

図2 アストロサイト刺激によるCa²⁺波伝播およびATP放出の画像化
 A: アストロサイト間に伝播するCa²⁺波。アストロサイトをガラスピペットで軽く触れると(白矢印)、刺激部位でまず[Ca²⁺]_i上昇が観察され、周辺へ同心円状に伝播するCa²⁺波が観察される(対照;上段)。アピラーゼ(apyrase)(gradeⅢ、20 units/ml)を添加して同様の実験を行うと、このCa²⁺波伝播は著しく抑制される(+Apyrase;下段)。
 B: アストロサイトからのATP放出。ルシフェリン-ルシフェラーゼ存在下で、海馬アストロサイトにガラスピペットで軽い機械刺激(位相差像参照;左端)を与え、発生する光子を高感度カメラで経時的にとらえた(露光時間500ms)。まず、刺激した部位で光子の増大が観察され、これは周辺へと広がった。
 C: Photon-ATP濃度相関。既知濃度のATP標準液を用いて光子-ATP濃度の相関を示した。両者には非常に高い相関が認められた($r^2 = 0.9996$)。
 D: Ca²⁺波とATP放出の相関。ATP伝播速度とCa²⁺波伝播速度を比較した。やはり、両者間には非常に高い相関が認められた。

が、ギャップ結合を形成するコネキシン 43 分子が ATP 放出能に強く影響すること¹⁵⁾、さらにコネキシンが ATP 放出チャンネルである可能性¹⁶⁾などから、コネキシン分子は意外にも ATP 放出能とも関係があるようである。ATP を介する異種グリア細胞間の gliotransmission の存在も報告されている。アストロサイトの Ca^{2+} 波は、培養細胞¹⁷⁾でもスライス標本¹⁸⁾でも ATP を介して近傍のミクログリアに伝播する。

3. アストロサイトーニューロン連関

1) アストロサイトの Ca^{2+} 波はニューロンに伝播する

ニューロンの活動がグルタミン酸を介してアストロサイトの Ca^{2+} 動態に影響することは、かなり以前から知られていた¹⁹⁾。しかし、海馬ニューロンを用いた実験では、各種グルタミン酸受容体の拮抗薬存在下でニューロンを興奮させても、約 20% の近傍アストロサイトでは遅い $[\text{Ca}^{2+}]_i$ 上昇が観察され、 Ca^{2+} 波が伝播していくのが観察され、これはアピラーゼおよび suramin で消失する。ニューロンからのシグナルは ATP によってもアストロサイトに伝わるのである²⁰⁾。

それでは、このアストロサイト間に伝播する Ca^{2+} 波はニューロンにも伝わるのだろうか。

アストロサイトを局所的に刺激すると、アストロサイト間 Ca^{2+} 波に加えて、近傍の約 30% のニューロンで $[\text{Ca}^{2+}]_i$ 上昇が認められ、これはアピラーゼおよび suramin で抑制される。そして、この Ca^{2+} 波のニューロンへの伝播は細胞外 Ca^{2+} 非依存的である。

前述したように、海馬には P2X2 または P2X4、P2X6 mRNA²¹⁾ が発現し、錐体細胞層には P2Y1 受容体蛋白質の強いシグナルが²²⁾、さらに介在ニューロンでは機能的 P2Y1 受容体が²³⁾ 発現している。ニューロンへの Ca^{2+} 波伝播が細胞外 Ca^{2+} に非依存的であったことを考慮すると、主に P2Y 受容体、特に P2Y1 受容体がこのアストロサイトーニューロン間の Ca^{2+} 波伝播の責任受容体であると考えられる。ATP を介する gliotransmission

transmission はグリア細胞ばかりでなく、ニューロンの Ca^{2+} 動態、さらにはニューロンの活動に影響し得ることが明らかとなった。

2) アストロサイトーシナプス連関

ATP 依存的なアストロサイト Ca^{2+} 波は、ニューロンのシナプス伝達をもダイナミックに制御する。海馬のニューロン・グリア共培養細胞では、グルタミン酸神経の興奮性シナプス伝達に起因する自発的な Ca^{2+} オシレーションが神経細胞で認められる²⁴⁾。外から ATP を加えると、神経終末の P2Y 様受容体を介してグルタミン酸放出を抑制することにより、この Ca^{2+} オシレーションを抑制する (図 3Ab、トレース 1、2)。この抑制メカニズムの詳細は不明な点が多いが、少なくとも後シナプスの応答に影響を与えないこと、グルタミン酸放出が抑制されること、イオノマイシンによるグルタミン酸放出には影響しないが、脱分極刺激による放出を抑制することから、神経終末の電位依存性 Ca^{2+} チャネルの抑制が関係しているものと考えられる²⁵⁾。アストロサイトに局所刺激を与えると、ATP が放出され、拡散し、これにより周辺のアストロサイトで $[\text{Ca}^{2+}]_i$ 上昇が Ca^{2+} 波伝播として観察される (図 3Bc、トレース 1、2)。このとき同時に、刺激したアストロサイト近傍ではニューロンの Ca^{2+} オシレーション、つまり興奮性シナプス伝達も抑制されるのである (図 3Bd (v)、トレース 3~5)。この抑制作用はアピラーゼ処置により消失する。つまり、ATP はアストロサイトーニューロン間の液性情報伝達物質として働き、シナプス伝達をきわめてダイナミックに抑制し得るのである。これは、これまでニューロンの前シナプスおよび後シナプスのみで完結されると考えられていたシナプス伝達を、アストロサイト (周辺シナプス) を加えた三者で、三者間シナプス (tripartite synapse) として考える必要性を提起するものである²⁾。このとき、ニューロンーアストロサイトをつなぐ重要な分子として、ATP が中心的な役割を果たしているのである (図 6 参照)。

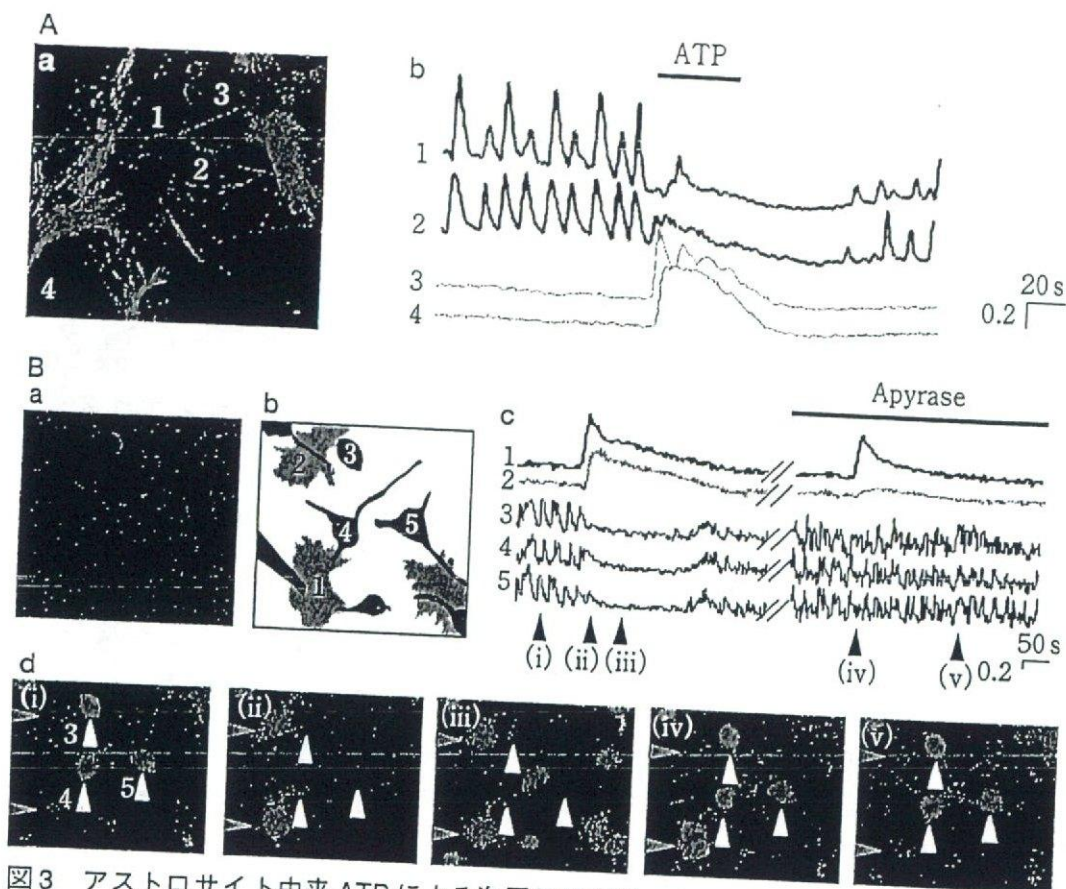


図3 アstrocyt由来ATPによる海馬神経細胞のシナプス伝達抑制(口絵カラー参照)

