流れセンシングにおけるFerの役割を明らかにする過程でこのモデルがさらに進化し，センシングの分子メカニズムを明らかにできることを期待したい．

追記：本研究の大半は増田と大澤正輝博士(現Duke大学)，藤原敬己博士（現Rochester大学）が中心になって行ったものである．本総説の当該部分の内容に関する責任は全て増田にある．

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Abstract – Crucial roles of PECAM-1 in shear stress sensing of vascular endothelial cells. Michitaka MASUDA, Naoko KOGATA, and Naoki MOCHIZUKI (Department of Structural Analysis, National Cardiovascular Center Research Institute, 5 Fujishiro-dai, Suita, Osaka 565-8565, Japan)
Folia Pharmacol. Jpn. (Nippon Yakurigaku Zasshi) **124**, 311～318 (2004)
Fluid shear stress (FSS) induces many forms of responses, including phosphorylation of ERK in endothelial cells (ECs). We have earlier reported that FSS and hyper-osmotic shock rapidly induce tyrosine phosphorylation of PECAM-1 (CD31). The phosphorylated PECAM-1 acts as a plasma membrane anchoring site for SHP2, a protein tyrosine phosphatase involved in the signal transmission from receptor tyrosine kinases to ERK. Osmotic shock also induces transient ERK activation in ECs. The osmotic-shock-induced ERK activation but not p38 MAP kinase activation was dependent on the PECAM-1 engagement and was blocked by its downregulation. When magnetic beads coated with antibodies against the extracellular domain of PECAM-1 were attached to ECs and tugged by magnetic force, PECAM-1 associated with the beads was tyrosine phosphorylated. ERK was also phosphorylated in these cells. Binding of the beads by itself or pulling on the cell surface using poly-L-lysine coated beads did not induce phosphorylation of PECAM-1 and ERK. These results suggest that PECAM-1 is a mechanotransduction molecule.
Keywords: vascular endothelial cells; fluid shear stress; PECAM-1; tyrosine phosphorylation; ERK

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◇研究テーマ：血管細胞生物学　接着分子，細胞骨格分子などの分子・細胞生物学研究から内皮細胞の流れ刺激受容の分子メカニズムを解明したい．
◇趣味：釣り，読書．

連載講座　DDS研究と癌治療

遺伝子と細胞のハイブリッド化による血管新生の制御と微小血管造影法による新生血管の可視化

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要旨　VEGFを治療薬として用いる血管新生療法と，一方で抗VEGF治療を行う癌治療はmirror imageの関係にあり，両領域の研究は相互に血管新生の機序解明に貢献している。

われわれの施設では，血管新生治療において血管増殖遺伝子を生分解性ゼラチンに封入し，貪食細胞（血管内皮前駆細胞など）に取り込ませるという新たな細胞・遺伝子ハイブリッド治療を開発した。これにより安全かつ高効率の遺伝子導入が可能となり，臨床応用への大きな期待が寄せられている。

固形腫瘍の自身への未熟な新生血管の誘導は一連のカスケードを形成しており，その各段階の因子に対するtarget-based therapyが臨床治験段階にあるが，その治療成績はまだ十分とはいえない。今後，組織局所での薬剤濃度上昇，つまりtissue targetingの要素や他の新たな阻害因子との協調的な抗腫瘍効果が必要となると考えられる。癌治療へのゼラチンを用いた遺伝子療法が，血管新生抑制治療へ応用可能であるかもしれない。また，新生血管に対する治療効果の評価法として，単色X線光を用いた微小血管造影法についても概説する。

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Control of Neovascularization by Cell-Gene Hybrid Therapy and Visualization of Angiogenic Vessels by Micro-Angiographic System

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Summary

VEGF is used as a therapeutic agent in regenerative therapy, while antibodies to VEGF are used as non-toxic anticancer agents in oncology. As this is a mirror-image relationship, academic achievements in both fields have mutually contributed to elucidating the mechanism of neovascularization. In regenerative therapy for cardiovascular disorders, we developed a novel cell-gene hybrid therapy. Functional gene embedded into biodegradable gelatin (gelatin-DNA complex) is induced into functional cells (EPCs, etc.) by phagocytosing action. This method achieves highly effective gene induction and expression non-virally. Moreover, intravenously injected functional cells not only serve as a protein-producing factory but also as a vector to the target tissue.

Tumor growth is dependent on new feeding vessels from preexisting vasculature, as postulated by Folkman in 1971. Progression of tumor-induced neovascularization consists of several sequential phases, in which there are some key molecules such as VEGF or MMPs to be targeted for anti-angiogenic therapy. However, results of numerical clinical research using antibody against VEGF or inhibitor of VEGFR are still unsatisfactory. We assume that the low density of anti-angiogenic agents in the target tissue is one of the limitations and that our new method (tissue targeted cell-gene hybrid therapy) could be also applied for anti-angiogenic therapy. Finally, we introduce novel synchrotron microangiography system, which

demonstrates vessels with a diameter of 50 to 500 μm and enables us to evaluate the effect of anti-angiogenic therapy.

Key words: VEGF, Cell-gene hybrid therapy, Angiogenesis, Cancer, Micro-angiography
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はじめに

1970年代初めにFolkmanらにより，腫瘍細胞の発育のためには栄養，酸素を供給する新生血管の誘導が必要であるという先駆的な仮説が提唱された[1]。以来分子生物学の発展に伴い，血管の初期発生（vasculogenesis），それに続く血管形成（angiogenesis）にかかわる血管内皮細胞増殖因子（vascular endothelial growth factor, VEGF）を代表とした様々な血管増殖因子，またはアンギオスタチンやエンドスタチンなどの内因性血管新生抑制因子などが発見された。最近の血管内皮細胞に関する研究の進歩に伴い，これら血管制御因子の複雑なcross talkが明らかになりつつある。固形腫瘍は急速な成長に見合う栄養，酸素供給を受けるために，VEGFの他にもmatrix metalloprotease（MMPs），thymidine phosphorylase（TP），hepatocyte growth factor（HGF）などの血管増殖にかかわる因子を産生し，にわか作りの未熟な血管を自己に誘導する。新生血管は平滑筋層，時には基底膜や周皮細胞の構造も不完全で，容易に腫瘍細胞の落剝を招き遠隔転移の原因ともなるといわれている。腫瘍による新生血管の誘導は一連のカスケードを形成しており，その各段階を制御する因子を治療標的として探索がなされ，新たな阻害剤開発が積極的に進められている。血管阻害治療薬は単独では抗癌作用を期待しにくいということがいわれてきたが，最近転移のない進行直腸癌の患者に対して，neoadjuvant chemotherapyに先んじて低用量の抗VEGF抗体（bevacizumab, Genentech社）を単独投与したところ，微小腫瘍血管数の減少および腫瘍血管のnormalizationを認めたという臨床報告もなされた[2]。

一方循環器領域では，1994年に米国タフツ大学で世界初の血管新生遺伝子治療が虚血肢へのVEGF遺伝子投与という形で行われた[3]。現在は重症虚血性心疾患，難治性閉塞性動脈硬化症などの観血的治療法で十分な効果が得られない症例に対して，遺伝子，細胞を用いた血管新生療法が試みられ良好な成績が報告されている。

上述のごとく循環器疾患に対する血管新生療法は，悪性腫瘍に対する抗腫瘍治療のmirror imageの関係といえる。本稿では，われわれが循環器疾患に対する血管新生療法に対して研究してきたことをまず概説し，癌治療への応用の可能性として愚見を述べたい。

I. ゼラチンによる遺伝子と細胞のハイブリッド化

循環器疾患に対する血管新生療法は当初，血管増殖因子の遺伝子を単独で用いる治療法が行われていたが，VEGFの過剰発現により腫瘍形成や血管透過性の高い異常新生血管の発育が報告された。そこで，機能的な血管系の再構築，つまり正常な血管新生と成熟した血管の発育には細胞の型と分子のバランスが必要であるというwell-tempered vesselの概念が提唱された[4]。そのためには相互に調節しあった血管新生因子の産生が必要である。われわれは，基地（base）となる機能細胞に補完的機能をもつ遺伝子を導入するという細胞遺伝子ハイブリッド治療を考案した。まず遺伝子を格子構造を有する生分解性ゼラチンに取り込ませ，このゼラチン-遺伝子複合体を貪食能を有する血管内皮前駆細胞（endothelial progenitor cells, EPCs）に導入させる方法である（図1）。

