCGA-3′ AS: 5′-CTGGTGCCTGGGGCG-3′ Probe: 5′-CCGCCGCCCGTCCACACCCGCC-3′	174

S, sense strand; AS, antisense strand.

with clinical variables and the synthesis of invasive and metastatic factor, VEGF-A and MMP-9. We also discussed the expression of AM2/IMD in the disorder.

2. Materials and methods

2.1. Tissue collection

Colorectal specimens were obtained from 23 Japanese patients with colorectal cancer (8 colon cancer and 15 rectal cancer) who underwent surgical resections at Miyazaki University Hospital between June 2007 and March 2009. The histological stages were determined according to Japanese General Rules for Clinical and Pathological Studies on Cancer of the Colon, Rectum and Anus, The 7th Edition. The specimens (0.2–1.0 g) included both cancerous tissue and normal-appearing colorectal tissue from the surrounding area. All specimens were immediately stored in liquid nitrogen until used for real-time quantitative PCR, immunoradiometric assays (IRMAs) and histological analysis. Written informed consent was obtained from all patients, and the study protocol was approved by the institutional review board (No. 519).

2.2. IRMA

The mature form of AM was measured using an AM mature IRMA kit (Shionogi & Co., Ltd., Osaka, Japan) [30]. Tissue samples boiled in 1.0 mol/L acetic acid were homogenized and centrifuged at 12,000 rpm for 20 min at 4 °C, after which the supernatants were stored at −30 °C until assayed.

2.3. Reverse-phase high performance liquid chromatography (RP-HPLC)

To characterize the molecular forms of immunoreactive AM in human colorectal tissues, collected samples (1.0 g) of cancer tissue and the surrounding normal tissue in one patient were immediately boiled for 10 min and then acidified by the addition of acetic acid to a final concentration of 1.0 mol/L to inactivate proteases. The samples were then homogenized and centrifuged at 12,000 rpm for 20 min, after which the supernatants were applied to Sep-Pak C18 cartridges (Nihon Waters K.K.). The resultant extracts were analyzed by RP-HPLC with a TSK ODS 120A column (4.6 × 150 mm; Tosoh) using a linear gradient from solvent A (H_2O–acetonitrile (CH_3CN)–100 mL/L trifluoroacetic acid (TFA, 90:10:1, by volume)) to solvent B (H_2O–acetonitrile–100 mL/L TFA (40:60:1, by volume)) at a flow rate 1 mL/min for 60 min. The immunoreactive AM in each fraction was measured using an IRMA [30] twice, and identical results were obtained. Synthetic human AM peptide was purchased from PEPTIDE INSTITUTE, INC. Osaka, Japan.

2.4. Real-time quantitative RT-PCR

Real-time quantitative RT-PCR was performed using TaqMan probes to quantify the mRNA levels of AM, AM2/IMD, CLR, RAMP2, RAMP3, VEGF-A, MMP-9 and β-actin. The primers and probes used in this study are listed in Table 1. To quantify the mRNA, standard curves were drawn using diluted cDNA made by human colon tissue. After extracting the total RNA using TRIzol (Life Technologies, Ltd.), 2-μg aliquots were used to synthesize cDNA as previously described [28]. Quantitative RT-PCR analysis was carried out using an Applied Biosystems Prism 7300 Sequence Detector. The PCR protocol entailed a starting cycle of 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The PCR products were electrophoresed and found to be of the expected molecular sizes, and β-actin mRNA was used as an internal normalization standard.

2.5. Immunohistochemical staining

Immunohistochemical staining was conducted with samples from all 23 patients after preparation of serial sections. Tissue sections (4 μm) that had been fixed in 10% formalin and embedded in paraffin were deparaffinized, hydrated in 0.01 mol/L phosphate-buffered saline (PBS), and incubated in 3% H_2O_2 in PBS for 20 min at room temperature to block endogenous peroxidase activity. For the detection of MMP-9 and AM2/IMD, prior antigen retrieval was performed by autoclaving the specimens at 121 °C for 15 min in citrate buffer (pH 6.0). The sections were then incubated with a mouse monoclonal antibody against human $AM_{[12-25]}$ (1:500 dilu-

Table 2
Patient characteristics.

Patient characteristics	Numbers
Patients	23
Median age (years) [range]	72 [35–91]
Male/female	15/8
Primary site	
Cecum/A/T/D/S/rectum	1/3/2/0/2/15
Histological stage[a]	
I/II/IIIa/IIIb/IV	1/8/5/4/5
Histological type[a] (all adenocarcinoma, partially overlap)	
tub1/tub2/muc/pap/sig	4/16/3/1/1
Liver/lung metastasis	4/1
Median longest diameter of tumor (mm) [range]	60 [25–122]

Abbreviations: A, ascending colon; T, transverse colon; D, descending colon; S, sigmoid colon; tub1, well differentiated type; tub2, moderately differentiated type; muc, mucinous type; pap, papillary type; sig, signet ring cell type.

[a] Japanese General Rules for Clinical and Pathological Studies on Cancer of the Colon, Rectum and Anus, The 7th edition.

tion; [26,30]) or a rabbit polyclonal antibody against human MMP-9 (1:50 dilution; #3852, Cell Signaling Technology, Inc.) and human $AM2/IMD_{[35-47]}$ (1:4000 dilution, originally made by Dr. Kitamura) for 24 h at 4 °C, followed by incubation for 30 min at room temperature with horseradish peroxidase-labeled polymer conjugated with a secondary antibody (Dako Envision+ System). The immunoreactivity was visualized using 3,3′-diaminobenzidine (Dako), and the sections were counterstained with Mayer's hematoxylin. The positive ratio of AM and MMP-9 immunostaining were analyzed by randomly selected 10 fields in 23 serial sections, and calculated as the percentage of the traced positive field, respectively.