A : aは海馬初代培養細胞の免疫染色像。細胞は、ニューロン特異的のマーカーMAP2(緑)およびアストロサイト特異的のマーカーglial fibrillary acidic protein (GFAP ; 赤)で同定した。ニューロン1および2、アストロサイト3および4の $[Ca^{2+}]_i$ 変動の時間経過をbに示した。ニューロンでは同期した興奮性シナプス伝達(Ca^{2+} オシレーション)が観察され、これはテトロドトキシン(TTX)、細胞外 Ca^{2+} 除去、さらにイオンチャンネル型グルタミン酸受容体拮抗薬で抑制されることから、グルタミン酸のシナプス伝達に起因していることがわかっている²⁴⁾。この Ca^{2+} オシレーションをシナプス伝達の指標として細胞外ATPの作用を検討した。ATP刺激($1\mu M$)により、ニューロンの Ca^{2+} オシレーションは消失し、アストロサイトでは $[Ca^{2+}]_i$ 上昇が認められた。

B : 単一アストロサイトを刺激すると、ニューロンの Ca^{2+} オシレーションは抑制される。aは位相差像を、bはニューロン(緑)およびアストロサイト(赤)の位置を模式的に示した。図b中左下に刺激ピペットをおいた。アストロサイト1を刺激した場合の、アストロサイト(1, 2)およびニューロン(3~5)の $[Ca^{2+}]_i$ 変動をcに示した。それぞれトレース番号は模式図の細胞番号に対応している。アストロサイト1の機械刺激(矢頭)によりアストロサイト間 Ca^{2+} 波(1, 2)およびニューロンの Ca^{2+} オシレーション抑制(3~5)が認められた。これらのアストロサイトによるシナプス伝達抑制作用はアピラーゼ(20 units/ml)より消失した(トレース右)。トレースの下に記した矢頭は模式図aの細胞番号と対応している。

3) アストロサイトからの自発的 ATP 放出による 恒常的なシナプス制御

アストロサイトの自発的な $[Ca^{2+}]_i$ 変動には、ニューロンの活動に依存しない成分が存在する。図4で示すように、テトロドトキシン (tetrodotoxin: TTX) で神経活動を完全に抑制した場合でも、アストロサイトは同期しない $[Ca^{2+}]_i$ 変動を呈した。この自発的な $[Ca^{2+}]_i$ 変動は、suramin、PPADS さらにアピラーゼによって抑制された (図4)。また、ニューロンを含まない精製したアストロサイトの培養系でも、自発的な

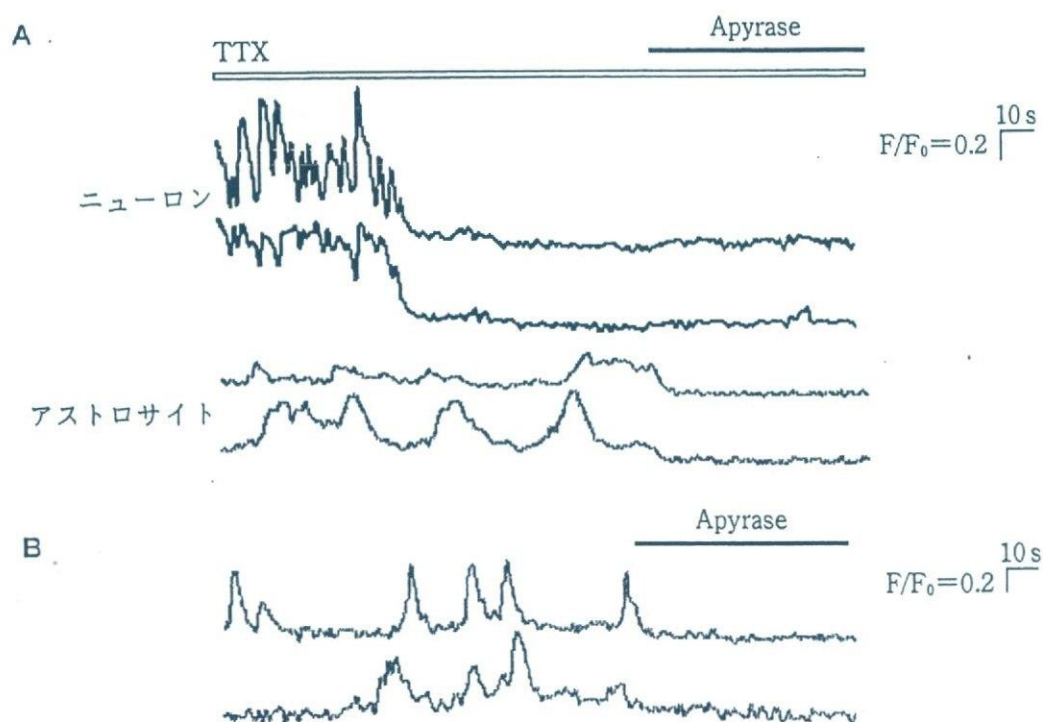


図4 アストロサイトのニューロン非依存的な Ca^{2+} 波の存在

A: ニューロン・グリア共培養系における $[Ca^{2+}]_i$ 変化。テトロドトキシン (TTX, $1\mu M$) によりニューロンの Ca^{2+} オシレーションは完全に抑制された。しかし、TTX 存在下でもアストロサイトの Ca^{2+} 波は観察された。

B: ニューロンを含まないアストロサイトのみ純培養系における $[Ca^{2+}]_i$ 変化。アストロサイトの純培養系でも、アストロサイトは低頻度で同期しない Ca^{2+} 波を呈し、これは suramin (データ未掲載) およびアピラーゼによりほぼ 90% のアストロサイトで抑制された。

[Ca²⁺]_i変動が観察された。さらに、海馬のスライス標本でもアストロサイトの自発的な [Ca²⁺]_i変動が認められ、これは TTX 非依存的であることが報告されている²⁶⁾。つまり、アストロサイトはニューロン活動に依存しない自発的な ATP 放出能をも有し、これにより自身の [Ca²⁺]_i変