本治療法は，以下の点で従来のgene therapyよりも優れている。

①細胞がbaseとしてだけでなく，治療要素としての働きをもつ。

②EPCsなどは血管内投与が可能である。

③*ex vivo*でウイルスベクターを用いることなく高効率の遺伝子導入効果がある。

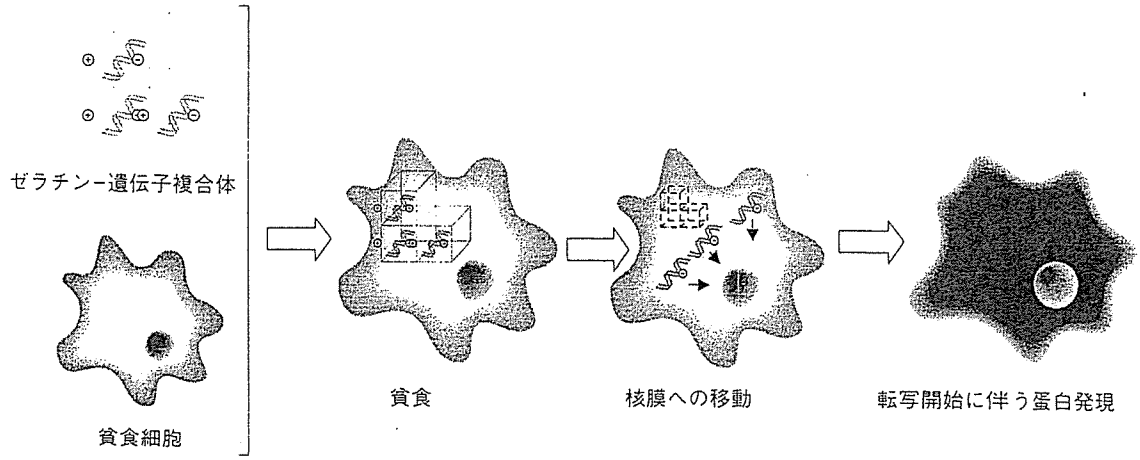

図1　貪食細胞によるゼラチン-遺伝子複合体の取り込み
貪食能をもつ細胞にゼラチン-遺伝子複合体を取り込ませた後，細胞を注入する。ゼラチンは分解され封入されていた遺伝子が核膜へ移動し，蛋白発現に向けたプロセスが開始される。

このハイブリッド治療を実現するkeyとなる物質がゼラチンである。このゼラチンの特徴として，

①陽性に帯電しているので，陰性に帯電している種々の物質（核酸や蛋白質）をイオン結合することができる。

②構造が三次元格子状なので結合物質をゲル内部に保護することにより，分解酵素の影響を受けにくくする。

③ゼラチンであるため生体内で徐々に分解を受けて，この分解に伴い結合物質を放出する。

④その分解速度は架橋度を変えることにより自由に調節できる。

⑤ゼラチン-遺伝子複合体は貪食細胞（EPCs，単球，マクロファージなど）に容易に貪食される。

⑥貪食細胞内で高率に遺伝子を発現する。

などがあげられる。われわれはゼラチンを，その構造や表面電化を変えることが容易である性質から，遺伝子の担体として利用することを着想した。遺伝子をあらかじめゼラチンの格子構造内へ封入して生体内へ投与することで核酸分解酵素による遺伝子の代謝が緩徐となり，結果として安全かつ高効率に遺伝子を導入することができると考えられる。実際に遺伝子をゼラチンと結合させて投与したところ，生体内における遺伝子の残存期間を飛躍的に延長させることに成功し（図2），遺伝子の発現率も従来の遺伝子単独投与と比較して約10倍の増加が認められた[5]。

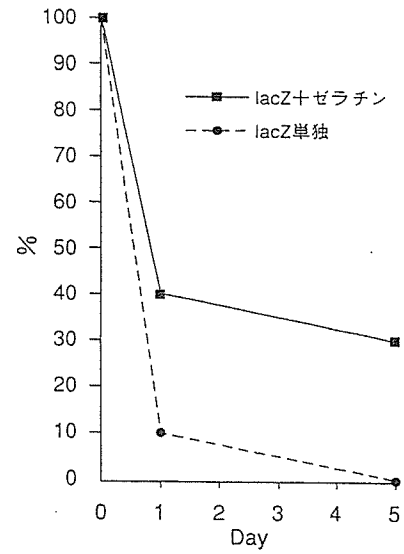

図2　生体内におけるlacZ遺伝子残存率
lacZ遺伝子をゼラチンへ結合させると，遺伝子単独の場合と比して長期にわたって残存する。

II.　肺高血圧症に対するEPCs-アドレノメデュリン遺伝子ハイブリッド治療

原発性肺高血圧症では，肺血管内皮の機能障害とそれに基づく血管作動性物質の不均衡が病態の主因と考えられるので，細胞遺伝子ハイブリッド治療に適していると考えられた。EPCsは生体内で虚血や血管内皮障害が起こった時に骨髄から動

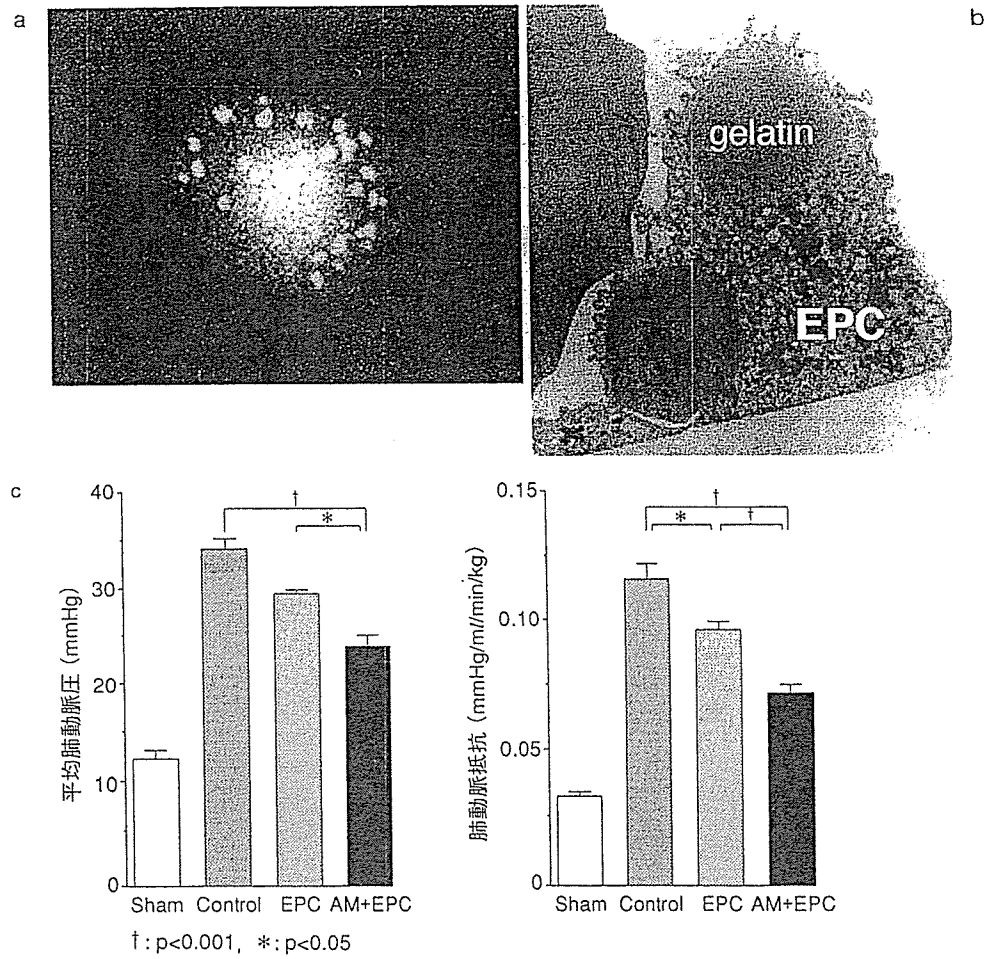

図 3 a：ゼラチンに結合した DNA（RITC-labeled）
b：ゼラチン-遺伝子複合体を貪食した EPCs
c：肺高血圧症モデルにおけるゼラチンを用いたハイブリッド治療の効果

員され，障害部位に遊走，付着し血管内皮細胞に分化して血管を形成する。また，VEGF などの血管新生因子を放出して局所の血管新生を促し，さらにマクロファージのような貪食能をも有する。

これを利用して，血管内皮細胞より産生される生体内で最も強力な血管拡張ペプチドである adrenomedullin（AM）の遺伝子をゼラチンに封入して *ex vivo* にて EPCs に取り込ませた（図 3 a, b）。この EPCs の貪食による *ex vivo* の遺伝子導入は，ウイルスベクターを用いずに EPCs 自身への 50～70％という高効率の遺伝子導入を可能としている。