2.6. Statistical analysis

Statistical analysis was carried out using GraphPad Prism 5 (MDF Co., Ltd.). Data are expressed as means ± standard deviation. The Wilcoxon matched-pair signed rank test was used to assess the association between two categorical variables. The Spearman rank correlation test was applied for the comparison of correlations among multiple groups. Difference between variables in clinical stage or type was analyzed by Kruskal–Wallis test. Values of $p < 0.05$ were considered significant.

3. Results

3.1. Patient characteristics

Table 2 shows the characteristics of the patients enrolled in this study. They included 15 males and 8 females, ranging in age from 35 to 91 years (median, 72 years). The primary site was the rectum by 65% of cases, and the histological characteristics varied widely. Among the five patients with stage IV disease, four exhibited liver metastasis and one exhibited lung metastasis.

3.2. Concentration and molecular form of AM in colorectal tumors and surrounding normal tissues

Fig. 1A shows that the concentration of AM was significantly ($p < 0.001$) higher in cancerous tissues (2.3 ± 1.0 pmol/g wet tissue) than in macroscopically normal colorectal tissue (0.7 ± 0.4 pmol/g wet tissue). On reverse phase HPLC, the AM immunoreactivity in both the cancerous tissue and surrounding normal tissue in a patient had one major peak, the elution position of which was identical to that of authentic human $AM_{[1-52]}$ (Fig. 1B).

3.3. Expression of preproAM, preproAM2/IMD, CLR, RAMP2, RAMP3, VEGF-A and MMP-9 mRNAs in colorectal tumors and surrounding normal tissues

Fig. 2 shows that the relative expression levels of the preproAM (A; +548%; $p < 0.0001$), preproAM2/IMD (B; +2674%; $p < 0.0001$), CLR (C; +518%; $p < 0.0001$), RAMP2 (D; +281%; $p < 0.0001$), RAMP3 (E; +178%; $p < 0.005$), VEGF-A (F; +277%; $p < 0.001$) and MMP-9 (G; +864%; $p < 0.0001$) mRNAs were significantly higher in cancerous tissues (Ca) than in the surrounding normal colorectal tissues (N).

3.4. Correlation between the expression of preproAM or preproAM2/IMD and VEGF-A or MMP-9 in colorectal tumors and surrounding normal tissues

The ratios of expression levels of VEGF-A mRNA in the cancerous and surrounding normal tissues (Ca/N) correlate the Ca/N ratios neither for preproAM mRNA ($r = 0.080$; $p = 0.213$, Fig. 3A) nor for preproAM2/IMD mRNA ($r = 0.042$; $p = 0.403$, Fig. 3B). On the other hand, the Ca/N ratios for MMP-9 mRNA did correlated significantly with those for preproAM mRNA ($r = 0.352$; $p = 0.005$, Fig. 3C), but not for preproAM2/IMD mRNA ($r = 0.041$, $p = 0.406$, Fig. 3D).

3.5. Localization of AM, AM2/IMD and MMP-9 immunoreactivity in colorectal tumors and surrounding normal tissues

In normal colorectal tissue, AM immunoreactivity was detected in endothelium, surface epithelium and some stromal cells (Fig. 4A and C). MMP-9 immunoreactivity was faintly detected in some stromal cells (Fig. 4B and D). In cancer tissue, strong AM immunoreactivity was distributed mainly in the cytoplasm of cancer cells and in some fibroblast-like stromal cells (Fig. 4E). Moreover, MMP-9

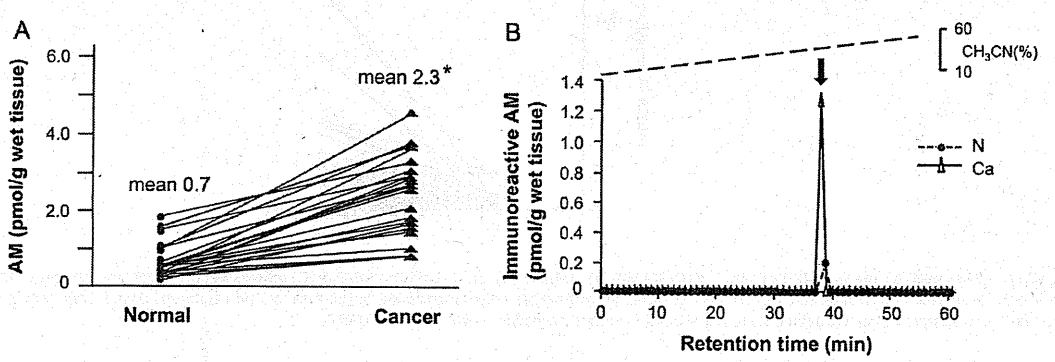

Fig. 1. (A) Concentration of immunoreactive AM in normal and cancerous colorectal tissues from 23 patients. Lines connect symbols indicating the AM concentrations in normal and cancerous tissue from each patient ($n = 23$). The absolute means are also shown in the figure. *$p < 0.001$. (B) RP-HPLC analysis of immunoreactive AM in normal and cancerous tissues from one patient. The arrow indicates the elution position of authentic human $AM_{[1-52]}$. N, Normal; Ca, Cancer tissue.