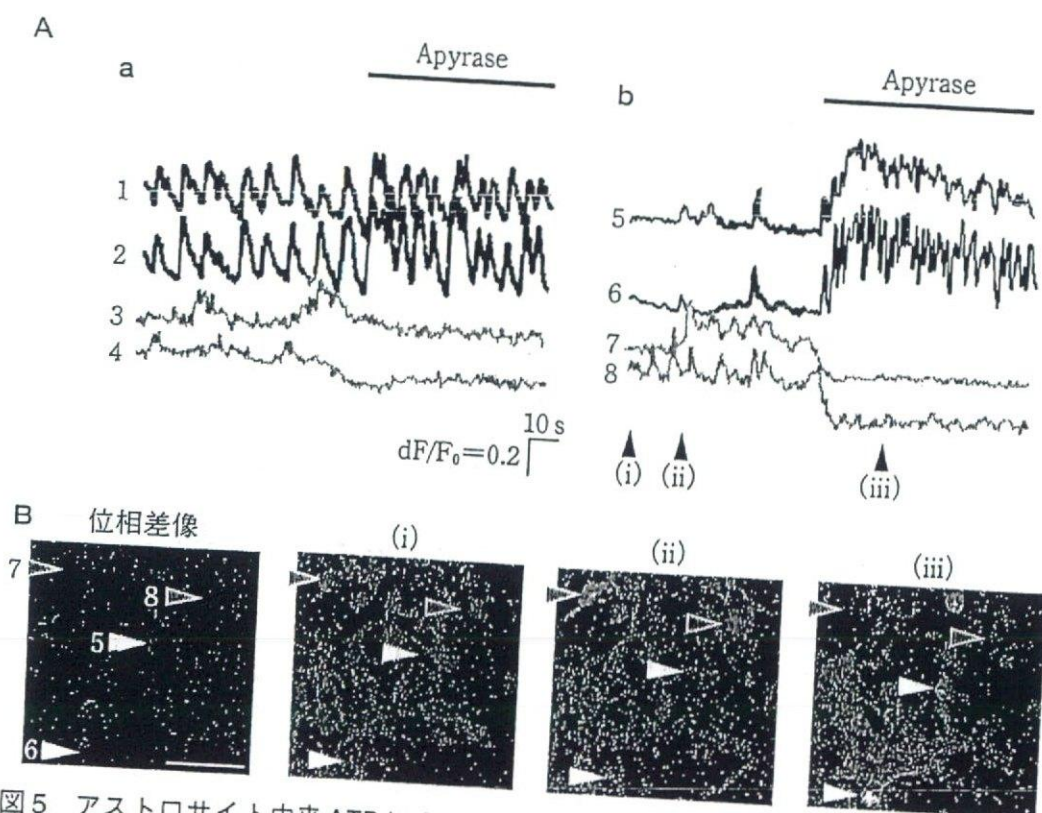


図5 アストロサイト由来 ATP による恒常的シナプス伝達制御 (口絵カラー参照)
 A : a はグルタミン酸によるシナプス伝達 (Ca²⁺オシレーション) に対するアピラーゼ (20 units/ml) の作用。アピラーゼを加えるとニューロンの Ca²⁺オシレーション (1、2) の頻度および振幅は増大する。一方、アストロサイトの Ca²⁺変動 (3、4) は消失した。特にニューロンの Ca²⁺オシレーション頻度が低いカバースリップにアピラーゼを加えると (b)、ニューロンの活動は爆発的に増大した (5、6)。このような細胞群ではアピラーゼ添加前のアストロサイトの [Ca²⁺]_i変化が比較的大きく (7、8)、これはやはりアピラーゼでその活動が消失する。
 B : Ab の Ca²⁺イメージを疑似カラーで示した。左端は位相差像、番号は Ab のトレースと対応している。疑似カラー上の番号は、Ab のトレースの下に記した矢頭 (i) ~ (iii) 各時間における Ca²⁺イメージを示す。(ii) ではアストロサイトのみが活動しており、アピラーゼ添加後の (iii) ではニューロンのみの [Ca²⁺]_i上昇が認められる。スケールバーは 50 μm。

化を引き起こしているのである。

では、この自発的なATP放出は近傍のニューロンの活動に影響していないのだろうか。

図5に示すように、海馬のニューロン・グリア共培養細胞にアピラーゼを添加してアストロサイトから放出されるATPを除去すると、シナプス伝達に起因するニューロンの Ca^{2+} オシレーションは、その頻度と振幅ともに増強された(図5Aa、トレース1、2)。特にニューロンの活動が小さい標本では、アピラーゼにより爆発的なシナプス伝達の増大が認められた(図5Ab、トレース5、6)。このような標本では、アストロサイトの自発的 Ca^{2+} 活動が大きく(図5Ab、トレース7、8)、これはアピラーゼにより完全に消失する。したがって、アストロサイトは自発的に細胞外ATP放出を調節することにより、シナプス伝達を恒常的にしかも強力に制御している可能性が明らかとなった。ニューロンの何倍もの数を有し、またシナプスを囲むように存在するアストロサイトは、シナプス伝達において非常に重要なニューロンのパートナーとして機能しているようである。

図6に、シナプス前部、シナプス後部および周辺シナプスのアストロサイトが形成する三者間シナプスの形態を、その制御因子ATPを中心に模式的に示した。シナプス伝達によって漏れでた神経伝達物質は周辺アストロサイトに作用して、アストロサイト間へ、もとのシナプスへ、さらにほかのシナプスへさまざまな情報をダイナミックに伝える。このようなアストロサイトを含めたグリア細胞-ニューロンコミュニケーションは、複雑な脳の情報処理・発信機能の素過程である可能性が高い。また、アストロサイトがニューロン活動に依存しないATP放出能を有していたことから、アストロサイトはいわゆるシナプス伝達が行われるニューロンの周辺環境を積極的に整備する役割も有しているようである。これらの知見は、今後のシナプス伝達研究はグリア細胞をも十分考慮して進めていく必要性を提起するものであると考えている。

おわりに

ATPがアストロサイト-ニューロン間の相互情報伝達物質として機能していることが明らかとなった。このようなグリア細胞によるシナプス伝達制御の生理的意義および病態との関連性解明には、今後の研究を待たなければならない。しかし最近、筆者らは、脊髄グリア細胞のATP受容体を介する情報伝達の変調が、神経因性疼痛を惹起することを明らかにし²⁷⁾、グリア細胞とニューロンのクロストークが重篤な疾患とリンクしている一例を示した。今後、このATPが介在するグリア細胞-ニューロン連関の研究は、中枢神経系の複雑な情報処理・発信機能を解明

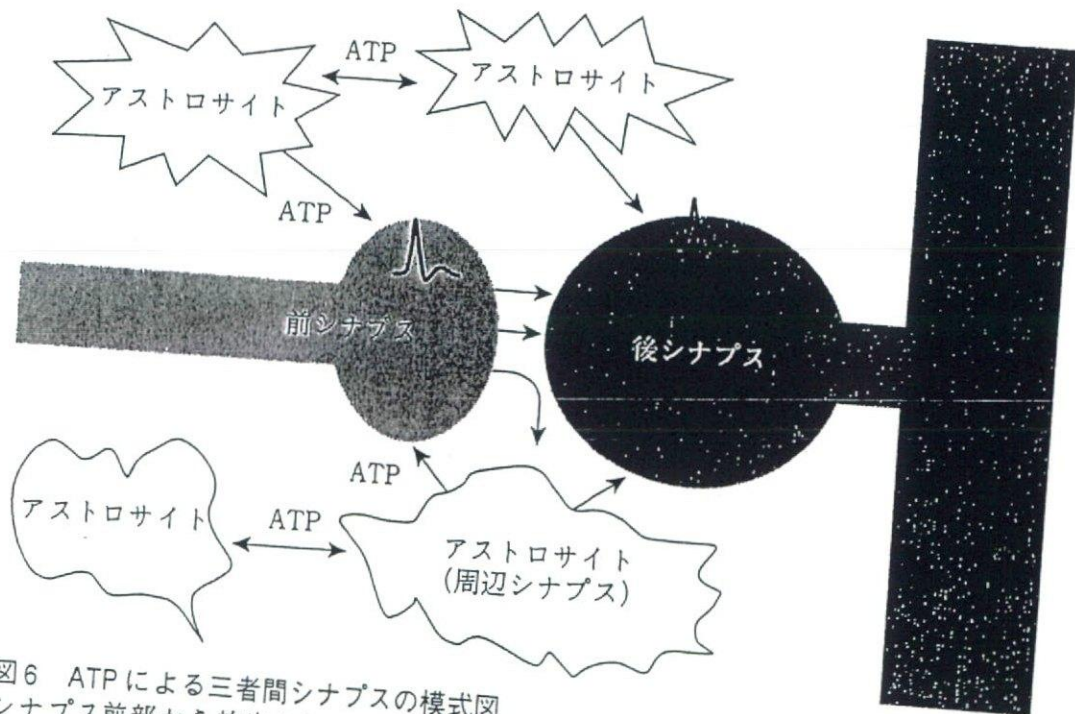


図6 ATPによる三者間シナプスの模式図
 シナプス前部から放出された神経伝達物質はシナプス後部の特異的受容体を刺激する。このとき、シナプス間隙から漏れた神経伝達物質は周囲を取り巻くアストロサイト(周辺シナプス)を刺激する。アストロサイトは刺激依存的に液性因子ATPを放出し、これはアストロサイト間の情報伝達物質として機能し、さらに神経細胞のシナプスへフィードバックされてシナプス伝達を即時的に制御する。