使用するゼラチンは豚の皮膚から抽出し，グルタルアルデヒドの架橋反応により格子構造とし，エチレンジアミンを加えることで正帯電ゼラチンが完成する。この正帯電ゼラチンは蛋白のみでなく，負に帯電した DNA と数時間接触することにより，容易に電気的複合体を形成する。このゼラチンは生体内で徐々に吸収されるため，ゼラチンに結合した蛋白や DNA の徐放，つまり長時間の発現が可能である。

肺高血圧ラットに AM 遺伝子を導入した EPCs を経静脈投与したところ，コントロール群に比べて平均肺動脈圧を有意に低下させ，生存率を改善させた。また，これらの効果は EPCs の単独投与よりも勝っていた（図 3c）[6]。

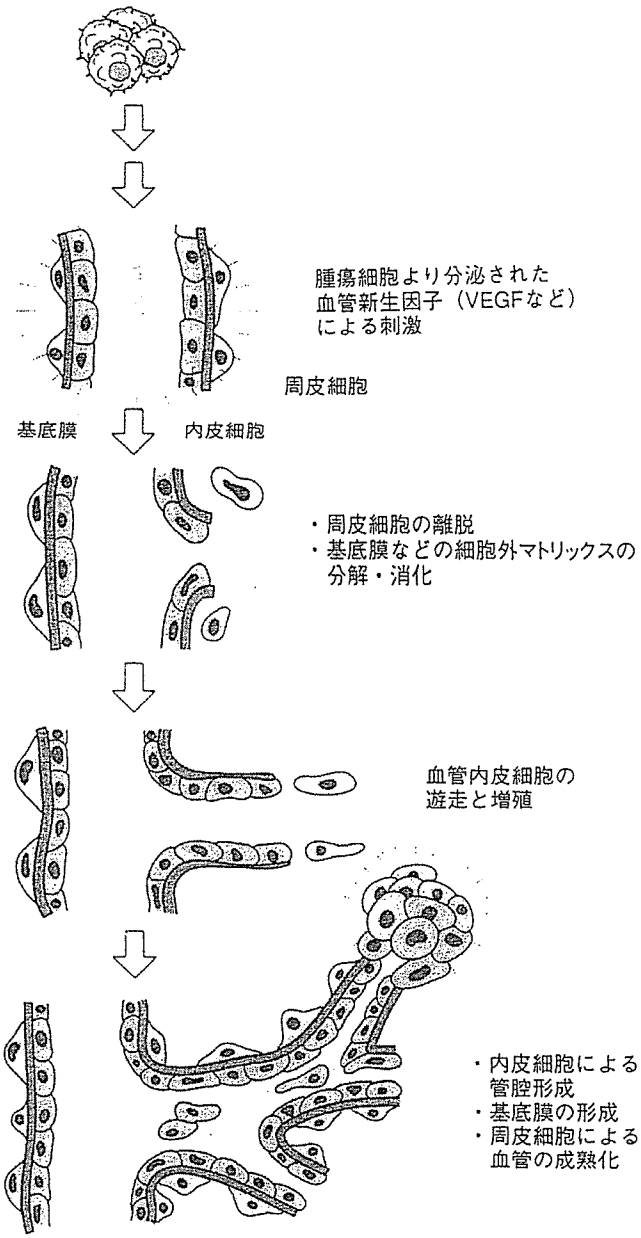

図 4　腫瘍細胞への新生血管誘導のカスケード概念図（文献[14]一部改変）

III.　細胞-遺伝子ハイブリッド治療の癌治療への応用

腫瘍血管新生は，主に腫瘍自身から分泌される血管新生因子による血管内皮細胞の活性亢進に始まる。活性化血管内皮細胞はプロテアーゼを産生し，基底膜をはじめとする細胞外マトリックスを破壊，それに続き遊走，増殖に伴い管腔形成をなす（図4）。これが腫瘍に到達することで新たな栄養，酸素供給源となって腫瘍が増大していくことが明らかにされている[7]。このいずれかの段階を阻害すれば腫瘍の退縮効果があると考えられているため，現在すでに様々な血管新生阻害剤が臨床治験段階にあり，その代表的なものが抗 VEGF

治療である。

1. 抗 VEGF 治療

VEGF は循環血液中の骨髄由来 EPCs の腫瘍局所への誘導，分化に重要であり，angiogenesis だけでなく vasculogenesis にも深く関与し，乳癌，大腸癌などの多くの固形腫瘍で産生が報告されている。腫瘍細胞が VEGF を発現した場合，発生臓器からの血管新生だけでなく，循環血液中からも血管内皮を動員できることを意味する。また，抗腫瘍免疫細胞である type 1 ヘルパー T 細胞や樹状細胞の免疫応答を制限する作用もあり，腫瘍における高 VEGF 発現は予後不良因子となるため，抗 VEGF 治療が抗腫瘍治療の一つの柱となる可能性が高い[8]。抗 VEGF 治療には VEGF の中和抗体（RhnMab など）と，VEGF 受容体アンタゴニスト（SU5416 など）の 2 種類がありいずれも臨床治験中であるが，現時点では cytotoxic な化学療法剤との併用がほとんどである。

福山らは腫瘍への targeting を実現するハイブリッド治療法としてマクロファージに遺伝子を導入する方法を提案している。つまり腫瘍に対する tissue-targeting 能と，貪食能をもつマクロファージに抗 VEGF 抗体遺伝子を導入し，血管内に投与することで高い腫瘍抑制効果を得るというものである[9]。血管内投与という一般的な投与法で局所効果が期待できる。

また，dormancy therapy の一種である腫瘍の血管新生阻害治療は腫瘍の退縮効果発現が緩徐である。有効性の判定には CT や PET が用いられているが，安価で再現性が高く，かつ厳密な定量性をもつ解析方法の開発が待たれている。最後に紹介する新世代の血管造影法はその評価法の一つとなり得るかもしれない。

2. 腫瘍免疫療法—樹状細胞への応用

腫瘍に Fas ligand を発現させ，樹状細胞と腫瘍細胞を共培養すると腫瘍・樹状細胞クラスターが形成される。この樹状細胞を分離した後，再度同じ腫瘍を混和して同系マウスに接種すると抗腫瘍効果が惹起される[10]。腫瘍に特異的な抗原ペプチドを利用して腫瘍ワクチンとして用いる腫瘍免疫療法では，強力な抗原提示能かつ抗腫瘍サイトカイン産生能をもつ樹状細胞が中心的な役割を果たしているといわれる。この抗原提示，活性化，サイトカイン分泌の過程を遺伝子導入にて協調的になすことが効果的な抗腫瘍反応を誘導し得ると考えられている。

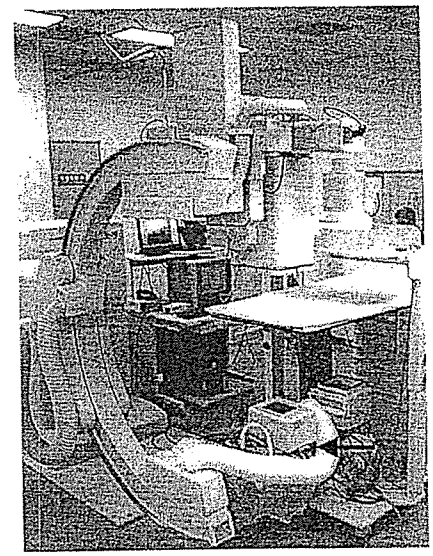

図 5 微小血管造影システム
C アーム下端の X 線管は，現在臨床で使用されている CT 用の高出力線源である（黒矢印）。上端には蛍光板を有した検出器とカメラを搭載している(白矢印)。

最近では骨髄より採取した樹状細胞と腫瘍細胞を融合させ，持続的な抗原提示能と腫瘍抗原自身の発現に伴う細胞傷害性 T リンパ球の活性化により，強力な腫瘍拒絶を誘導する手法や樹状細胞を遺伝子導入にて操作し，特定の機能を修飾，強化する研究も試みられている。貪食，遊走，分泌，抗原提示という多機能細胞である樹状細胞は，細胞，遺伝子ハイブリッド治療の cell-base として，非常に魅力的なものと思われる。樹状細胞への高効率の遺伝子導入の目的にて，ゼラチンを用いた遺伝子導入が応用可能であるかもしれない。

IV. 微小血管造影法による新生血管の可視化

再生医療や癌治療において新生血管の挙動を解明し，臨床的効果を評価するために微小血管の可視化は不可欠であるが，既存の血管造影法では 200 μm 以下の微小血管の映像化は困難である。近年シンクロトン放射光施設から得られる単色 X 線を利用した微小血管造影法による再生血管の定量的かつ形態的評価が試みられている[11]。国立循