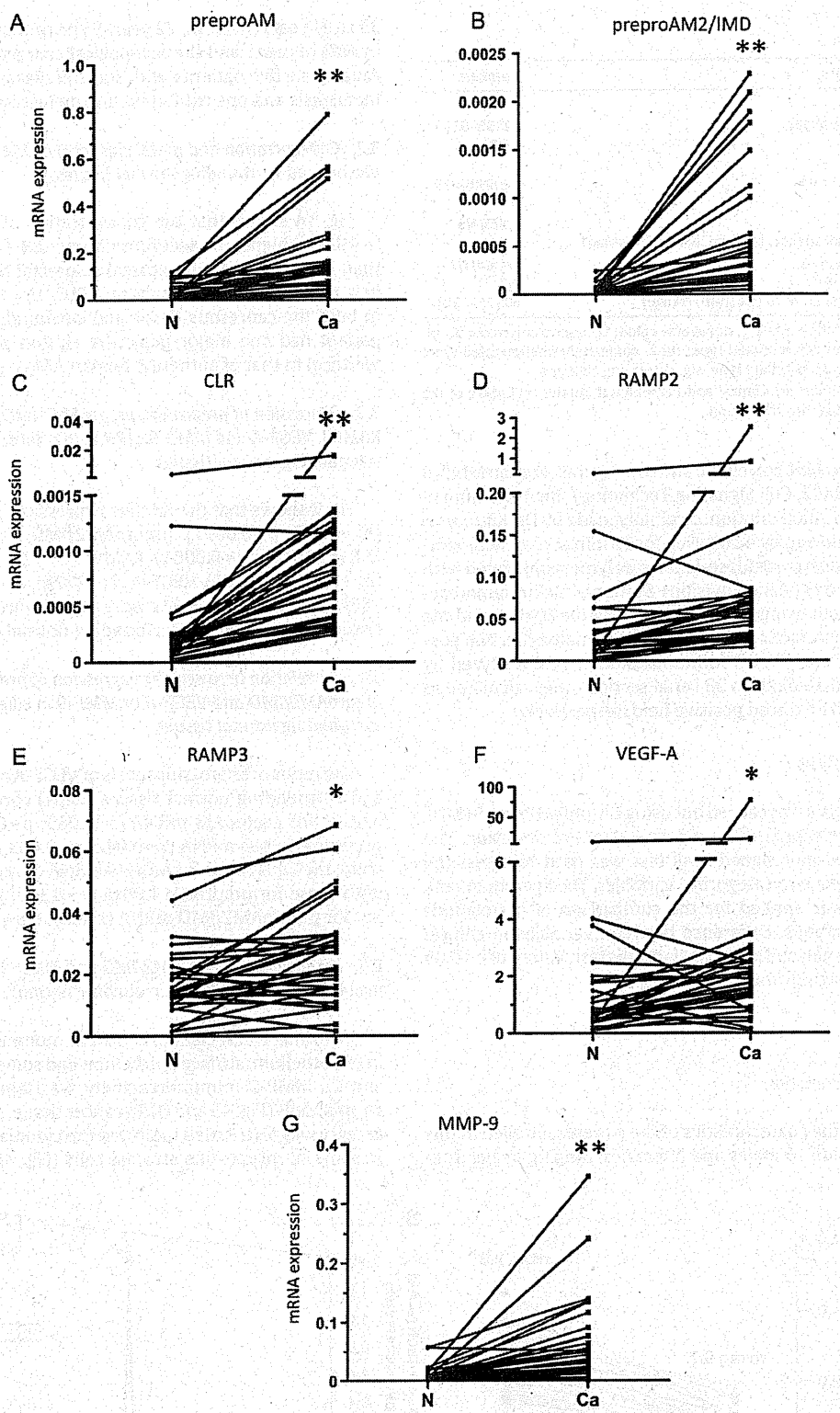

Fig. 2. Expression of preproAM (A), preproAM2/IMD (B), CLR (C), RAMP2 (D), RAMP3 (E), VEGF-A (F) and MMP-9 (G) mRNAs in normal (N) and cancerous tissues (Ca). β-actin served as an internal normalization standard. Lines connect symbols indicating the mRNA expression in the normal and cancerous tissue from each patient ($n = 23$). The *Y*-axes in C, D and F are separated into two segments to make details of the data visible. $^{*}p < 0.001$, $^{**}p < 0.0001$.