するひとつのキーワードになると思われる。

謝 辞

稿を終えるにあたり、終始懇篤なるご指導、ご鞭撻をいただきました九州大学大学院薬学研究院教授・井上和秀先生に深く感謝申し上げます。また、実験にご協力くださいました国立医薬品食品衛生研究所・小濱とも子、藤下加代子、最上(重本)由香里、津田 誠(現・九州大学大学院助手)ほか共同研究者の方々、さらに的確なご助言をいただき自由な研究環境をつくっていただきました同研究所副所長・大野泰雄先生に深甚なる謝意を表します。

参 考 文 献

- 1) Ransom B, Behar T, and Nedergaard M : *Trends Neurosci.* 26, 520-522, 2003
- 2) Haydon PG : *Nat. Rev. Neurosci.* 2, 185-193, 2001
- 3) Webb TE, Simon J, Krishek BJ, Bateson AN, Smart TG, King BF, Burnstock G, and Barnard EA : *FEBS Lett.* 324, 219-225, 1993
- 4) Burnstock G : *Pharmacol. Rev.* 24, 509-581, 1972
- 5) Torres GE, Egan TM and Voigt MM : *J. Biol. Chem.* 274, 6653-6659, 1999
- 6) Chambers JK, Macdonald LE, Sarau HM, Ames RS, Freeman K, Foley JJ, Zhu Y, McLaughlin MM, Murdock P, McMillan L, Trill J, Swift A, Aiyar N, Taylor P, Vawter L, Naheed S, Szekeres P, Hervieu G, Scott C, Watson JM, Murphy AJ, Duzic E, Klein C, Bergsma DJ, Wilson S, and Livi GP : *J. Biol. Chem.* 275, 10767-10771, 2000
- 7) Abbracchio MP, Boeynaems JM, Barnard EA, Boyer JL, Kennedy C, Miras-Portugal MT, King BF, Gachet C, Jacobson KA, Weisman GA, and Burnstock G : *Trends Pharmacol. Sci.* 24, 52-55, 2003
- 8) Pintor J, Torres M, Castro E, and Miras-Portugal MT : *Br. J. Pharmacol.* 103, 1980-1984, 1991
- 9) Giaume C, and McCarthy KD : *Trends Neurosci.* 19, 319-325, 1996
- 10) Guthrie PB, Knappenberger J, Segal M, Bennett MV, Charles AC, and Kater SB : *J. Neurosci.* 19, 520-528, 1999
- 11) Franke H, Krugel U, and Illes P : *Glia* 28, 190-200, 1999
- 12) Abbracchio MP, Saffrey MJ, Hopker V, and Burnstock G : *Neuroscience* 59, 67-76, 1994
- 13) Neary JT, Kang Y, Bu Y, Yu E, Akong K, and Peters CM : *J. Neurosci.* 19, 4211-4220, 1999

- 14) Koizumi S, Saito Y, Nakazawa K, Nakajima K, Sawada J I, Kohsaka S, Illes P, and Inoue K : *Life Sci.* 72, 431-442, 2002
- 15) Cotrina ML, Lin JH, Alves-Rodrigues A, Liu S, Li J, Azmi-Ghadimi H, Kang J, Naus CC, and Nedergaard M. : *Proc. Natl. Acad. Sci. USA* 95, 15735-15740, 1998
- 16) Stout CE, Costantin JL, Naus CC, and Charles AC : *J. Biol. Chem.* 277, 10482-10488, 2002
- 17) Verderio C, and Matteoli M : *J. Immunol.* 166, 6383-6391, 2001
- 18) Schipke CG, Boucsein C, Ohlemeyer C, Kirchhoff F, and Kettenmann H. : *Faseb. J.* 16, 255-257, 2002
- 19) Cornell-Bell AH, Finkbeiner SM, Cooper MS, and Smith SJ. : *Science* 247, 470-473, 1990
- 20) Koizumi S, Fujishita K, Tsuda M, and Inoue K : *Drug Development Research* 59, 88-94, 2003
- 21) Rubio ME, and Soto F. : *J. Neurosci.* 21, 641-653, 2001
- 22) Moore D, Chambers J, Waldvogel H, Faull R, and Emson P. : *J. Comp. Neurol.* 421, 374-384, 2000
- 23) Bowser DN, Williams SR, and Khakh BS : *Society for Neuroscience Abstract* 251.5, 2003
- 24) Koizumi S, and Inoue K. : *Br. J. Pharmacol.* 122, 51-58, 1997
- 25) Koizumi S, Fujishita K, Tsuda M, Shigemoto-Mogami Y, and Inoue K. : *Proc. Natl. Acad. Sci. USA* 100, 11023-11028, 2003
- 26) Nett WJ, Oloff SH, and McCarthy KD. : *J. Neurophysiol.* 87, 528-537, 2002
- 27) Tsuda M, Shigemoto-Mogami Y, Koizumi S, Mizokoshi A, Kohsaka S, Salter M W, and Inoue K. : *Nature* 424, 778-783, 2003

小泉 修一 (こいずみ・しゅういち)

国立医薬品食品衛生研究所 薬理部室長

1992年九州大学大学院薬学研究科博士課程修了、(財)ヒューマンサイエンス振興財団博士研究員を経て、95年厚生省入省。96年英国ケンブリッジ大学 Babraham 研究所博士研究員。98年帰国後、国立医薬品食品衛生研究所主任研究官、2003年より同室長。
専門分野は、中枢神経系薬理・生理。

Inhibition of Transforming Growth Factor- β Production in Brain Pericytes Contributes to Cyclosporin A-Induced Dysfunction of the Blood-Brain Barrier

Fuyuko Takata,^{1,2} Shinya Dohgu,¹ Atsushi Yamauchi,¹ Noriko Sumi,¹ Shinsuke Nakagawa,^{2,3} Mikihiro Naito,⁴ Takashi Tsuruo,⁴ Hideki Shuto,¹ and Yasufumi Kataoka^{1,2,5}

Received July 24, 2006; accepted October 5, 2006

SUMMARY

1. The present study was designed to clarify whether brain pericytes and pericyte-derived transforming growth factor- β 1 (TGF- β 1) participate in cyclosporin A (CsA)-induced dysfunction of the blood-brain barrier (BBB).

2. The presence of brain pericytes markedly aggravated CsA-increased permeability of MBEC4 cells to sodium fluorescein and accumulation of rhodamine 123 in MBEC4 cells.

3. Exposure to CsA significantly decreased the levels of TGF- β 1 mRNA in brain pericytes in pericyte co-cultures. Treatment with TGF- β 1 dose-dependently inhibited CsA-induced hyperpermeability and P-glycoprotein dysfunction of MBEC4 cells in pericyte co-cultures.

4. These findings suggest that an inhibition of brain pericyte-derived TGF- β 1 contributes to the occurrence of CsA-induced dysfunction of the BBB.

KEY WORDS: Cyclosporin A; brain pericytes; transforming growth factor- β ; blood-brain barrier; permeability; P-glycoprotein; mouse brain endothelial cells.

INTRODUCTION

Cyclosporin A (CsA), a cyclic 11-amino acid peptide, is widely used as a potent immunosuppressant to prevent allograft rejection in solid organ transplantation and in fatal graft-versus-host disease after bone marrow transplantation; it is also used to treat various autoimmune diseases including rheumatoid arthritis (Ka-

¹ Department of Pharmaceutical Care and Health Sciences, Faculty of Pharmaceutical Sciences, Fukuoka University, Jonan-ku, Fukuoka, 814-0180, Japan.

