Genetic studies have been conducted in the past 30 years. In the first phase, the association of MMD with HLA was investigated extensively, but the opinions remained equivocal. In the second phase, linkage analysis was performed, which demonstrated multiple loci—3p24.2—26, 6q, 8q23, 12p12, and 17q25. None of these studies were replicated. A large genome-wide linkage analysis using 3 generation families that has been performed in 2008 has resolved the enigma of MMD and revealed a single locus on17q25. This locus is expected to provide a clue to the genetic basis for MMD, paving a way to comprehensive understanding of molecular consequences in MMD.

Key words : moyamoya disease, genetic factor, pathophysiological investigations, HLA analysis, genetic analysis

はじめに

もやもや病は，両側の内頸動脈終末部の狭窄もしくは閉塞および，いわゆる「もやもや血管」と呼ばれる閉塞部近傍の異常血管網の形成を認める，慢性進行性の疾患である[1,2]。

1997年の報告によれば，罹患率0.35人/10万人年，有病率3.16人/10万人といわれていたが[3]，2007年Babaらは北海道で悉皆疫学調査を行い，罹患率0.94人/10万人年，有病率10.5人/10万人と報告している[4]。また2008年Kuriyamaらは，罹患率は0.54人/10万人年，有病率10.5人/10万人と報告している[5]。このほかIkedaらの報告[6]など，もやもや病の罹患率および有病率は，核磁気共鳴装置の普及により増加している[7,8]。

家族内で発症のみられる，いわゆる家族性もやもや病の割合は10%以上であり，東アジア人に多くの患者が認められていること[9]，一卵性双生児がともに発症する確率が80%と高いこと[10]など，遺伝背景が強いと示唆される[11,12]。しかし，同時に浸透率が低く，一卵性双生児でも完全に罹患状態は一致しないことから，環境要因の関与も無視できない。本報告では，2000年以前の総説[13,14]以降を中心に，2008年の段階における遺伝子研究の経過および現状を，筆者らの検討も含め報告する。

Ⅰ．種々の血管関連因子の関与（Table 1）

もやもや病の特徴的な病理学的所見として，内膜の肥厚や内弾性板の屈曲・中膜の菲薄化が認められる[15]。1990年以降，もやもや病の病理学的所見や，関連遺伝子の発現の評価や関連遺伝子の変異を同定し，発症の要因や原因遺伝子の特定を試みる研究が行われ，platelet-derived growth factor（PDGF）およびそのreceptorやbasic fibroblast growth factor（bFGF），hepatocyte growth factor（HGF），vascular endothelial growth factor（VEGF），cellular retinoic acid-binding protein 1（CRABP-1）などの成長因子の検討や，prostaglandin E2（PGE2）・cyclooxygenase-2（Cox2）などの炎症関連蛋白の検討により，もやもや病の病態との相関性が報告されてきた[16-31]。

Yamamotoら[22]はAoyagiら[20]の報告により，smooth muscle cell（SMC）内のelastin生合成およびtransforming growth factor-β1（TGFβ1）の検討を行い，elastinのmRNAおよび蛋白の発現およびTGFβ1の増加を認めた。

Hojoら[24]は，脳脊髄液でbFGFが上昇しているという報告をもとに[16,21]，SMCと血清中のbFGFとTGFβ1の遺伝子の発現，および血清中のTGFβ1の量を検討し，TGFβ1の発現の上昇を示した。Uenoら[32]は，Hojoら[24]の報告をもとに，TGF関連遺伝子のうち*TGFβ1*および*TGFβR2*の多型解析を行ったが，もやもや病と関連のある多型は認めなかった。

上記のように，さまざまな関連蛋白およびその遺伝子の発現の評価により，血管の結合組織の異常や細胞増殖因子増加が見出され，SMC増殖の理解に迫る所見が得られてきた。

また組織標本も，硬膜や脳脊髄液を用いた研究[18,19,27,28,31]やsuperficial temporal artery（STA）のSMCを用いた研究[16,18,22,24,26]やautopsyからの標本を用いた報告[25]がされていたが，Takagiらは，MCAを用いてcaspase3依存性のapoptosis[29]や，hypoxia-inducing factor-1（HIF-1α），endoglin，transforming growth factor-β3（TGFβ3）が，コントロール群に比して有意に発現していることなど[30]，生体のmiddle cerebral artery（MCA）における血管関連因子の動態について，より生体に近い状態での観察を報告した。