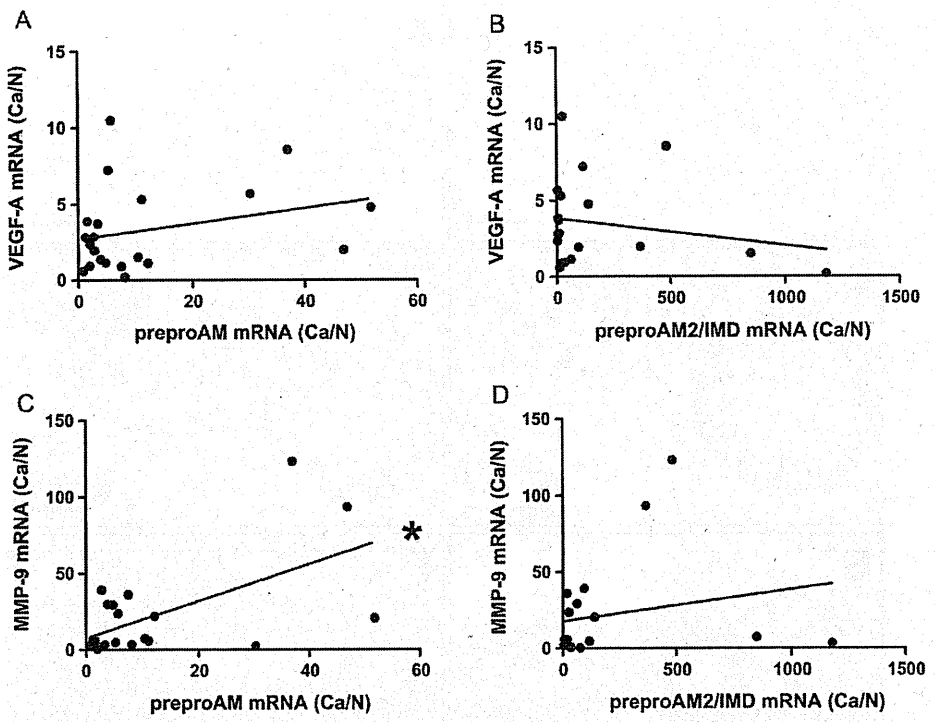

Fig. 3. (A and B) Correlation between the expression ratios of VEGF-A and preproAM mRNAs (A, $r = 0.080$, $p = 0.213$) or preproAM2/IMD mRNAs (B, $r = 0.042$, $p = 0.403$) in the cancerous and surrounding normal colorectal tissues (Ca/N) from each patient. (C and D) Correlation between the expression ratios of MMP-9 and preproAM mRNAs (C, $r = 0.352$, $p = 0.005$) or preproAM2/IMD mRNAs (D, $r = 0.041$, $p = 0.406$) in the cancerous and surrounding normal colorectal tissues (Ca/N) from each patient. $^*p < 0.01$.

immunoreactivity was intensely present in the stroma of cancer tissue (Fig. 4F). MMP-9-positive cells were often found surrounding the AM-positive cells in the serial section, and the positive ratios of AM and MMP-9 in the tissue sections were 14.4% and 11.1% in the fields of cancer tissue specimens, respectively. AM2/IMD immunoreactivity was scattery found in stroma of normal colorectal tissue (Fig. 4G), while it was intensely detected in cancer cells (Fig. 4H).

4. Discussion

AM and its specific receptors, CLR/RAMP2 and CLR/RAMP3, are expressed in various cancer cell lines and in cancers of the pancreas, lung, kidney, breast, ovary and prostate [3,7,27,33]. In addition, AM has been shown to promote tumor growth by stimulating cell proliferation and angiogenesis and inhibiting apoptosis [9,13,18] under hypoxic conditions that lead to AM activation [20]. Conversely, neutralizing AM activity using a specific antibody reduces the growth in glioblastoma cells [31] and pancreatic [18] and colorectal adenocarcinoma cells [19]. However, there have been few studies examining the site-specific expression profiles of the genes encoding AM and its receptors, or the levels of the translated peptides in patients with colorectal cancer. In the present study, we found that colorectal cancers exhibited higher levels of AM synthesis than the surrounding normal tissues.

Most colonic cancer tissue contains substantial amounts of MMP-9 [6] and VEGF [5], co-activation of which is thought to facilitate invasion and metastasis [16]. Consistent with that idea, we found that cancerous colorectal tissue contains significantly higher levels of both MMP-9 and VEGF-A than the surrounding normal tissues. However, the precise mechanism of AM on angiogenesis is unclear; Iimuro et al. [17] reported that AM enhances angiogenesis mediated by VEGF, whereas Fernandes-Sauze et al. [11] found that the proangiogenic action of AM is VEGF-independent. In the present study, expression of preproAM mRNA did not correlate with VEGF mRNA expression. On the other hand, magnitude of MMP-9 mRNA significantly correlated with that of preproAM mRNA. More interestingly, AM appeared to localize specifically in cancer cells, while MMP-9 localized in surrounding stromal cells. AM modulates the production of MMP-2 in rheumatoid synovial fibroblasts [24], aortic adventitial fibroblasts [37], and hepatic stellate cells [38]. On the other hand, amino-acid sequence of AM is reported to be cleaved by MMP-2, but not by MMP-9 [25]. Our finding implicates a cross-talk between cancer cells and the microenvironment during tumor progression through activation of AM and MMP-9. We also found that AM-related peptide, AM2/IMD was highly expressed in colorectal cancer tissue as well. Importantly, the percent increase of AM2/IMD synthesis in cancer tissues to the surrounding normal tissues was greater than AM. The pathological role of AM2/IMD remains to be elucidated in cancer development. AM2/IMD mRNA is up-regulated along with AM under hypoxic conditions [4]. However, we did not find any relationship between preproAM2/IMD mRNA and VEGF-A or MMP-9 synthesis in the present study, implicating a distinct role of these peptides in the tumor progression. Further experiments will be necessary to precisely characterize the pathophysiological roles of AM and AM2/IMD in colorectal cancer development.

Although it has been suggested that plasma AM level appears to be predictive in tumor progression [32], our data are consistent with a report by Buyukberber et al. [3] that AM expression did not associate with any clinical variables, such as histological type ($\chi^2 = 1.77$, $p = 0.78$) or stage ($\chi^2 = 3.17$, $p = 0.53$). We speculate that AM does not directly contribute to tumor progression, but that they more likely have mixed biological effects supporting colon cancer survival in association with matrix degradation.