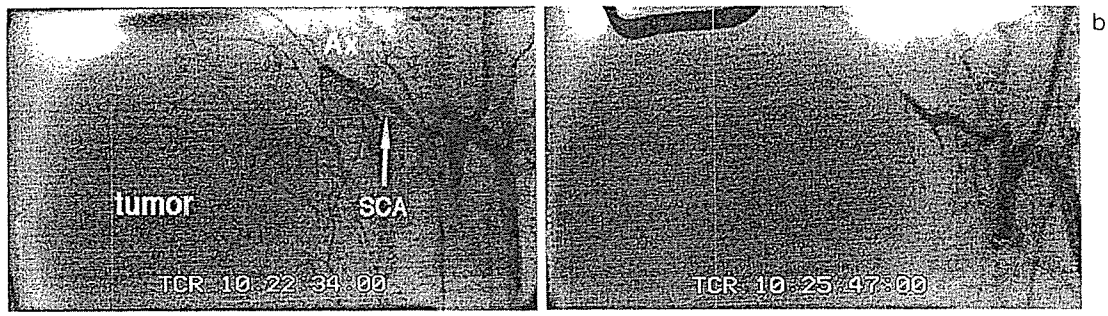

図6　癌移植マウスの微小血管造影像（移植後4週間目，腫瘍の直径は約15 mm）
a：未治療群。
b：抗VEGF抗体投与群。

環器病センターを中心とする研究グループは従来の巨大なX線発生装置であるシンクロトン放射光施設を使用せずに，通常規模の病院に設置可能な微小血管造影装置の開発に世界で初めて成功した（図5）[12]。

50～100 μm程度の微小血管を造影検査で評価するためには，微量の造影剤を検出できる装置が必要となり，その要素としては高輝度で単色性と平行性に富んだX線源をもち，高感度，高解像度の検出構造が必要である。新エネルギー・産業技術総合開発機構（NEDO）の支援，浜松ホトニクス株式会社およびNHKエンジニアリングサービスの協力を得て本装置は共同開発された。2004年3月に国立循環器病センターに移設され，4月から血管再生治療の評価手法として臨床応用が開始されている。

X線源として既存の大容量出力をもつCT用X線管を改変して用い，ランタノイド系の金属を複合した特殊フィルターにて疑似単色X線を得た。X線の平行化には多数の微細孔を有するキャピラリープレートを用い，検出装置にはCCDの100倍の超高感度をもつアバランシェ型ハイビジョンモノクロ新Super-Harpカメラを使用することで約50 μmの高解像度が得られる。

マウスの腋窩に大腸癌を移植し，抗VEGF抗体を用いて治療した群とそうでない群に4週間にわたって複数回の微小血管造影検査を施行，新生血管の可視定量化を試みる実験を行った。control群では週数を経るごとに，腋窩および乳腺動脈より誘導される新生血管の増大を認め，治療群では4週目に新生血管網の形成が阻害されている様子が鮮明に観察された（図6a, b）。なお，微小血管造影法では腫瘍へのprimary branchから四分枝レベルまでの血管の性状観察が可能であったが，従来の血管造影装置ではいずれの新生血管も可視化することは不可能であった[13]。

微小血管造影法は病理学的診断法などとは異なり，治療前後で血管の変化を検討することができる利点がある。動脈硬化，糖尿病における微小血管疾患治療のみでなく，病的血管新生の制御が極めて重要な役割をもつ癌治療の評価や早期診断に有用である可能性がある。

おわりに

われわれが考案したゼラチンを用いた遺伝子導入法は安全性，遺伝子の徐放化による持続発現の点で従来法よりも優れている。貪食能をもつ細胞であれば基本的に適応可能であると考えられ，血管制御の臨床応用に大きな期待が寄せられている。また，今後はこれに加えて標的組織での効率のよい機能発現，つまり遊走能をもつ細胞によるtissue-targetingの要素も重要になってくると思われる。当施設での最近の研究結果とともに，癌治療への応用の可能性について述べてみた。また，最新の新生血管の評価法として，微小血管造影法についても紹介させていただいた。

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Inhibition of cholinesterase elicits muscarinic receptor-mediated synaptic transmission in the rat adrenal medulla

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Abstract

To determine the role of acetylcholinesterase in cholinergic synaptic transmission in the adrenal medulla in vivo, we applied a dialysis technique to the adrenal medulla of anesthetized rats and examined the effect of acetylcholinesterase inhibitor on the contribution of nicotinic and muscarinic receptors to catecholamine release. Exogenous acetylcholine-induced epinephrine release was inhibited by atropine (a muscarinic receptor antagonist) as well as hexamethonium (a nicotinic receptor antagonist). Endogenous acetylcholine (nerve stimulation)-induced epinephrine release was inhibited by hexamethonium but not atropine. In the presence of neostigmine (an acetylcholinesterase inhibitor), both exogenous and endogenous acetylcholine-induced catecholamine release was enhanced. In either case, epinephrine release was inhibited by atropine as well as hexamethonium. In the presence of eserine (another acetylcholinesterase inhibitor), endogenous acetylcholine-induced epinephrine release was also inhibited by atropine. Exogenous or endogenous acetylcholine-induced norepinephrine release was primarily inhibited by hexamethonium regardless of whether neostigmine was absent or present. In the rat adrenal medulla, the inhibition of acetylcholinesterase not only enhanced cholinergic synaptic transmission but also elicited muscarinic receptor-mediated synaptic transmission for epinephrine release.

Keywords: Microdialysis; Acetylcholine; Norepinephrine; Epinephrine; Nicotinic receptors

1. Introduction

In vivo catecholamine release from adrenal medulla is controlled by the central nervous system through the sympathetic pathway via splanchnic nerves, which make synaptic contacts with chromaffin cells (Coupland, 1965). It has been reported that catecholamine release induced by splanchnic nerve stimulation is predominantly inhibited by nicotinic receptor antagonists and is resistant to muscarinic receptor antagonists in dogs (Kennedy et al., 1991; Kimura et al., 1992), cats (Alamo et al., 1991) and rats (Wakade and Wakade, 1983). Acetylcholine released from splanchnic nerve endings evokes catecholamine release mainly by activation of nicotinic receptors on the surface of chromaffin cells. On the other hand, both nicotinic and muscarinic receptors are present on the surface of chromaffin cells of various species because both receptor agonists evoke a substantial catecholamine release (Tsujimoto and Nishikawa, 1975; Kirpekar et al., 1982; Role and Perlman, 1983; Ballesta et al., 1989; Chen and Dixon, 1990; Chritton et al., 1991; Zhou et al., 1991). These findings have suggested that nicotinic receptors are primarily concentrated in the synaptic regions of chromaffin cells and muscarinic receptors localize on the extra-synaptic regions of chromaffin cells (Wakade and Wakade, 1983; Kimura et al., 1992). This anatomical distribution of receptors may explain the predominance of nicotinic receptors in physiological synaptic transmission. However, here, questions arise. Can acetylcholine go in and out at synaptic connections? What conditions are relevant to the extra-synaptic muscarinic receptor-mediated transmission in the in vivo adrenal medulla?

The action of released neurotransmitters is generally terminated by reuptake mechanisms through neuronal transporters or by enzymatic destruction. Released acetylcholine is degraded to choline and acetate by acetylcholinesterase, and choline but not acetylcholine is carried into the nerve

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endings through neuronal transporters. Thus, acetylcholinesterase plays a critical role in terminating the action of released acetylcholine. Under physiological conditions, there is enough acetylcholinesterase activity in splanchnic nerve endings, chromaffin cells and interstitial cells (Coupland, 1965; Palkama, 1967; Lewis and Shute, 1969; Somogyi et al., 1975). Acetylcholine released from splanchnic nerve endings may be rapidly degraded before reaching the extra-synaptic muscarinic receptors. It has been, however, reported that exposure to chronic or acute stresses reduces acetylcholinesterase activity in the adrenal gland (Gabriel and Soliman, 1983). If acetylcholinesterase activity is reduced in the adrenal medulla, released acetylcholine may not only accumulate in synaptic regions of chromaffin cells but also spill over to the extra-synaptic regions. Then, we considered that acetylcholinesterase plays an important role in determining cholinergic receptors involved in the in vivo synaptic transmission.

In the present study, we applied the microdialysis technique to the adrenal medulla of anesthetized rats and tried to examine the role of acetylcholinesterase in the in vivo cholinergic synaptic transmission. First, we investigated the contribution of nicotinic and muscarinic receptors to exogenous or endogenous acetylcholine-induced catecholamine release. Second, we investigated the influence of acetylcholinesterase inhibitor on their contributions to exogenous or endogenous acetylcholine-induced catecholamine release.