現在の知見では，血管平滑筋細胞の増殖シグナルが常に高い状態に保たれ，その一方でapoptosisの亢進など全体として平滑筋細胞の回転の亢進があるものと考えられる。

Table 1　2008年までに行われてきた血管関連因子の要約

研究者	因子	対象	試料	結果
Yamamoto, et al (1997)[22]	elastin	case　6名, control　4名＋7名	STA	caseにてelastinのmRA, protein levelともに高値
Aoyagi, et al (1993)[16]	PDGF receptor	case　4名, control　4名	STA	caseにてPDGF receptorのdown-regulation
Sakamoto, et al (2008)[31]	VEGF	case　7名, control　4名	dura	VEGF-positive cellsの発現の上昇
Hoshimaru, et al (1991)[18]	bFGF	case　4名, control　3名	STA & dura	caseにてSTA, duraともに, 強く発現
Takahashi, et al (1993)[19]	bFGF	case　15名, control　19名	CSF	CSF内のbFGFの発現の上昇
Houkin, et al (1998)[25]	bFGF	case　2名, control　2名	ICA & MCA	bFGFが内皮細胞に限局的な発現 apototic cellの発現
Hojo, et al (1998)[24]	TGFβ1	STA：case　6名, control　4名 serum：case　14名, control　10名	STA, serum	caseにてbFGF, TGFβ1のmRNAおよび血清中TGFβ1の上昇
Ueno, et al (2000)[23]	TGFβ1	case　61名, control　99名	blood	遺伝子多型と疾患に明らかな相関なし
Nanba, et al (2004)[28]	HGF	case　39名, control　6名	CSF	caseにてHGFの上昇
Nanba, et al (2004)[29]	c-Met	case　2名, control　2名	ICA & MCA	caseにてHGFおよびc-Metの発現の上昇
Kim, et al (2003)[27]	CRABP-I	case　20名, control　4名	CSF	caseにてCRABP-Iの特異的発現
Takagi, et al (2007)[30]	HIF-1α endoglin	case　12名, control　12名	MCA	HIF-1αのendothelium, intimaでの発現の上昇, endoglin, TGF-β3のendotheliumでの発現の上昇
Yamamoto, et al (1999)[26]	PGE2, COX2	case　12名, control　8名	STA	IL-1βの刺激により, PGE2, COX2ともに有意に上昇
Takagi, et al (2006)[29]	ssDNA　cleaved caspase-3	case　10名, control　10名	MCA	caseにてssDNAはintima, mediaともに, cleaved caspase-3はmediaにて上昇

一方，平滑筋細胞への分裂増殖のシグナルの亢進がどのようなメカニズムで生じるのか，あるいは亢進そのものがもやもや病の本体であるのかについては依然不明であり，今後明らかにする必要がある。特に血管新生において中心的な役割を担うもう1つの重要な細胞構成成分である血管内皮細胞の関与について評価する必要がある。特にもやもや病では，血管内皮細胞の細胞回転も亢進している可能性が高く[33]，内皮細胞のストレスに対する脆弱性なども評価する必要がある。もやもや病の原因遺伝子が同定された際，もやもや病に特徴的な内膜の肥厚や，新生血管の発現などの病態解明において，非常に重要な知見となりうるため，今後も継続的な研究が必要である。

II. Human leukocyte antigen (HLA) との相関研究 (Table 2)

HLAタイプはBehçet病や，高安病，リウマトイド血管炎などの血管と関連の高い疾患や，自己免疫性疾患や感染性疾患，ウイルス関連腫瘍など多様な疾患との相関が指摘される報告がある。もやもや病の除外診断として，髄膜炎や，自己免疫性疾患があるように[15]，炎症性疾患[34]やsystemic lupus erythematosus (SLE)[35]，antiphospholipid antibodies (APS)[36-38]，Graves病[39]などの自己免疫疾患に伴い，もやもや血管を認める類もやもや病の報告は，過去にも多数報告されている。特に環境要因として感染性病原体や自己免疫の関与を考える際にHLAは重要な示唆を与えてくれるため[40-42]，もやもや