² PharmaCo-Cell Company Ltd., Nagayo-machi, Nagasaki, 851-2127, Japan.

³ Department of Pharmacology 1, Nagasaki University School of Medicine, Sakamoto, Nagasaki, 852-8501, Japan.

⁴ Institute of Molecular and Cellular Biosciences, University of Tokyo, Bunkyo-ku, Tokyo, 113-0032, Japan.

⁵ To whom correspondence should be addressed; e-mail: ykataoka@fukuoka-u.ac.jp.

han, 1989). Despite its high efficacy, CsA has adverse effects including nephrotoxicity, cardiovascular disorders, gastrointestinal disorders and neurotoxicity. CsA-associated neurotoxicity occurs with a relatively high frequency (20–40%) in organ-transplanted patients with high blood drug levels or within the therapeutic range (The U.S. Multicenter FK506 Liver Study Group, 1994; Pirsch *et al.*, 1997; Gijtenbeek *et al.*, 1999). However, the mechanism of CsA-induced neurotoxicity remains obscure.

The entry of CsA into the brain is usually prevented by the tight junctions and P-glycoprotein (P-gp), a multi-drug efflux pump, of brain microvascular endothelial cells. But CsA-associated neurotoxicity, including tremors, seizures and encephalopathy, strongly suggests the possibility that CsA is transported across the blood-brain barrier (BBB). We previously reported that CsA produced convulsions by inhibiting γ -aminobutyric acid (GABA)ergic neural activity and the binding properties of the GABA_A receptor (Shuto *et al.*, 1999). The inhibition of GABAergic neurotransmission by CsA may lead to an activation of serotonergic neural activity and, consequently, produce tremors (Shuto *et al.*, 1998). These *in vivo* findings are considered to be due to a direct action of CsA transported across the BBB rather than an indirect effect of CsA in the periphery. Indeed, we previously demonstrated that a high concentration of CsA decreased the function and expression of P-gp in brain capillary endothelial cells (Kochi *et al.*, 1999, 2000). The BBB is primarily formed from these cells, which are closely sealed by tight junctions (Pardridge, 1999). P-gp is abundantly expressed in brain endothelial cells and limits the accumulation of many hydrophobic molecules and toxic substances in the brain (Schinkel, 1999). Brain capillary endothelial cells are surrounded by two other cellular components of the BBB, astrocytes and brain pericytes. We also previously reported that the presence of astrocytes markedly aggravated CsA-induced hyperpermeability of, and P-gp dysfunction in, MBEC4 cells, through the acceleration of NO production (Dohgu *et al.*, 2004a). Brain pericytes are important for the growth and migration of endothelial cells and the integrity of microvascular capillaries (Thomas, 1999; Ramsauer *et al.*, 2002). Brain capillary endothelial cells communicate closely with brain pericytes to maintain the BBB (Hori *et al.*, 2004; Dohgu *et al.*, 2005). Transforming growth factor- β (TGF- β) is a cytokine produced by pericytes (Antonelli-Orlidge *et al.*, 1989). We previously reported that brain pericytes contribute to the up-regulation of barrier function and P-gp activity in brain endothelial cells through production of TGF- β 1 (Dohgu *et al.*, 2005).

The present study was designed to clarify whether brain pericytes and pericyte-derived TGF- β participate in CsA-induced dysfunction of the BBB. We first evaluated the effect of CsA on the permeability of, and P-gp function in, mouse brain capillary endothelial (MBEC4) cells, either alone or co-cultured with human brain pericytes. Next, the effect of CsA on TGF- β 1 mRNA expression in brain pericytes and the effect of TGF- β 1 on CsA-decreased BBB function were examined in a co-culture system containing MBEC4 cells and brain pericytes.

72

MATERIALS AND METHODS

73

Materials

74

75

76

77

78

CsA was kindly supplied by Novartis Pharma (Basel, Switzerland). Sodium fluorescein (Na-F, MW 376), rhodamine 123 and human TGF- β 1 were purchased from Sigma (St. Louis, MO). Culture medium and subculture reagents were obtained from Invitrogen (Carlsbad, CA). All remaining reagents of analytical grade were purchased from Wako (Osaka, Japan).

79

Cell Culture

80

81

82

83

84

85

86

87

88

89

90

91

92

MBEC4 cells, isolated from BALB/c mouse brain cortices and immortalized by SV40-transformation (Tatsuta *et al.*, 1992), were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 units/mL penicillin and 100 μ g/mL streptomycin. Human brain pericytes (CS-ABI-499, Cell Systems Corporation, Kirkland, WA) were cultured in CS-C Complete Medium Kit (Cell Systems Corporation). They were grown in a humidified atmosphere of 5% CO₂/95% air at 37°C. To make an *in vitro* BBB model, brain pericytes (20,000 cells/cm²) were first cultured in the wells of a 12-well culture plate. After 2 days, MBEC4 cells (42,000 cells/cm²) were seeded on the inside of the collagen-coated polycarbonate membrane (1.0 cm², 3.0 μ m pore size) of a Transwell[®]-Clear insert (12-well type, Costar, MA) placed in the plate containing layers of brain pericytes (pericyte co-culture). A monolayer system was also generated with MBEC4 cells alone (MBEC4 monolayer).

93

94

95

96

Cell viability was assessed using a WST-8 assay (Cell Counting Kit, DOJINDO, Kumamoto, Japan). The absorbance of a highly water-soluble formazan dye (WST-8), reduced by mitochondrial dehydrogenase, was measured in each sample at wavelengths of 450-nm (test wavelength) and 700-nm (reference wavelength).

97

Treatment with CsA and TGF- β 1

98

99

100

101

102

103

104

105

106

107

TGF- β 1 was dissolved in 4 mM HCl containing 1 mg/mL of bovine serum albumin; CsA was dissolved in ethanol. Each original solution was then diluted with serum-free medium. The final concentrations in the test media were 4 μ M HCl/1 μ g/mL bovine serum albumin or 0.1% ethanol. MBEC4 cells were cultured for 3 days, and the inserts were washed three times with serum-free medium. Cells were then exposed for 1–12 h to 5 μ M of CsA injected into the inside of the insert (luminal side). Alternatively, TGF- β 1 (0.01–1 ng/mL) was loaded on the luminal side. In parallel, cells were treated with serum-free medium containing the corresponding amount of ethanol and/or HCl and bovine serum albumin as the vehicle.

Transcellular Transport of Na-F

108

To initiate the transport experiments, the medium was removed and MBEC4 cells were washed three times with Krebs–Ringer buffer (118 mM NaCl, 4.7 mM KCl, 1.3 mM CaCl₂, 1.2 mM MgCl₂, 1.0 mM NaH₂PO₄, 25 mM NaHCO₃, and 11 mM D-glucose, pH 7.4). Krebs–Ringer buffer (1.5 mL) was added to the outside of the insert (abluminal side). Krebs–Ringer buffer (0.5 mL) containing 100 μg/mL of Na-F was loaded on the luminal side of the insert. Samples (0.5 mL) were removed from the abluminal chamber at 10, 20, 30 and 60 min and immediately replaced with fresh Krebs–Ringer buffer. Aliquots (5 μL) of the abluminal medium were mixed with 200 μL of Krebs–Ringer buffer and the concentration of Na-F was determined using a fluorescence multiwell plate reader (Ex(λ) 485 nm; Em(λ) 530 nm) (CytoFluor Series 4000, PerSeptive Biosystems, Framingham, MA). The permeability coefficient and clearance were calculated according to the method described by Dehouck *et al.* (1992). Clearance was expressed as microliters (μL) of tracer diffusing from the luminal to abluminal chamber and was calculated from the initial concentration of tracer in the luminal chamber and final concentration in the abluminal chamber: clearance (μL) = [C]_A × V_A/[C]_L where [C]_L is the initial luminal tracer concentration, [C]_A is the abluminal tracer concentration and V_A is the volume of the abluminal chamber. During the 60-min period of the experiment, the clearance volume increased linearly with time. The average volume cleared was plotted against time, and the slope was estimated by linear regression analysis. The slope of clearance curves for the MBEC4 monolayer or co-culture systems was denoted by PS_{app}, where PS is the permeability–surface area product (in μL/min). The slope of the clearance curve with a control membrane was denoted by PS_{membrane}. The real PS value for the MBEC4 monolayer and the co-culture system (PS_{trans}) was calculated as 1/PS_{app} = 1/PS_{membrane} + 1/PS_{trans}. The PS_{trans} values were divided by the surface area of the Transwell inserts to generate the permeability coefficient (P_{trans}, in cm/min).