In summary, this study highlights that expressions of AM and AM2/IMD are increased in human colorectal cancer coinciding with

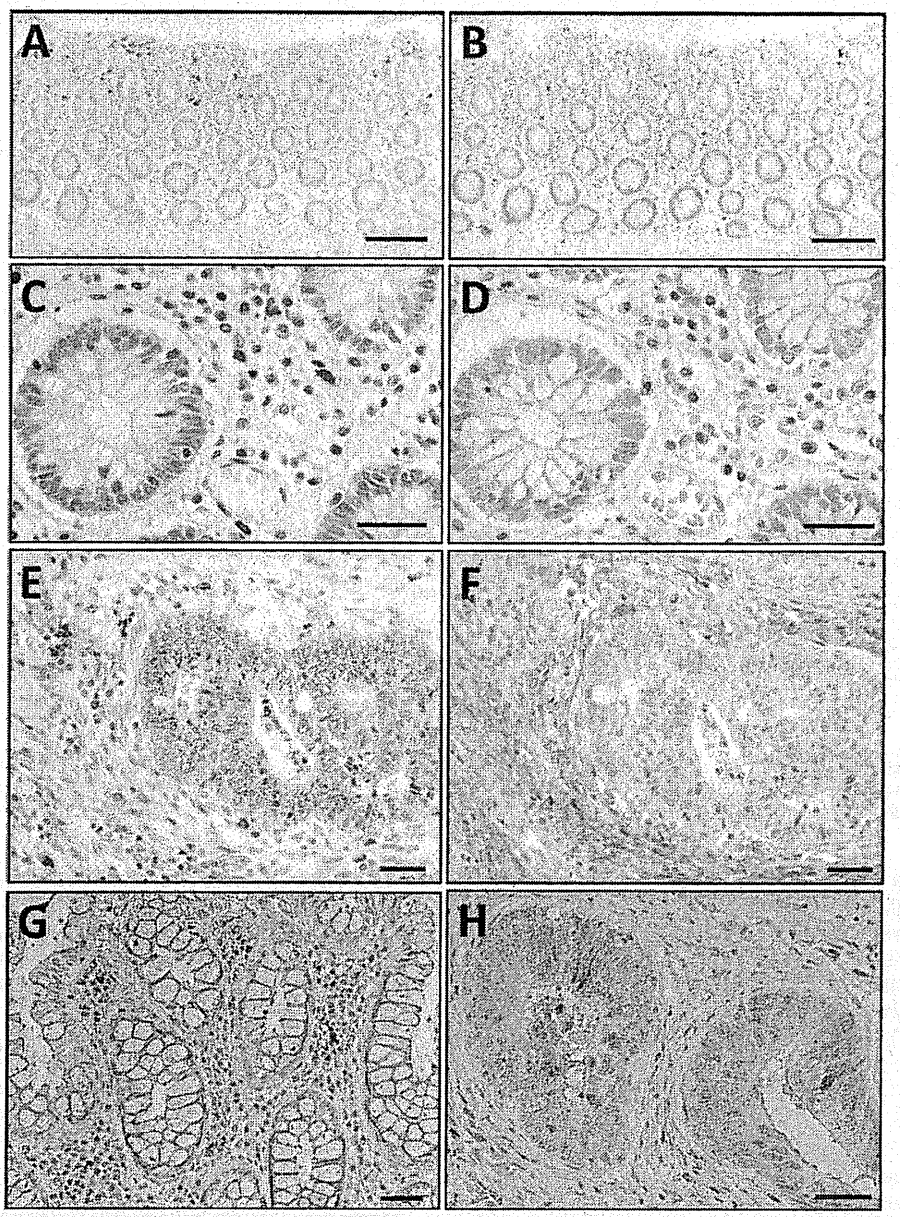

Fig. 4. Representative immunohistochemical staining of AM, MMP-9 and AM2/IMD in sections of human colorectal tissue. (A–D) Immunostaining of AM (A and C) and MMP-9 (B and D) in normal colorectal tissue collected from a patient with colorectal cancer. (E and F) Immunostaining of AM (E) and MMP-9 (F) in moderately differentiated adenocarcinoma tissue. (G and H) Immunostaining of AM2/IMD in normal colorectal tissue (G) and moderately differentiated adenocarcinoma tissue (H). Bars: A, B = 20 μm; C–H = 5 μm.

MMP-9 and VEGF-A in surrounding stroma, suggesting a possible involvement in tumor survival/progression.

Conflicts of interest

None declared.

Acknowledgments

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Structure–function analysis of helix 8 of human calcitonin receptor-like receptor within the adrenomedullin 1 receptor

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ABSTRACT

Adrenomedullin 1 (AM_1) receptor is a heterodimer composed of calcitonin receptor-like receptor (CLR) – a family B G protein-coupled receptor (GPCR) – and receptor activity-modifying protein 2 (RAMP2). Both family A and family B GPCRs possess an eighth helix (helix 8) in the proximal portion of their C-terminal tails; however, little is known about the function of helix 8 in family B GPCRs. We therefore investigated the structure–function relationship of human (h)CLR helix 8, which extends from Glu430 to Trp439, by separately transfecting nine point mutants into HEK-293 cells stably expressing hRAMP2. Glu430, Val431, Arg437 and Trp439 are all conserved among family B GPCRs. Flow cytometric analysis revealed that Arg437Ala or Trp438Ala mutation significantly reduced cell surface expression of the receptor complex, leading to a ~20% reduction in specific ^{125}I-AM binding but little change in their IC_{50} values. Both mutants showed 6–8-fold higher EC_{50} values for AM-induced cAMP production and ~50% reductions in their maximum responses. Glu430Ala mutation also reduced AM signaling by ~45%, but surface expression and ^{125}I-AM binding were nearly the same as with wild-type CLR. Surprisingly, Glu430Ala and Val431Ala mutations significantly enhanced AM-induced internalization of the mutant receptor complexes. Taken together, these findings suggest that within hCLR helix 8, Glu430 is crucial for Gs coupling, and Arg437 and Trp439 are involved in both cell surface expression of the hAM_1 receptor and Gs coupling. Moreover, the Glu430–Val431 sequence may participate in the negative regulation of hAM_1 receptor internalization, which is not dependent on Gs coupling.