Table 2　2008年までに行われてきたHLAとの相関研究の要約

研究者	対象	方法	結果
Kitahara, et al (1982)[43]	日本人 case 18名 control 106名	HLA class I	RR：HLA-AW24＝3.83（P＜0.05），Bw46＝6.50（P＜0.05），Bw54＝3.58（P＜0.025）
Aoyagi, et al (1995)[44]	日本人 case 32名 control 178名	HLA class I，HLA class II（DR，DQ）	HLA-B51，B67，DR1，Cw1に正の相関　Aw24，Bw46，Bw54に相関関係は認めない
Inoue, et al (1997)[45]	日本人 case 71名 control 525名	HLA class II	HLA-DRB1*0502に正の相関，HLA-DRB1*0405，DRB1*0401に負の相関
Han, et al (2003)[46]	韓国人 case 28名 control 198名	HLA class I，class II	HLA-B35相関あり。HLA-B35は女性，壮年発症において有意に相関。HLA-B51，DRB1*0502，DRB1*0405，DRB1*0401では明らかな相関なし

Table 3　2008年までに行われてきた遺伝解析の要約

	対象	領域	方法	結果
〔連鎖解析〕				
Ikeda, et al (1999)[47]	16家系　77名（男性28名，女性49名）うち患者37名（男性14名，女性23名）	Whole genome	Multipoint Non-parametric method	3p24.2-26（NPL＝3.46 P＜0.000238）
Inoue, et al (2000)[48]	19家系	6番染色体	Affected sib pair analysis	D6S441にてIBD（0：12：8）と連鎖不平衡を認めた。
Yamauchi, et al (2000)[49]	24家系　103名（男性44名，女性59名）うち患者56名（男性19名，女性37名）	17番染色体長腕	Parametric analysis (AD mode, AR mode)	17q25でAD mode AR modeともに，D17s939にてmaximum LOD score 4.58
Sakurai, et al (2004)[50]	12家系　46名	Whole genome	Affected sib pair analysis	8q23.1（MLS＝3.6 NPL＝3.3　P＜0.000072），12p12（MLS＝2.3 NPL＝2.5　P＜0.004）
〔相関解析〕				
Kang, et al (2006)[51]	家族性もやもや病の患者11名と50名のnormal control，50名の孤発性もやもや病	3p24.2-26，TIMP4，17q25，TMP2	Association study	家族性もやもや病においてTIMP2 promotor-418にてG/C多型　OR＝18.00と高値。Exon3＋853にてG/A多型　OR＝3.20
〔その他解析〕				
Nanba, et al (2005)[11]	Yamauchi, et al (2000)にて17q25の連鎖が確認された1家系	17q25限定	9の遺伝子に対して，sequence analysis，2,100個のESTを用いてbioinformatics	明らかな既知および未知の原因遺伝子は認められなかった。

病との相関性についてもこれまでにいくつかの研究が行われてきた。

Kitaharaら[43]は日本人18例のcaseと106例のcontrolにおいて相対危険度を解析し，HLA-AW24，BW46，BW54にて有意に危険度が高いと報告した。また同時に以前の研究デザインで使用した31名のcaseを追加し，同様に解析を行い，HLA-BW54＝3.80（P＜0.005）と有意な相関があったことを報告している。

Aoyagiら[44]は日本人32例のcaseと178例のcontrolを用いて，HLA-B51，B67，DR1，Cw1において相関を指摘した。また同時にAW24，BW46，BW54においては明らかな相関関係は認められなかったことを報告した。

Inoueら[45]が日本人71例のcaseおよび525例のcontrolを用いてHLA　class II遺伝子の解析を行い，DRB1*0502にて正の相関を認めており，一方DRB1*0405やDRB1*0401において負の相関をしていることを示した。