2. Materials and methods

2.1. Animal preparation

The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Adult male Wistar rats weighing 340–480 g were anesthetized with pentobarbital sodium (50–55 $mg \cdot kg^{-1}$ i.p.). The level of anesthesia was maintained with a continuous intravenous infusion of pentobarbital sodium (15–25 $mg \cdot kg^{-1} \cdot h^{-1}$ i.v.). A cervical midline incision was made to expose the trachea, which was then cannulated. The rats were ventilated with a constant-volume respirator using room air mixed with oxygen. A thermostatic heating pad was used to keep the esophageal temperature within a range of 37–38 °C. Heart rate, arterial pressure and electrocardiogram were monitored and recorded continuously. With the animal in the lateral position, the left adrenal gland and left splanchnic nerve were exposed by a flank incision, and the left splanchnic nerve was transected in all protocols. In protocols requiring nerve stimulation, shielded bipolar stainless steel electrodes were applied to the distal end of the nerve, which was then stimulated with a digital stimulator (SEN-7203, Nihon Kohden, Japan) with a rectangular pulse (10 V and 1 ms in duration).

2.2. Dialysis probe construction

The materials of the dialysis probe were the same as those used in our previous cardiac dialysis experiments (Akiyama and Yamazaki, 2001). Briefly, each end of the dialysis fiber (0.31 mm OD and 0.20 mm ID; PAN-1200 50,000 MW cutoff, Asahi Chemical, Japan) was inserted into the polyethylene tube (25-cm length, 0.5 mm OD and 0.2 mm ID; SP-8) and glued. The length of the dialysis fiber exposed was 3 mm. When a dialysis probe was bathed in 38 °C Ringer's solution containing norepinephrine (100 $ng \cdot ml^{-1}$) and epinephrine (100 $ng \cdot ml^{-1}$) and perfused with Ringer's solution at a speed of 10 $\mu l \cdot min^{-1}$, in vitro recovery rates of norepinephrine and epinephrine were $1.7 \pm 0.2\%$ and $1.9 \pm 0.3\%$, respectively (number of dialysis probes = 3).

2.3. In vivo dialysis technique

The left adrenal gland was gently lifted, and the dialysis probe was implanted in the medulla of the left adrenal gland along the long axis by using a fine guiding needle (10 mm length, 0.51 mm OD and 0.25 mm ID). The dialysis probe was perfused with Ringer's solution or Ringer's solution containing pharmacological agents at a speed of 10 $\mu l \cdot min^{-1}$ using a microinjection pump (CMA/100, Carnegie Medicin, Sweden). Ringer's solution consisted of (in mM) 147.0 NaCl, 4.0 KCl and 2.25 $CaCl_2$. All pharmacological agents tested were locally administered by perfusion through the dialysis probe after being dissolved in Ringer's solution. The concentrations of pharmacological agents were determined based on their concentrations used in earlier in vitro studies and the in vitro recovery rate of the dialysis probe. One sampling period was 1 min (1 sample volume = 10 μl). Each sample was collected in a microtube containing 2 μl of 0.1N hydrochloric acid to prevent the oxidation of amine. In this study, catecholamine release was evoked by 1-min local administration of cholinergic receptor agonists or 1-min electrical stimulation of left splanchnic nerves. We continuously collected five dialysate samples per pharmacological or electrical stimulation: one before, one during and three after stimulation.

We measured the dead space volume for local administration or dialysate sampling. Taking this space into account, we locally administered pharmacological agents and sampled dialysate. Dialysate norepinephrine and epinephrine concentrations were high immediately after probe implantation, but decreased rapidly and stabilized within 3–4 h. After basal dialysate norepinephrine and epinephrine concentrations reached steady levels, we started the protocols.

Using high-performance liquid chromatography with electrochemical detection, dialysate norepinephrine and epinephrine concentrations were measured as indices of norepinephrine and epinephrine release. The dialysate sample from the adrenal medulla was injected directly into the high-performance liquid chromatography using an autosampler

(CMA/200, 240, Carnegie Medicin). Details of the high-performance liquid chromatography system have been previously described (Yamazaki et al., 1995).

2.4. Preliminary experiments

Preliminarily, to ensure that catecholamine release was reproducible on repetition of the same stimulation, we examined dialysate catecholamine levels in response to the repeated pharmacological or electrical stimulation: 1-min local administration of acetylcholine (1 mM) ($n=2$) or 1-min electrical stimulation of left splanchnic nerves (4 Hz) ($n=2$). Both stimulations were repeated six times at 30-min intervals. Substantial amounts of norepinephrine and epinephrine were observed in dialysate before stimulation. These basal levels were maintained throughout the experiment. Each stimulation elicited almost identical dialysate catecholamine responses on repetition. Dialysate norepinephrine and epinephrine levels reached peak levels during stimulation and declined after the stimulation was stopped. Based on these results, in the following main experiments, we repeated 1-min local administration of cholinergic receptor agonists or 1-min nerve stimulation and examined the dialysate catecholamine responses in various conditions.

2.5. Main experiments

2.5.1. Protocol 1

To ensure the presence of nicotinic or muscarinic receptors on the surface of rat chromaffin cells, we examined the dialysate catecholamine responses to a nicotinic or muscarinic receptor agonist. In six rats, the nicotinic receptor agonist, dimethylphenylpiperazinium or the muscarinic receptor agonist, pilocarpine was locally administered for 1 min at 30-min intervals. We tested 10 and 100 μM of dimethylphenylpiperazinium and 10 μM, 100 μM and 1mM of pilocarpine.

2.5.2. Protocol 2

To determine the involvement of nicotinic and muscarinic receptors in catecholamine release, we examined the effect of cholinergic receptor antagonists on dialysate catecholamine responses to exogenous or endogenous acetylcholine: 1-min local administration of acetylcholine (1 mM) ($n=6$) or 1-min nerve stimulation at 2 ($n=4$) and 4 Hz ($n=6$). Each stimulation was repeated four times at 30-min intervals. The first and third stimulations were done as a control, and the second and fourth stimulations were done at 20-min local administration of the nicotinic receptor antagonist, hexamethonium (1 mM) or the muscarinic receptor antagonist, atropine (10 μM), respectively. Administration of each cholinergic receptor antagonist was stopped immediately after sampling. In addition, we examined the effect of combined nicotinic and muscarinic receptor antagonists on dialysate catecholamine responses to exogenous or endogenous acetylcholine: 1-min local administration of acetylcholine (1 mM) ($n=4$) or 1-min nerve stimulation at 2 ($n=3$) and 4 Hz ($n=4$). Each stimulation was repeated twice at 30-min intervals. The first stimulation was done as a control, and the second stimulation was done at 20-min local administration of combined hexamethonium and atropine.

2.5.3. Protocol 3

To determine the role of acetylcholinesterase in cholinergic synaptic transmissions, we examined the effects of acetylcholinesterase inhibitors on dialysate catecholamine responses to exogenous or endogenous acetylcholine: 1-min local administration of acetylcholine (10 μM) ($n=6$) or 1-min nerve stimulation at 2 Hz ($n=6$). The concentration of administered acetylcholine or the frequency of nerve stimulation was chosen to obtain similar dialysate catecholamine responses to those in protocol 2. Each stimulation was repeated five times at 30-min intervals. The first stimulation was done as a control in the absence of the acetylcholinesterase inhibitor. The remaining stimulations were done in the presence of the acetylcholinesterase inhibitor, neostigmine (10 μM), and the sequence of stimulations was the same as that in protocol 2. In four other rats, 1-min nerve stimulation (2 Hz) was similarly repeated, and another acetylcholinesterase inhibitor, eserine (100 μM) was tested to exclude the nonspecific effects of neostigmine.

At the end of the experiment, the rats were killed with pentobarbital sodium, and the implant sites were examined. The dialysis probes had been implanted in the adrenal gland medulla; no bleeding or necrosis was found macroscopically.

2.6. Statistical methods

To examine the effect of pharmacological agents and nerve stimulation, we analyzed heart rate and mean arterial blood pressure, and dialysate norepinephrine and epinephrine responses, by using one-way analysis of variance with repeated measures. When statistical significance was detected, the Newman–Keuls test was applied (Winer, 1971). Statistical significance was defined as $P<0.05$. The percentage was calculated for each animal and then averaged. Values are presented as means ± S.E.

3. Results

Local administration of pharmacological agents did not influence heart rate or mean arterial pressure in any of the protocols. In protocol 2, the first nerve stimulation (4 Hz), at its maximum effect, decreased heart rate from 430 ± 7 to 414 ± 9 beats·min^{-1} and increased mean arterial blood pressure from 118 ± 7 to 133 ± 8 mm Hg ($P<0.05$). Mean arterial blood pressure and heart rate recovered after cessation of the nerve stimulation. Repeated nerve stimulation evoked the same responses of heart rate and mean arterial pressure.

Basal dialysate norepinephrine and epinephrine levels were not affected by cholinergic receptor antagonists or acetylcholinesterase inhibitors. In all sampling sequences, the peak dialysate norepinephrine or epinephrine levels were obtained during stimulation. We subtracted the basal value from the peak value of dialysate norepinephrine or epinephrine levels and expressed this value as an index of the dialysate norepinephrine or epinephrine response to stimulation.