1. Introduction

Adrenomedullin (AM) receptors are heterodimers composed of calcitonin receptor-like receptor (CLR) – a family B G protein-coupled receptor (GPCR) – and receptor activity-modifying protein 2 (RAMP2) or RAMP3, both of which are single membrane-spanning domain accessory proteins [19]. CLR/RAMP2 (AM_1 receptor) has proven to be the most highly specific AM receptor and is particularly sensitive to the AM receptor antagonist AM-(22-52) in all species tested [20]. By contrast, CLR/RAMP3 (AM_2 receptor) binds calcitonin gene-related peptide (CGRP) at lower concentrations than it binds AM and is more sensitive to the CGRP receptor antagonist CGRP-(8-37) than is the AM_1 receptor, particularly in rodents [20]. Although intermedin (AM2), like AM, belongs to the calcitonin/CGRP family and is more closely related to AM than to CGRP [23,29], intermedin elicits cAMP production in cells expressing CLR/RAMP3, but not CLR/RAMP2 [23,29].

AM, the endogenous agonist, is a novel vasodilator also shown to be necessary for development of the fetal cardiovascular system and able to powerfully act against various vascular diseases including hypertension, atherosclerosis and secondary lymph edema [7,11,16,18,30]. All of these effects are mediated via the AM_1 receptor [7,11,18,30]; the in vivo function of the AM_2 receptor remains unclear.

When acting as chaperones, RAMP2 and -3 transport CLR molecules from the endoplasmic reticulum to the cell surface, where the CLR/RAMP complex mediates AM-induced intracellular cAMP production and Ca^{2+} mobilization [17]. Upon AM binding, both AM receptors undergo rapid internalization via a clathrin- and β-arrestin-dependent pathway without dissociation of the CLR and RAMP molecules [8,17].

At present the crystal structures of four family A GPCRs are available: rhodopsin, β_1-adrenergic receptor, β_2-adrenergic receptor and adenosine A_{2A} receptor [21,24]. The structural findings revealed the presence of an eighth helix (helix 8), oriented per-

Abbreviations: AM, adrenomedullin; CGRP, calcitonin gene-related peptide; C-tail, cytoplasmic C-terminal tail; CLR, calcitonin receptor-like receptor; GPCR, G protein-coupled receptor; GRK, GPCR kinase; h, human; HEK, human embryonic kidney; Hyg, hygromycin; RAMP, receptor activity-modifying protein; RCP, CGRP-receptor component protein; WT, wild-type.

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pendicular to the seven transmembrane helixes, in the proximal portion of the C-terminal tail (C-tail). To date, helix 8 of family A GPCRs has been shown to play key roles in the cell surface expression of GPCRs, stabilization of GPCRs at the cell-surface, the conformational switch involved in GPCR activation, Gs coupling, Gq coupling and activation of GPCR kinases [4,25,28,31,34]. On the other hand, no crystal structures of family B GPCRs are currently available, and little is known about the function of helix 8 within the receptors. Recently, Conner et al. [2] used mutagenesis and various synthetic peptides to characterize helix 8 in human (h)CLR. They showed that putative hCLR helix 8 extends from Glu430 to Trp439 and contains four residues, Glu430, Val431, Arg437 and Trp439, which are strictly conserved among family B GPCRs. In addition, putative hCLR helix 8 possesses an Arg436–Arg437 sequence, which an earlier study suggests may be part of a consensus motif involved in Gs coupling [32]. But to our knowledge, there is still no detailed information concerning the structure–function relationship of putative hCLR helix 8 complexed within AM receptors. To address that issue, we examined the effects of nine hCLR helix 8 point mutants separately expressed in human embryonic kidney (HEK)-293 cells stably expressing hRAMP2 [13], which enables hCLR to function as a hAM_1 receptor.

2. Materials and methods

2.1. Reagents and antibodies

Human AM was kindly donated by Shionogi & Co. (Osaka, Japan). FITC-conjugated mouse anti-V5 monoclonal antibody (anti-V5-FITC antibody) was from Invitrogen. All other reagents were of analytical grade and obtained from various commercial suppliers.

2.2. Expression constructs

Double V5-tagged hCLR (V5-hCLR) [14] was cloned into pIRES1/Hyg, yielding pIRES1-V5-hCLR [13]. Nine point mutations within putative hCLR helix 8 were introduced using a QuikChange® kit (Stratagene) according to the manufacturer's instructions, with pIRES1-V5-hCLR serving as the template. For each mutation, two complementary 30- to 40-mer oligonucleotides (sense and antisense) were designed with the mutation in the middle. The resultant mutants were all sequenced using an Applied Biosystems 310 Genetic Analyzer.

2.3. Cell culture and DNA transfection

Several hygromycin (Hyg)-resistant HEK 293 cell clones stably expressing hRAMP2 were isolated previously [15]. Among them, the clone that most efficiently promoted AM-mediated V5-hCLR internalization [15] was selected and maintained in DMEM supplemented with 10% FBS, 100 U/ml penicillin G, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B and 100 μg/ml Hyg at 37 °C under a humidified atmosphere of 95% air/5% CO_2.

Transient transfection of HEK-293 cells stably expressing hRAMP2 was performed using LipofectAMINE as previously described [13]. Briefly, the cells were seeded into 12-well plates (for flow cytometric analysis) or 24-well plates (for binding and cAMP assays) and, upon reaching 70–80% confluence, were transfected with empty vector (pIRES1/Hyg) (*Mock*) or V5-tagged wild-type (WT) or mutant constructs; at all times, V5-hCLR was included in each transfection set. This was accomplished by incubating the cells for 4 h in OptiMEM 1 medium containing plasmid DNAs (0.2 μg/well for 24-well plates; 0.4 μg/well for 12-well plates), Plus regent (2 μl/well for 24-well plates; 2.5 μl/well for 12-well plates) and LipofectAMINE (2 μl/well for 24-well plates; 2.5 μl/well for 12-well plates), and all experiments were performed 48 h after transfection. Separate transfections were carried out with different passage number (1–5) hRAMP2-expressing cells; the intra- and inter-assay coefficients of variance were less than ~10%.