Hanら[46]は韓国人28例のcaseおよび198例のcontrolを用いてHLA class I遺伝子，class II遺伝子の解析を行っている。結果，HLA-B35に有意な相関があると報告している。またHLA-B35は女性，壮年発症において

Table 4　狭義の家族性もやもや病と，広義の家族性もやもや病の定義

狭義分類	1997年のRCMJにより定義された[38]以下の所見のすべてを満たすもの (1)診断上，脳血管撮影は必須であり，少なくとも次の所見がある。 ①頭蓋内内頸動脈終末部，前および中大脳動脈近位部に狭窄または閉塞がみられる。 ②脳血管撮影上その付近にもやもや血管が動脈相においてみられる。 もしくはMRI上，大脳基底核部に少なくとも一側で2つ以上の明らかなflow voidを認める ③これらの所見が両側性にある。 (2)特別な基礎疾患に伴う類似の脳血管病変を除く。 ①動脈硬化，②自己免疫疾患，③髄膜炎，④脳腫瘍，⑤Down症候群，⑥Recklinghausen病，⑦頭部外傷，⑧頭部放射線照射，⑨その他
広義分類	以下の所見を満たす狭窄・閉塞性病変 ①頭蓋内内頸動脈終末部近傍に狭窄または閉塞がみられる。 ②もやもや血管の存在は不可欠ではない。 ③両側性であることは不可欠ではない。 ④特別な基礎疾患に伴う類似の脳血管病変を除く。

［略称］RCMJ：Research Committee on Spontaneous Occlusion of the Circle of Willis (Moyamoya Disease) of the Ministry of Health and Welfare, Japan.

有意に相関があることも同時に報告している。一方で，日本人で指摘されたHLA-B51，DRB1*0502，DRB1*0405，DRB1*0401では明らかな相関が認められなかったと述べている。

これまでのHLAの解析において，確定的な結果は得られていない。また以下に述べるようにInoueら[48]は19家系で6番染色体に対して連鎖解析を施行したが，HLA遺伝子の存在する6pに連鎖は認めなかった。以上から，HLAの評価については，確定的な結果が得られておらず，今後の確認が必要である。

III. 遺伝解析 (Table 3)

Ikedaら[47]は，16家系77名(男/女=28/49)を対象に解析を行った。そのうち，もやもや病は37名に認められた。同胞対から3世代まで多様な家系を含んでおり，さらに世代のとび越え現象(skipping)が認められたため，遺伝形式を仮定しないで遺伝解析の行えるnon-parametric method (疾患関連遺伝子頻度：0.00035)にて解析を行った。結果，有意な連鎖を3p24.2-26で認めた。

Inoueら[48]は，19家系で6番染色体に対してNon-parametric methodを用いて連鎖解析を施行した。結果D6S441（6q）にてIBD（0：12：8）と連鎖不平衡を認めた。一方，HLA遺伝子の存在する6pに連鎖は認めなかった。

Yamauchiら[49]はneurofibromatosis type 1 (NF1)が類もやもや病を発症することに注目し，24家系103名(うち患者56名)を対象に，常染色体17番長腕に焦点を当てparametric method (AD mode, AR mode)およびnon-parametric methodを用いて連鎖解析を行った。結果，17q25に連鎖を認めた。