3.1. Protocol 1

Local administration of dimethylphenylpiperazinium or pilocarpine increased dialysate norepinephrine and epinephrine concentrations, with these responses being dependent on the concentration of dimethylphenylpiperazinium or pilocarpine (Fig. 1). The ratios of norepinephrine response to epinephrine response were 79 ± 11% at 100 μM of dimethylphenylpiperazinium and 6 ± 1% at 1 mM of pilocarpine. None of the ratios were affected by the concentration of dimethylphenylpiperazinium or pilocarpine.

3.2. Protocol 2

3.2.1. Administration of acetylcholine

Local administration of acetylcholine increased dialysate norepinephrine and epinephrine concentrations (Fig. 2). The ratio of norepinephrine response to epinephrine response was 43 ± 5%. In dialysate norepinephrine and epinephrine responses, there was no difference between the first and third stimulations. This indicated that the effect of hexamethonium had already disappeared 30 min after the cessation of local administration. Dialysate norepinephrine response was inhibited by hexamethonium but not by atropine. Dialysate epinephrine response was inhibited by atropine as well as hexamethonium. Both dialysate norepinephrine and epinephrine responses were completely inhibited by the combination of hexamethonium and atropine, and this inhibition of dialysate epinephrine response was almost equivalent to the sum of the individual inhibition by hexamethonium and atropine.

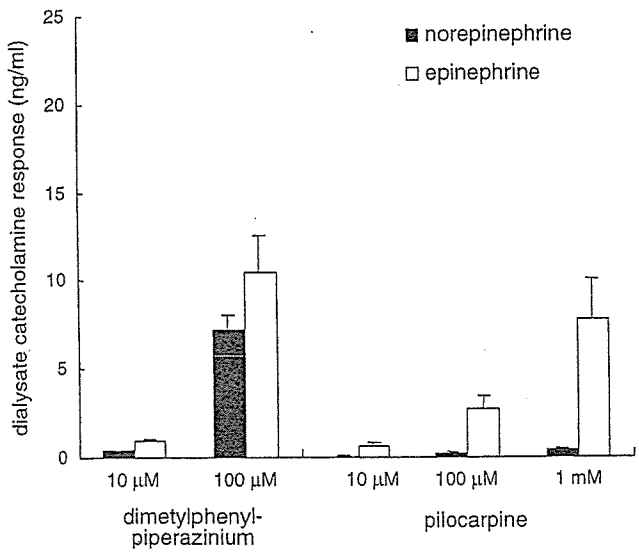

Fig. 1. Dialysate catecholamine response to dimethylphenylpiperazinium or pilocarpine. Values are means ± S.E.

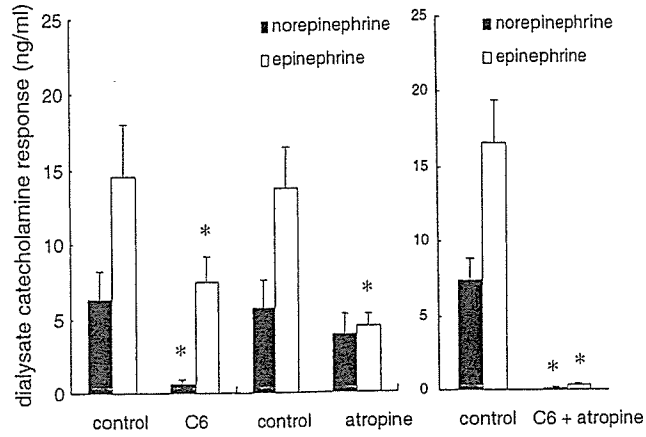

Fig. 2. Effects of hexamethonium (C6) and atropine on dialysate catecholamine response induced by administration of acetylcholine. Values are means ± S.E. *$P<0.05$ vs. dialysate norepinephrine or epinephrine response just before administration of C6 and/or atropine.

3.2.2. Nerve stimulation at 2 and 4 Hz

Nerve stimulation at both frequencies increased dialysate norepinephrine and epinephrine concentrations (Fig. 3). The ratio of norepinephrine response to epinephrine response was 14 ± 3% at 2 Hz and 24 ± 3% at 4 Hz stimulation. At both frequencies, the dialysate norepinephrine response was inhibited by hexamethonium but not by atropine. Dialysate epinephrine response was also inhibited by hexamethonium but not by atropine. The inhibition by the combination of hexamethonium and atropine was almost the same as that by hexamethonium alone.

3.3. Protocol 3

3.3.1. Administration of acetylcholine in the presence of neostigmine

Neostigmine enhanced dialysate norepinephrine and epinephrine responses by about 30–40-fold, but did not change the ratio of norepinephrine response to epinephrine response (Fig. 4). Dialysate norepinephrine response was inhibited by hexamethonium but not by atropine. Dialysate epinephrine response was inhibited by atropine as well as hexamethonium. Neostigmine did not alter the inhibitory action of hexamethonium or atropine.

3.3.2. Nerve stimulation in the presence of neostigmine and eserine

Neostigmine enhanced the dialysate norepinephrine and epinephrine responses by about two- to threefold, but did not change the ratio of norepinephrine response to epineph-

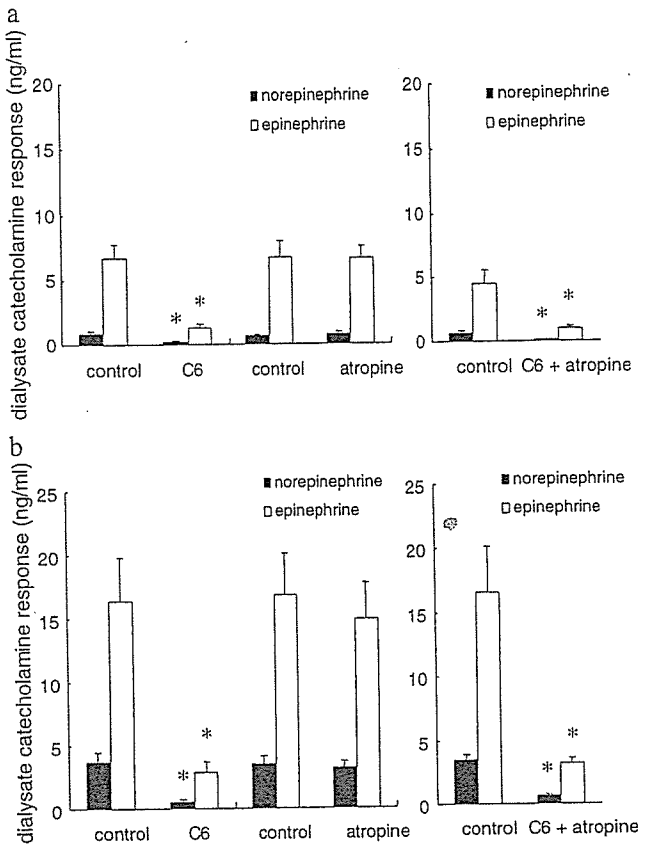

Fig. 3. Effects of hexamethonium (C6) and atropine on dialysate catecholamine response induced by nerve stimulation at 2 (a) and 4 Hz (b). Values are means ± S.E. *$P<0.05$ vs. dialysate norepinephrine or epinephrine response just before administration of C6 and/or atropine.

rine response (Fig. 5a). Dialysate norepinephrine response was inhibited by hexamethonium but not by atropine. Dialysate epinephrine response was inhibited by atropine as well as hexamethonium. Neostigmine elicited the mus-

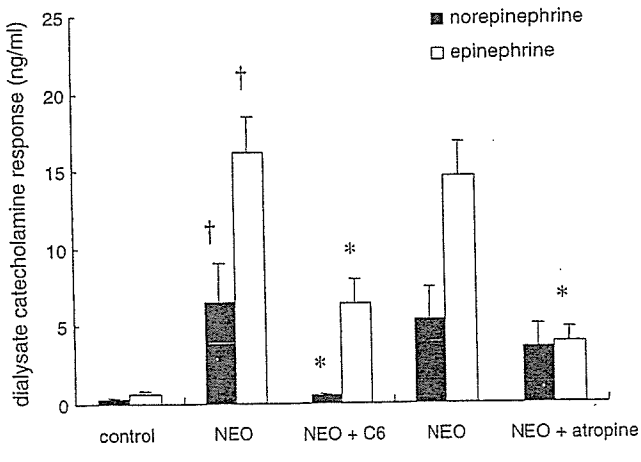

Fig. 4. Effect of hexamethonium (C6) or atropine on dialysate catecholamine response induced by administration of acetylcholine in the presence of neostigmine (NEO). Values are means ± S.E. †$P<0.05$ vs. dialysate norepinephrine or epinephrine response in the absence of NEO. *$P<0.05$ vs. dialysate norepinephrine or epinephrine response just before administration of C6 or atropine.