109
110
111
112
113
114
115
116
117
118
119
120
121
122
123
124
125
126
127
128
129
130
131
132
133
134
135

Functional Activity of P-gp

136

The functional activity of P-gp was determined by measuring the cellular accumulation of rhodamine 123 (Sigma) according to the method of Fontaine *et al.* (1996). MBEC4 cells were washed three times with assay buffer (143 mM NaCl, 4.7 mM KCl, 1.3 mM CaCl₂, 1.2 mM MgCl₂, 1.0 mM NaH₂PO₄, 10 mM HEPES, and 11 mM D-glucose, pH 7.4), and then incubated in 0.5 mL of assay buffer containing 5 μM rhodamine 123 for 60 min. The solution was then removed and the cells were washed three times with ice-cold phosphate-buffered saline and solubilized in 1 M NaOH (0.2 mL). The solution was neutralized with 1 M HCl (0.2 mL) and the rhodamine 123 content was determined using a fluorescence multiwell plate reader (Ex(λ) 485 nm; Em(λ) 530 nm, CytoFluor Series 4000). Protein concentration was measured by the method of Bradford (Bradford, 1976).

137
138
139
140
141
142
143
144
145
146
147

148 **Expression of TGF- β 1 Receptor mRNA in MBEC4 Cells and Human Brain**
149 **Pericytes**

150 Reverse-transcription polymerase chain reaction (RT-PCR) was employed
151 to determine the level of mRNA expression for TGF- β 1 receptor I and II in
152 MBEC4 cells and brain pericytes. Total RNA was extracted from cultured cells us-
153 ing TRIzolTM reagent (Invitrogen, Carlsbad, CA) and 1 μ g of RNA was reverse-
154 transcribed and amplified by PCR using a SuperScript One-Step RT-PCR sys-
155 tem (Invitrogen). Amplification was performed in a DNA thermal cycler (PC707;
156 ASTEC, Fukuoka, Japan). The primers used and PCR conditions are summarized
157 in Table I. Ten microliters of each PCR product was analyzed by electrophoresis on
158 a 2% agarose (Sigma) gel with ethidium bromide staining. Gels were visualized on
159 a UV light transilluminator and photographed using a DC290 Zoom digital camera
160 (Kodak, Rochester, New York).

161 **Relative Quantitation of TGF- β 1 mRNA by Real-Time RT-PCR**

162 Real-time RT-PCR was employed to determine the level of TGF- β 1 gene ex-
163 pression in brain pericytes with CsA-treated pericyte co-culture. Total RNA was
164 extracted from brain pericytes using TRIzolTM reagent (Invitrogen) and 2 μ g RNA
165 was reverse-transcribed using a SuperScriptTM III First-Strand Synthesis System
166 (Invitrogen) in a total volume of 20 μ L, according to the manufacturer's protocol.

167 Real-time PCR was conducted on an Mx3000PTM Multiplex Quantitative PCR
168 System (Stratagene, La Jolla, CA) with 2 μ L of reverse-transcription product,
169 BrilliantTM SYBR[®] Green QPCR Master Mix (Stratagene), primers at 150 nM
170 and reference dye, in a total volume of 50 μ L as per the manufacturer's pro-
171 tocol. The following PCR conditions were employed: 95°C for 10 min, followed
172 by cycles of 95°C for 30 s, 54°C for 60 s and 72°C for 90 s. The sequences of
173 primers were as follows: sense primer 5'-CCCTGGACACCAACTATTG-3' and
174 antisense primer 5'-CCGGGTTATGCTGGTTGTA-3' for TGF- β 1 (Untergasser
175 *et al.*, 2005); sense primer 5'-GAGTCAACGGATTGGTCGT-3' and antisense
176 primer 5'-TTGATTTGGAGGGATCTCG-3' for glyceraldehyde-3-phosphate de-
177 hydrogenase (GAPDH; GenBank Accession Number, M33197). After amplifica-
178 tion, a melting curve was obtained by heating at 55°C and fluorescence data were
179 collected at 0.2°C/s.

180 Relative quantitative analysis was performed employing Mx3000PTM Multi-
181 plex Quantitative PCR System software (Stratagene). We used the expression of
182 GAPDH to normalize the expression data for the TGF- β 1 gene. For a comparative
183 analysis, values from vehicle treated brain pericytes were arbitrarily set as 1. Each
184 sample was analyzed in triplicate.

185 **Statistical Analysis**

186 Values are expressed as means \pm SEM. Statistical analysis was performed us-
187 ing Student's *t*-test. One-way and two-way analyses of variance (ANOVAs) fol-
188 lowed by Tukey-Kramer's tests or Dunnett tests were applied to multiple com-

Table 1 Nucleotide Sequences of Probes Used in RT-PCR Assays and Amplification Conditions

Gene	Source (reference)	Sequence	Product (bp)	Amplification conditions
TGF- β R	Mouse (Machida <i>et al.</i> , 2000)	Sense 5'-ATCCATCACTAGATCGCCCT-3'	824	94°C for 30 s, 57°C for 30 s, 72°C for 30 s
	Human (GenBank Accession Number, L11695)	Antisense 5'-CGATGGATCAGAAAGGTACAAGA-3' Sense 5'-GATGGGCTCTGCTTTGTCTC-3'	214	94°C for 30 s, 54°C for 30 s, 72°C for 30 s
TGF- β R	Mouse (Machida <i>et al.</i> , 2000)	Antisense 5'-CAAGGCCAGGTGATGACTTT-3' Sense 5'-CGTGTGGAGGAAGAAACA-3'	560	94°C for 30 s, 57°C for 30 s, 72°C for 30 s
	Human (GenBank Accession Number, E10743)	Antisense 5'-TCTCAAACTGCTCTGAGGTG-3' Sense 5'-TTTCCACCTGTGACAACCA-3'	185	94°C for 30 s, 54°C for 30 s, 72°C for 30 s
		Antisense 5'-GGAGAAGCAGCATCTTCCAG-3'		

189 parisons. The differences between means were considered to be significant when
 190 *P* values were less than 0.05.

191

RESULTS

192 To obtain molecular evidence for the expression of TGF- β receptor I and II in
 193 MBEC4 cells and human brain pericytes, RT-PCR was carried out with a primer
 194 pair specific to each type of TGF- β receptor, from either mouse (for use on MBEC4
 195 cells) or human (for use on human brain pericytes). As shown in Fig. 1A, RT-PCR
 196 with mRNA obtained from either MBEC4 cells or brain pericytes yielded a single
 197 product. The size of these products was as expected from the primer positions.