2.4. Flow cytometric analysis

Following transfection of the indicated V5-tagged cDNAs into hRAMP2-expressing HEK-293 cells in 12-well plates, the cells were exposed to selected concentrations of hAM in prewarmed serum-free DMEM containing 20 mM Hepes and 0.5% bovine serum albumin for 60 min at 37 °C. Receptor internalization was stopped by adding ice-cold PBS, after which the cells were harvested, resuspended in ice-cold buffer for flow cytometric analysis [17] and labeled with anti-V5 FITC antibody (1:1000 dilution) for 60 min at 4 °C in the dark. Following two successive washes, the cells were subjected to flow cytometry in an EPICS XL flow cytometer (Beckman Coulter), and cell surface expression of each V5-tagged receptor, before and after exposing cells to AM, was analyzed using EXPO 2 software (Beckman Coulter) [17].

2.5. Radioiodination and radioligand binding

^{125}I-hAM (specific activity 5 μCi/pmol) was produced in our laboratory, as previously described [12]. Briefly, hAM (10 μg) in 25 μl 0.4 M sodium acetate buffer (pH 5.6) was introduced into a tube followed by the addition of Na^{125}I (0.5 mCi/5 μl, MP Biomedicals). Lactoperoxidase (Calbiochem: 600 ng/10 μl of 0.1 M sodium acetate, pH 5.6) and H_2O_2 (70 ng/5 μl of water) were then added. After letting the tube stand for 10 min at 33 °C, additional H_2O_2 (100 ng/5 μl of water) was added, and the tube was left for another 10 min at 33 °C. Immediately thereafter, the mixture was submitted to reverse-phase high-performance liquid chromatography, and mono-iodinated hAM was purified and used for the following binding assay.

To assess whole-cell radioligand binding, transfected HEK-293 cells in 24-well plates were washed twice with prewarmed PBS and then incubated with 20 pM ^{125}I-hAM for 5 h at 4 °C in the presence (for nonspecific binding) or absence (for total binding) of 1 μM unlabeled hAM in modified Krebs–Ringers–Hepes medium [17]. After washing the cells twice with ice-cold PBS, they were harvested in 0.5 M NaOH, and the associated cellular radioactivity was measured in a γ-counter. Specific binding was defined as the difference between the total binding (Bo, in the absence of hAM) and nonspecific binding (N, measured in the presence of 1 μM AM). The *Y*-axis for calculating IC_{50} values for hAM in competition with ^{125}I-hAM for binding to transfected HEK-293 cells is $(B-N)/(Bo-N)\times 100$ (%), where B is the ^{125}I-hAM bound.

2.6. Measurement of intracellular cAMP

Transfectants in 24-well plates were incubated for 15 min at 37 °C in Hanks' buffer containing 20 mM Hepes, 0.2% bovine serum albumin, 0.5 mM 3-isobutyl-1-methylxanthine (Sigma) and the indicated concentrations of hAM. The reactions were terminated by addition of lysis buffer (GE Healthcare), after which the cAMP content was determined using a commercial enzyme immunoassay kit according to the manufacturer's instructions (GE Healthcare) for a non-acetylation protocol.

2.7. Data analysis and statistics

Competitive binding data (IC_{50} values) and cAMP concentration–response data (EC_{50} values and maximum responses) were analyzed using a four-parameter logistic equation with MasterPlex ReaderFit software (Hitachi Software Engineering America Ltd., USA). Results are expressed as means ± SEM of