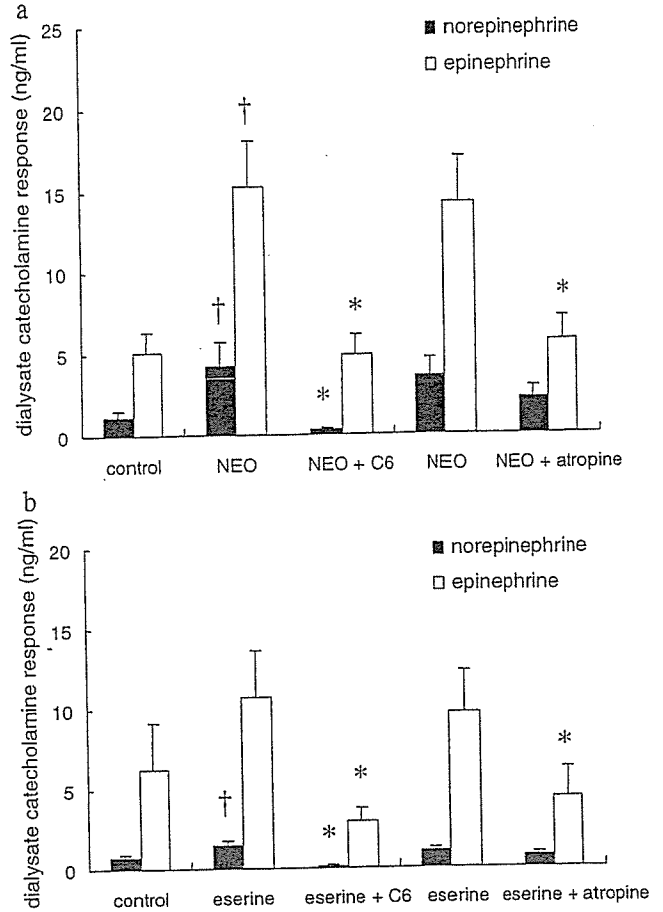

Fig. 5. Effect of hexamethonium (C6) or atropine on dialysate catecholamine response induced by nerve stimulation in the presence of neostigmine (NEO) (a) or eserine (b). Values are means ± S.E. †$P<0.05$ vs. dialysate norepinephrine or epinephrine response in the absence of NEO or eserine. *$P<0.05$ vs. dialysate norepinephrine or epinephrine response just before administration of C6 or atropine.

carinic receptor-mediated synaptic transmission, and the inhibitory action of hexamethonium or atropine became identical with that of exogenous acetylcholine. Enhancement of dialysate norepinephrine and epinephrine responses by eserine was smaller than that by neostigmine, but changes in the inhibitory actions of hexamethonium or atropine were similar to that by neostigmine (Fig. 5b).

3.3.3. Time course of dialysate catecholamine levels in the absence and presence of neostigmine

To examine the influence of neostigmine on the decay of catecholamine release induced by exogenous or endogenous acetylcholine, we compared the time course of dialysate catecholamine levels in the absence and presence of neostigmine (Fig. 6). In 10 μM acetylcholine with neostigmine, the peak value and decay-slope of dialysate catecholamine levels corresponded to those in 1 mM acetylcholine. Similarly, the peak value and decay-slope of dialysate catecholamine levels were almost identical between 2 Hz stimulation with neostigmine and 4 Hz stimulation. Neostigmine en-

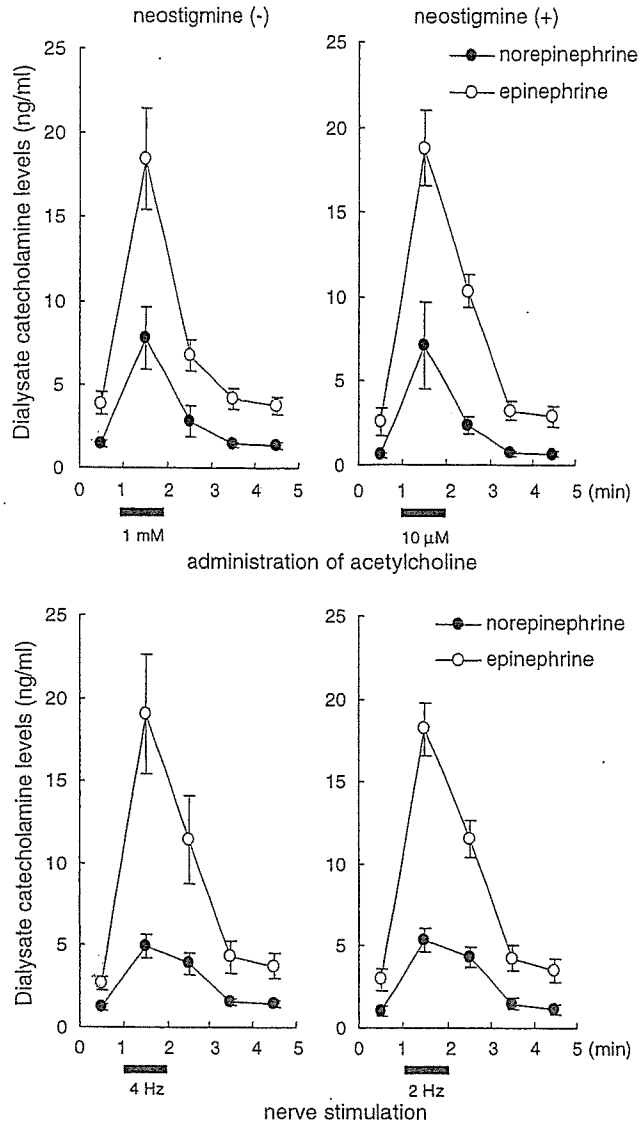

Fig. 6. Comparison of exogenous and endogenous acetylcholine-induced changes in dialysate catecholamine levels in the absence and presence of neostigmine.

hanced the exogenous or endogenous acetylcholine-induced dialysate catecholamine response, but did not change the decay-slope in dialysate catecholamine levels.

4. Discussion

Microdialysis technique with high-performance liquid chromatography made it possible to continuously monitor norepinephrine and epinephrine release at 1-min intervals without blood sampling in the in vivo rat adrenal medulla. Moreover, we were able to locally administer pharmacological agents and observe their effects on catecholamine release without systemic influence. Thus, dialysate sampling with local administration enabled us to precisely assess the cholinergic transmission in the adrenal medulla.

4.1. Presence of nicotinic and muscarinic receptors on the surface of chromaffin cells

In our study of rat adrenals, a nicotinic receptor agonist (dimethylphenylpiperazinium) substantially evoked both norepinephrine and epinephrine release, while a muscarinic receptor agonist (pilocarpine) primarily evoked epinephrine release only. Our observations are consistent with earlier studies in the perfused rat adrenal gland (Wakade and Wakade, 1983; Chen and Dixon, 1990; Zhou et al., 1991). It is well known that adrenal chromaffin cells are divided into two types of populations: norepinephrine- and epinephrine-storing cells (Coupland, 1984). Therefore, in the rat adrenal medulla, both nicotinic and muscarinic receptors substantially exist on the surface of epinephrine-storing cells, while on the surface of norepinephrine-storing cells, nicotinic receptors are primarily present, but muscarinic receptors may be nearly absent.

4.2. Contribution of nicotinic and muscarinic receptors to catecholamine release

Exogenous acetylcholine-induced norepinephrine release was predominantly mediated by nicotinic receptors, while exogenous acetylcholine-induced epinephrine release was mediated by muscarinic as well as nicotinic receptors. These data also support the findings from earlier studies (Wakade and Wakade, 1983; Chen and Dixon, 1990; Zhou et al., 1991) and our agonist study in protocol 1. On the other hand, endogenous acetylcholine-induced norepinephrine and epinephrine release was exclusively mediated by nicotinic receptors. Involvement of muscarinic receptors was not significant in either norepinephrine or epinephrine release.