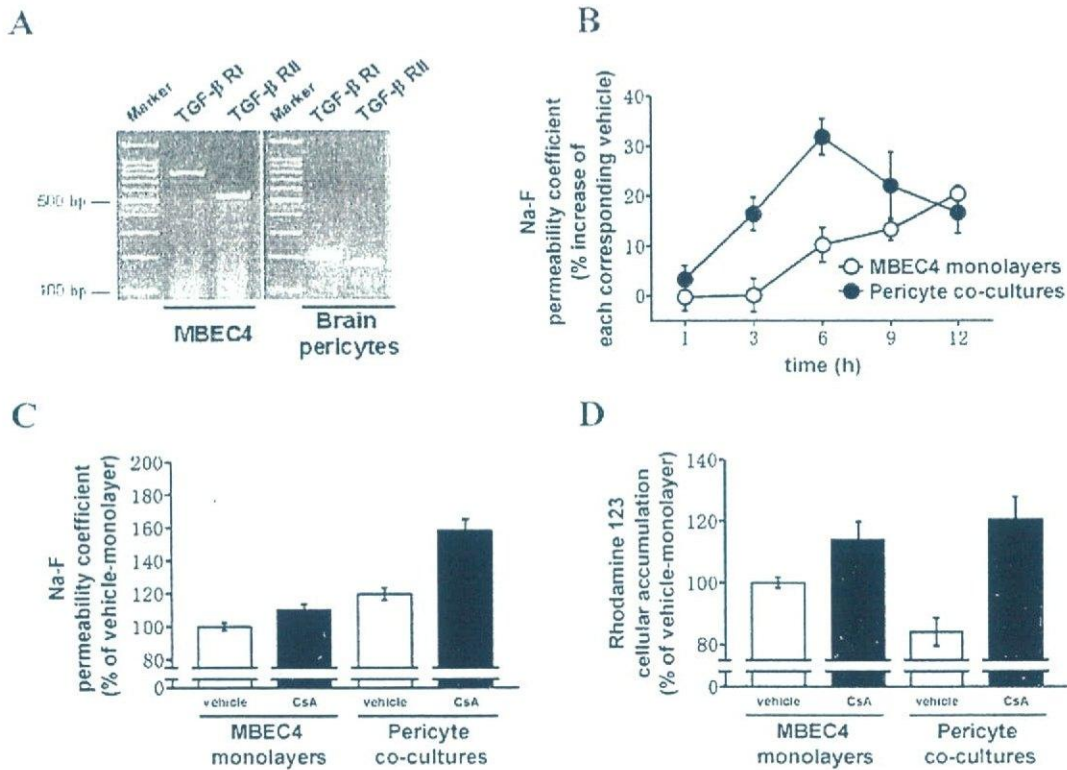


Fig. 1 (A) Expression of TGF- β receptor I and II mRNA in MBEC4 cells and human brain pericytes by RT-PCR analysis. (B) Time-course of the effect of CsA ($5 \mu\text{M}$) on the Na-F permeability of MBEC4 cells in MBEC4 monolayers and pericyte co-cultures. Transport experiments were performed after 1, 3, 6, 9 and 12 h of exposure to CsA. Results are expressed as % increase of each corresponding vehicle treatment (MBEC4 monolayers; $2.78 \pm 0.10 \times 10^{-4}$ to $3.47 \pm 0.14 \times 10^{-4}$ cm/min, pericyte co-cultures; $3.24 \pm 0.10 \times 10^{-4}$ to $4.47 \pm 0.20 \times 10^{-4}$ cm/min). Values are the means \pm SEM ($n = 7-16$). (C) Effect of treatment with CsA ($5 \mu\text{M}$) for 6 h on the Na-F permeability of MBEC4 cells in MBEC4 monolayers and pericyte co-cultures. Results are expressed as % of vehicle-treated MBEC4 monolayers (% of vehicle-monolayer ($3.26 \pm 0.13 \times 10^{-4}$ cm/min)). Values are the means \pm SEM ($n = 7$). (D) Effect of treatment with CsA ($5 \mu\text{M}$) for 6 h on the rhodamine 123 accumulation of MBEC4 cells in MBEC4 monolayers and pericyte co-cultures. Results are expressed as % of vehicle-treated MBEC4 monolayers (% of vehicle-monolayer (0.51 ± 0.12 nmol/mg protein)). Values are the means \pm SEM ($n = 8$).

When MBEC4 cells were co-cultured with pericytes, the Na-F permeability of MBEC4 cells was increased from $3.26 \pm 0.13 \times 10^{-4}$ cm/min in monolayers to $3.86 \pm 0.23 \times 10^{-4}$ cm/min (Fig. 1C). The presence of pericytes decreased rhodamine 123 accumulation in MBEC4 cells from 0.51 ± 0.12 nmol/mg protein in monolayers to 0.43 ± 0.10 nmol/mg protein (Fig. 1D). A 12 h-exposure to CsA at 5 or 10 μ M showed no effect on cell viability as determined by mitochondrial dehydrogenase activities (WST-8 assay) of MBEC4 cells in MBEC4 monolayers (100.0 ± 1.8 and $103.1 \pm 7.8\%$ of vehicle, respectively, $n = 4$ inserts) and pericyte co-cultures (99.2 ± 2.5 and $106.8 \pm 2.3\%$ of vehicle, respectively, $n = 4$ inserts). Figure 1C and D shows the effect of CsA (5 μ M) on the permeability of MBEC4 cells to Na-F and the accumulation of rhodamine 123 in MBEC4 cells, respectively, in both MBEC4 monolayers and pericyte co-cultures. The Na-F permeability of MBEC4 cells was time-dependently increased during a period of 1–6 h after the addition of CsA (5 μ M), reaching a peak at 6 h (Fig. 1B). A significant difference in CsA-induced hyperpermeability was observed between MBEC4 monolayers and pericyte co-cultures, with the effect being most apparent after a 6 h-exposure [$F(1, 24) = 10.51, P < 0.01$] (Fig. 1C). At the peak time, CsA increased the Na-F permeability of MBEC4 cells by 10.3 ± 3.4 and $31.9 \pm 3.6\%$ of each corresponding vehicle treatment in MBEC4 monolayers and pericyte co-cultures, respectively (Fig. 1C). Following exposure of cells to CsA for 9–12 h, permeability of the MBEC4 monolayers was gradually increased. However, the effect of CsA on pericyte co-cultures permeability became more moderate, reducing to the same level seen in MBEC4 monolayers (Fig. 1B). To clarify the role of brain pericytes in CsA-induced dysfunction of BBB, a 6 h-exposure of cells to CsA (5 μ M) was employed in the following experiment. The accumulation of rhodamine 123 in MBEC4 cells was increased by 13.9 ± 5.9 and $43.4 \pm 8.5\%$ of each corresponding vehicle treatment in MBEC4 monolayers and pericyte co-cultures, respectively, after a 6 h-exposure to CsA (Fig. 1D). A significant difference in CsA-induced decrease in P-gp activity was observed between MBEC4 monolayers and pericyte co-cultures [$F(1, 28) = 4.65, P < 0.05$]. CsA-induced inhibition of P-gp function was more potent in pericyte co-cultures than in MBEC4 monolayers.

When pericyte co-cultures were treated with CsA (5 μ M) for 6 h, the levels of TGF- β 1 mRNA in brain pericytes were significantly decreased to $81.0 \pm 7.3\%$ of vehicle (Fig. 2A) [$F(2, 13) = 5.05, P < 0.05$]; however, a 12 h exposure to CsA failed to decrease levels of TGF- β 1 mRNA in brain pericytes ($103.8 \pm 10.8\%$ of vehicle). In pericyte co-cultures, TGF- β 1 (0.01–1 ng/mL) dose-dependently inhibited the elevation of Na-F permeability (Fig. 2B), and rhodamine 123 accumulation (Fig. 2C), in MBEC4 cells, induced by CsA (132.9 ± 11.4 to $105.4 \pm 2.5\%$ and 140.3 ± 9.9 to $98.6 \pm 6.4\%$ of vehicle, respectively).