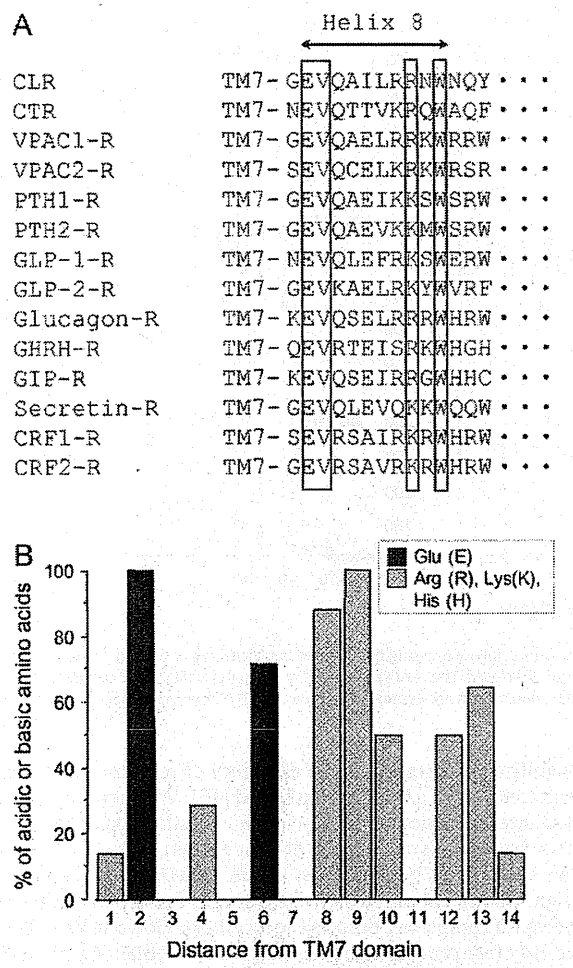

Fig. 1. (A) Alignment of the first 14 residues of the C-terminal tails of 14 family B hGPCRs. Four conserved residues, E (Glu), V (Val), R (Arg) and W (Trp), are *boxed*. CTR, calcitonin receptor; VPAC-R, vasoactive intestinal polypeptide, pituitary adenylyl cyclase activating peptide receptor; PTH, parathyroid hormone; GLP, glucagon-like peptide; GHRH, growth hormone releasing hormone. (B) Distribution of charged amino acid residues within the first residues of 14 family B hGPCR C-tails. Putative helix 8 of family B GPCRs is situated between positions 2 and 11. Acidic residues (E (Glu)) occur most frequently at positions 2 and 6, while dibasic residues (R (Arg) and K (Lys)) occur most frequently at positions 8 and 9.

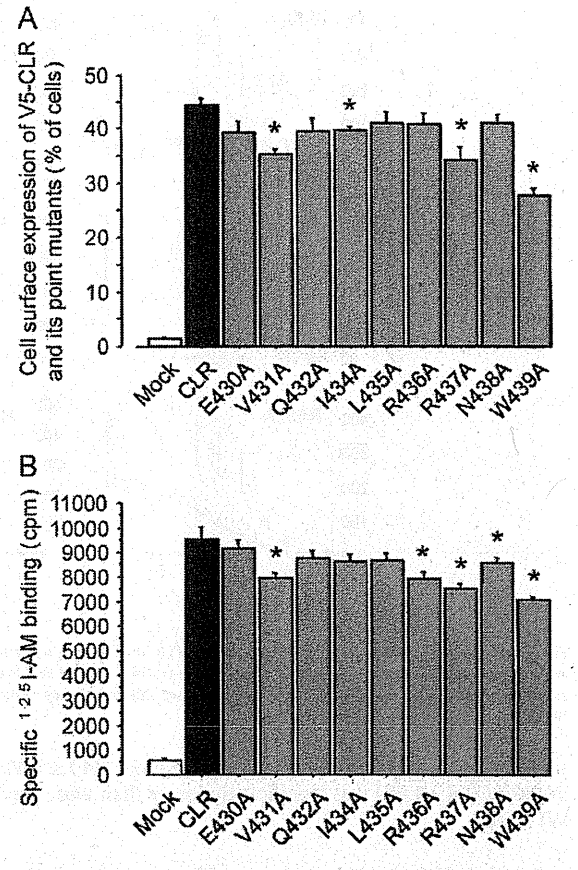

Fig. 2. Characterization of hCLR helix 8 point mutants in HEK-293 cells stably expressing hRAMP2. (A) Flow cytometric analysis of the cell surface expression of the indicated V5-tagged proteins. Transfected cells were incubated for 1 h at 4 °C with anti-V5-FITC antibody; mock incubation with the antibody served as the control. Surface V5-FITC-labeled proteins are expressed as % of cells. Cell surface expression of WT-hCLR and all hCLR helix 8 point mutants was significantly higher than that seen with mock ($p < 0.05$). Bars represent means ± SEM of six independent experiments. *$p < 0.05$ vs. hCLR. (B) Specific binding of ^{125}I-hAM. Transfected cells were incubated for 5 h at 4 °C with ^{125}I-AM (20 pM) in the presence or absence of 1 μM unlabeled hAM. Specific ^{125}I-AM binding to WT-hCLR and all hCLR helix 8 point mutants was significantly higher than that seen with mock ($p < 0.05$). Bars represent means ± SEM of six experiments. *$p < 0.05$ vs. hCLR.

at least five independent experiments; only bars in the cAMP dose–response curves (Fig. 3) are expressed as means ± SD due to limitations of the software. Differences between two groups were evaluated using Student's *t*-tests; differences among multiple groups were evaluated using one-way analysis of variance followed by Scheffe's tests. Values of $p < 0.05$ were considered significant.

3. Results

3.1. Amino acid sequence alignment of putative helix 8 in family B GPCRs

Fig. 1A is based on results from Conner et al. [2] and shows the alignment of the first 14 residues of the C-terminal tails of 14 family B hGPCRs. Putative helix 8 is situated between positions 2–11 [2]. Among the 14 residues shown, the four boxed residues, E (Glu), V (Val), R (Arg) and W (Trp), all of which are within helix 8, are strictly conserved. It is also noteworthy that acidic residues (E (Glu)) occur most frequently at positions 2 and 6, while dibasic residues (R (Arg) and K (Lys)) occur most frequently at positions 8 and 9 (Fig. 1B).

3.2. Cell surface expression of point mutants of putative hCLR helix 8

We recently established a line of HEK-293 cells stably expressing hRAMP2 [13], which, prior to transfection with hCLR, lack AM receptors (Fig. 3). This enabled us to use these cells to examine the role of helix 8 in cellular trafficking and receptor signaling mediated via hCLR within the AM_1 receptor complex (hCLR/RAMP2). We accomplished this by testing the effects of nine helix 8 point mutations on receptor function.

Fig. 2A shows the cell surface expression of V5-tagged WT protein and the nine point mutants following their separate transfection into hRAMP2-expressing HEK-293 cells. When the cells were transfected with empty vector (*Mock*), surface binding of anti-V5-FITC antibody was within the 2% limit of resolution characteristic of flow cytometric analysis. Similar to WT-hCLR (44.2 ± 1.5%), the E430A, Q432A, I434A, L435A, R436A and N438A mutants appeared at the surface of 39–42% of cells. By contrast, cell