There was a differential contribution of nicotinic and muscarinic receptors between exogenous and endogenous acetylcholine-induced epinephrine release. Cholinergic synaptic transmission by endogenous acetylcholine was exclusively mediated by nicotinic receptors. Our observations agree with earlier studies in rats (Wakade and Wakade, 1983) and dogs (Kimura et al., 1992). Splanchnic nerves densely innervate the adrenal medulla, and their endings make synaptic contacts with chromaffin cells (Coupland, 1965). If exogenous acetylcholine equally reaches the synaptic and extra-synaptic regions of chromaffin cells while endogenous acetylcholine acts and terminates in the synaptic regions, nicotinic receptors could be primarily localized at synaptic regions and muscarinic receptors could be present on the extra-synaptic regions of the epinephrine-storing chromaffin cells. Actually, nicotinic receptors are concentrated at the neuromuscular junction of innervated skeletal muscle (Apel and Merlie, 1995) and at synaptic zones of postsynaptic membranes in sympathetic ganglia (Marshall, 1981) and brain neurons (Arroyo-Jiménez et al., 1999). Moreover, immunohistochemical analysis of guinea-pig chromaffin cells recently revealed that nicotinic receptors were mainly concentrated at synaptic

regions (Inoue et al., 2000). Thus, this anatomical localization of receptors may explain the difference in the contribution of cholinergic receptors between exogenous and endogenous acetylcholine-induced catecholamine release. Here, we focused on whether synaptic connection is so tight that released acetylcholine cannot spill over to extra-synaptic regions and on what conditions are relevant to the muscarinic receptor-mediated cholinergic transmission in the in vivo state. We concentrated on the enzymatic degradation by acetylcholinesterase.

4.3. Influence of acetylcholinesterase inhibitor on catecholamine release

Acetylcholinesterase inhibitors enhance the exogenous or endogenous acetylcholine-induced catecholamine release response in the adrenal medulla (Tsujimoto and Nishikawa, 1975; Orts et al., 1987). This enhancement may be ascribed to the elevation of acetylcholine levels on the surface of chromaffin cells, the reduction in the decay-slope of acetylcholine levels, and/or the spread of the action site of acetylcholine. In the present study monitoring at 1-min intervals, neostigmine enhanced the exogenous or endogenous acetylcholine-induced catecholamine release response, but did not change the decay-slope of catecholamine release. Thus, it is unlikely that enhancement of the catecholamine release response is attributable to the slow decay of acetylcholine levels on the surface of chromaffin cells.

Neostigmine caused a 30–40-fold increase in the exogenous acetylcholine-induced catecholamine release. This enhancement was much greater than that in the endogenous acetylcholine-induced catecholamine release response. There is abundant acetylcholinesterase activity on splanchnic nerve endings, chromaffin cells and interstitial cells (Coupland, 1965; Palkama, 1967; Lewis and Shute, 1969; Somogyi et al., 1975). Our data suggest that about 97% of administered acetylcholine was degraded by tissue acetylcholinesterase without reaching cholinergic receptors on the surface of chromaffin cells, and only about 3% of administered acetylcholine was bound to cholinergic receptors, leading to catecholamine release. Moreover, the catecholamine release response and the manner of contribution of cholinergic receptors were almost identical between 10 μM acetylcholine in the presence of neostigmine and 1 mM acetylcholine in the absence of neostigmine. Therefore, in the case of exogenously administered acetylcholine, acetylcholinesterase inhibitor equally elevates acetylcholine levels on the surface of chromaffin cells regardless of synaptic or extra-synaptic regions.

Neostigmine caused a three- to fivefold increase in the endogenous acetylcholine-induced catecholamine release and changed the manner of contribution of cholinergic receptors to epinephrine release. Epinephrine release by nerve stimulation in the absence of neostigmine was exclusively mediated by nicotinic receptors, but epinephrine release by nerve stimulation in the presence of neostigmine was mediated by muscarinic as well as nicotinic receptors. In the presence of neostigmine, the manner of contribution of cholinergic receptors to endogenous acetylcholine-induced catecholamine release was similar to that to exogenous acetylcholine-induced catecholamine release. We also obtained similar results in the presence of another acetylcholinesterase inhibitor, eserine. Thus, acetylcholinesterase inhibitors not only enhanced the endogenous acetylcholine-induced catecholamine release response but also elicited muscarinic receptor-mediated epinephrine release. The epinephrine release response induced by 2 Hz stimulation in the presence of neostigmine was almost identical to that induced by 4 Hz stimulation in the absence of neostigmine. Thus, the amount of acetylcholine contributing to epinephrine release could be also similar between the two stimulations. The manner of contribution of cholinergic receptors was, however, different between the absence and presence of neostigmine. This difference could not be explained by elevation of the acetylcholine level in the synaptic regions. Recently, an electrophysiological study in the rat ganglion suggested that synaptically released acetylcholine activated the receptors located extra-synaptically in the presence of eserine (Callister and Sah, 1997). Considering that muscarinic receptors are mainly present on the extra-synaptic regions of epinephrine-storing cells, our data indicate that acetylcholinesterase inhibitors extend the binding sites of endogenous acetylcholine to the extra-synaptic regions rather than elevate the acetylcholine level in the synaptic regions and elicit muscarinic receptor-mediated synaptic transmission.

4.4. Other possible mechanisms

It has been reported that ganglionic acetylcholine release receives presynaptic autoinhibition through muscarinic receptors (Dujic et al., 1990; Myers and Undem, 1996). Moreover, an electrophysiological study suggested that the acetylcholine release from splanchnic nerves received presynaptic inhibition through muscarinic receptors in rat adrenal gland (Barbara et al., 1998). If so, acetylcholinesterase inhibitors would induce the activation of presynaptic muscarinic receptors by increasing the acetylcholine level in synaptic regions (Brehm et al., 1992). In such a condition, a muscarinic receptor antagonist would facilitate acetylcholine release by blocking presynaptic autoinhibition and acting to facilitate norepinephrine and epinephrine release. Therefore, the inhibition by muscarinic receptor antagonists in the presence of acetylcholinesterase inhibitors cannot be explained by the presynaptic regulatory mechanism on splanchnic nerve endings.

It has been reported that cholinesterase inhibitors at high concentrations block nicotinic receptors in adrenal chromaffin cells (Mizobe and Livett, 1982), striatal synaptosomes (Clarke et al., 1994) and sympathetic neurons (Zheng et al., 1998). Moreover, it has been speculated that inactivation of nicotinic receptors enhances the sensitivity of muscarinic

receptors in the process of catecholamine release from the adrenal gland (Lee and Trendelenburg, 1967; Tsujimoto and Nishikawa, 1975). Neostigmine might block nicotinic receptors on the chromaffin cells and enhance the sensitivity of muscarinic receptors. This action might be involved in the cholinergic transmission through muscarinic receptors in the presence of acetylcholinesterase inhibitors. In our study, however, 10 μM neostigmine did not affect the contribution of nicotinic and muscarinic receptors to exogenous acetylcholine-induced catecholamine release. Thus, under our experimental conditions, it is unlikely that neostigmine blocks nicotinic receptors and enhances the sensitivity of muscarinic receptors.

In conclusion, in the in vivo state, acetylcholinesterase prevents the spillover of released acetylcholine to extra-synaptic regions and restricts its action to synaptic regions. Acetylcholinesterase inhibitor extends the action of released acetylcholine to the extra-synaptic regions and elicits the muscarinic receptor-mediated synaptic transmission. On the other hand, acetylcholinesterase prevents the administered acetylcholine from reaching the surface of chromaffin cells. Acetylcholinesterase inhibitor prevents degradation of administered acetylcholine and potentiates the effect of administered acetylcholine on nicotinic and muscarinic receptors on the surface of chromaffin cells regardless of location. Thus, acetylcholinesterase plays a crucial role in the differential contribution of cholinergic receptors to exogenous and endogenous acetylcholine-induced catecholamine release.

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Selective Inhibition of Vascular Endothelial Growth Factor Receptor-2 (VEGFR-2) Identifies a Central Role for VEGFR-2 in Human Aortic Endothelial Cell Responses to VEGF

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ABSTRACT

Vascular endothelial growth factor receptors (VEGFR) are considered essential for angiogenesis. The VEGFR-family proteins consist of VEGFR-1/Flt-1, VEGFR-2/KDR/Flk-1, and VEGFR-3/Flt-4. Among these, VEGFR-2 is thought to be principally responsible for angiogenesis. However, the precise role of VEGFRs1–3 in endothelial cell biology and angiogenesis remains unclear due in part to the lack of VEGFR-specific inhibitors. We used the newly described, highly selective anilinoquinazoline inhibitor of VEGFR-2 tyrosine kinase, ZM323881 (5-[[7-(benzyloxy) quinazolin-4-yl]amino]-4-fluoro-2-methylphenol), to explore the role of VEGFR-2 in endothelial cell function. Consistent with its reported effects on VEGFR-2 [IC(50) < 2 nM], ZM323881 inhibited activation of VEGFR-2, but not of VEGFR-1, epidermal growth factor receptor (EGFR), platelet-derived growth factor receptor (PDGFR), or hepatocyte growth factor (HGF) receptor. We studied the effects of VEGF on human aortic endothelial cells (HAECs), which express VEGFR-1 and VEGFR-2, but not VEGFR-3, in the absence or presence of ZM323881. Inhibition of VEGFR-2 blocked activation of extracellular regulated-kinase, p38, Akt, and endothelial nitric oxide

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