DISCUSSION

In the present study, CsA (5 μ M) time-dependently increased the Na-F permeability of MBEC4 cells in pericyte co-cultures, this effect reaching a peak at 6 h

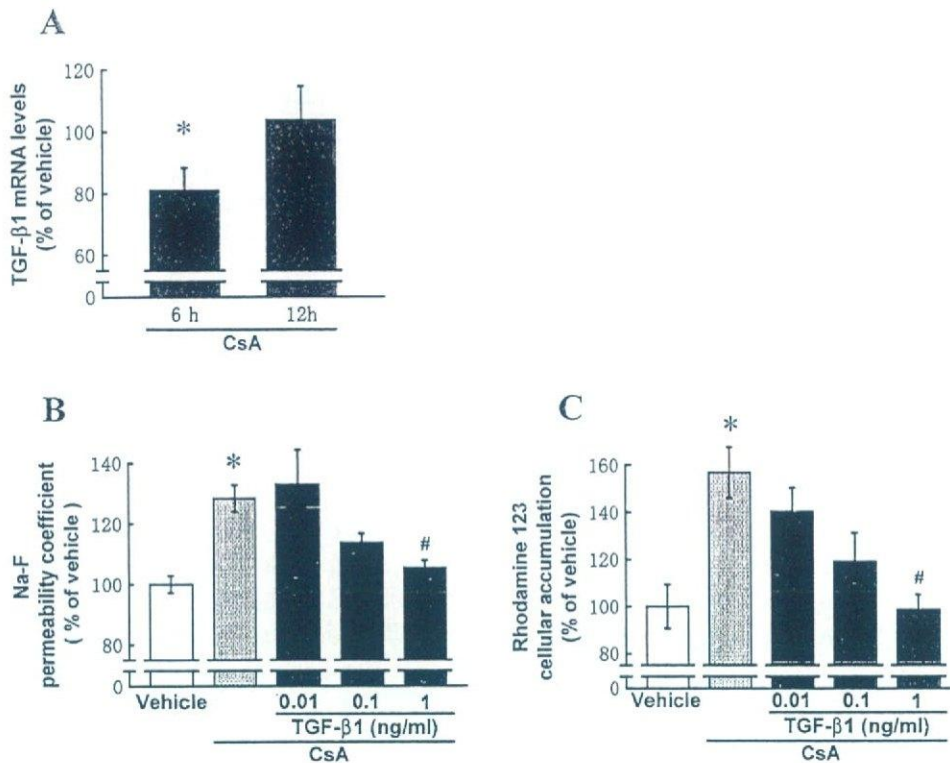


Fig. 2 (A) Effect of CsA ($5 \mu\text{M}$) on TGF- β 1 mRNA expression in brain pericytes at 6 and 12 h after the addition of CsA in pericyte co-cultures. Total RNA of brain pericytes was extracted and subjected to real-time PCR analysis. Fold changes in TGF- β 1 mRNA are normalized to GAPDH and compared with each corresponding vehicle treatment. Values are the means \pm SEM ($n = 3-5$). * $p < 0.05$, significant difference from vehicle. (B) Effect of TGF- β 1 on CsA-increased Na-F permeability of MBEC4 cells in pericyte co-cultures. Results are expressed as % of vehicle (vehicle; $3.28 \pm 0.22 \times 10^{-4}$ cm/min). Values are the means \pm SEM ($n = 8-16$). * $p < 0.05$, significant differences from vehicle. # $p < 0.05$, significant difference from CsA treatment. (C) Effect of TGF- β 1 on CsA-increased rhodamine 123 accumulation of MBEC4 cells in pericyte co-cultures. Results are expressed as % of each corresponding vehicle treatment (vehicle; 1.16 ± 0.24 nmol/mg protein). Values are the means \pm SEM ($n = 4-12$). * $p < 0.05$, significant differences from vehicle. # $p < 0.05$, significant difference from CsA treatment.

240 after the addition of CsA (Fig. 1B and C). A 6 h exposure to CsA also decreased P-
 241 gp function in MBEC4 cells in pericyte co-cultures, this effect being more apparent
 242 than that in MBEC4 monolayers (Fig. 1D). These findings suggest that CsA-induced
 243 hyperpermeability of, and P-gp dysfunction in, MBEC4 cells, was aggravated in per-
 244 icyte co-cultures. We previously reported that treatment with CsA ($0.5-10 \mu\text{M}$) for
 245 24 h dose-dependently decreased the viability of MBEC4 cells (Kochi *et al.*, 1999).
 246 The present study demonstrated that a 12 h-exposure to CsA at concentrations up
 247 to $10 \mu\text{M}$ had no effect on the viability of MBEC4 cells in MBEC4 monolayers and
 248 pericyte co-cultures. Thus, the exposure time (6 h) and the submaximum concentra-
 249 tion ($5 \mu\text{M}$) of CsA without cytotoxicity were selected here.

250 The significance of brain pericytes in the regulation of the BBB was suggested
 251 by our previous study using a primary culture of rat brain pericytes. We reported

that brain pericyte-derived TGF- β 1 contributed to the induction and up-regulation of BBB function (Dohgu *et al.*, 2004b, 2005). In the present study, the presence of human brain pericytes decreased the function of tight junctions and increased the function of P-gp, in MBEC4 cells, by about 20%. An interjunctional property of MBEC4 cells was lowered by co-culturing with human brain pericytes, this event being inconsistent with our previous report using a primary culture of rat brain pericytes. The different backgrounds of human brain pericytes commercially supplied and species-different cell type may be compounding factors in this discrepancy (Lai and Kuo, 2005); however, further studies are required to confirm this. Here, we employed a convenient *in vitro* model with MBEC4 cells and human brain pericytes.

To test whether TGF- β 1 production of the brain pericytes participates in the mediation of CsA-induced dysfunction of the BBB, we examined the effect of CsA on the expression of TGF- β 1 in brain pericytes. A 6 h exposure to CsA (5 μ M) significantly decreased the levels of TGF- β 1 mRNA in brain pericytes in pericyte co-cultures (Fig. 2A). Treatment with TGF- β 1 dose-dependently inhibited CsA-induced hyperpermeability and P-gp dysfunction in MBEC4 cells in pericyte co-cultures (Fig. 2B and C). These findings suggest that CsA decreases BBB function by inhibiting TGF- β 1 production in the brain pericytes. In pericyte co-cultures, an aggravation of CsA-induced hyperpermeability of MBEC4 cells occurred with a peak at 6 h after the addition of CsA, becoming more moderate at 9–12 h (Fig. 1B). In parallel with these events, TGF- β 1 mRNA levels were significantly decreased by a 6 h exposure to CsA but not by a 12 h exposure (Fig. 2A), suggesting that CsA-induced hyperpermeability is ameliorated by the compensatory secretion of TGF- β 1 from brain pericytes during the later period of CsA exposure. These data further support a critical role of pericyte-derived TGF- β 1 in mediating CsA-induced BBB dysfunction.

RT-PCR analysis demonstrated the expression of TGF- β receptor I and II in MBEC4 cells and brain pericytes (Fig. 1A). TGF- β 1 mRNA was also detected in brain pericytes (Fig. 2A) and MBEC4 cells (data not shown). Considering these observations, TGF- β is likely to participate in the up-regulation of BBB function through an autocrine and/or paracrine pathway in brain endothelial cells and pericytes. Autoinduction of TGF- β 1 is mediated by binding of the transcription factor activator protein-1 (AP-1) complex to homologous elements in two regions of the TGF- β 1 promoter (Kim *et al.*, 1990). In this positive autoregulation process, CsA is known to directly inhibit activation of the JunD isoforms in the AP-1 complex responsible for TGF- β signaling in lung fibroblasts (Eickelberg *et al.*, 2001). Calcineurin, a molecular target of CsA, has been shown to be involved in AP-1 activation in immune cells (Pfeuffer *et al.*, 1994; Tsuboi *et al.*, 1994). Based on this evidence, CsA may be interpreted as lowering TGF- β 1 expression in brain pericytes due, at least in part, to an inhibition of the AP-1 activation step during TGF- β 1 autoinduction. We previously reported that CsA increased NO production in MBEC4 cells (Dohgu *et al.*, 2004a) and elevated levels of NO have been known to reduce TGF- β 1 production in the heart (Smith *et al.*, 2005). Therefore, CsA may also act on MBEC4 cells to lower TGF- β 1 expression in brain pericytes.