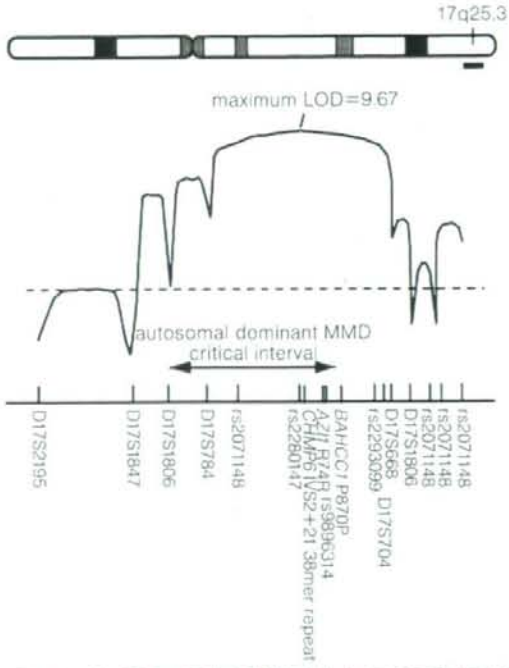

Fig. 1　17q25.3に存在する家族性もやもや病の遺伝子座

Sakuraiら[50]は12家系46人の核家族に対して，全ゲノムに対して連鎖解析を施行し，遺伝形式を仮定しないnon-parametric methodを行い，8q23.1のD8S546にてMLS=3.6　NPL=3.3，2p12のD12S1690にて

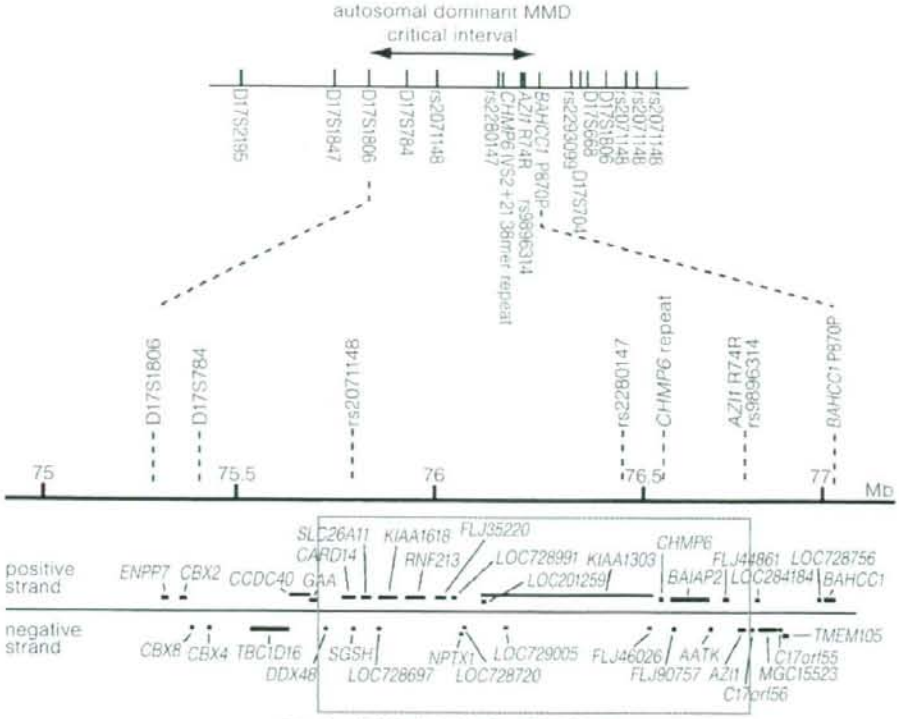

Fig. 2　家族性もやもや病の候補遺伝子

MLS＝2.3　NPL＝2.5と連鎖を示唆する結果を認めた。一方で報告があった領域に関しては3p，6q，17qそれぞれにおいて有意な連鎖を示唆する結果は得られなかった。

Nanbaら[31]はYamauchiらが指摘したD17S785とD17S836の間の領域に存在する65の遺伝子のうち，病因論上関係があると思われる9の遺伝子（*DNAI2*，*AANAT*，*PSR*，*HCNGP*，*HN1*，*SGSH*，*SYNGR2*，*EVPL*，*TIMP2*）に対して，翻訳領域の配列決定を行うと同時に，NCBIの2,100個のexpressed sequence tag（EST）を用いて，未知の遺伝子の精査を行ったが，明らかな責任遺伝子の同定には至らなかった。

Kangら[33]はSMCの増殖制御を行う*MMP2*や*MMP9*について検討し，候補領域である3p24.2-26[47]の*TIMP4*および17q25[49]の*TIMP2*に注目し，家族例11名，孤発例もやもや病50名，対照例50名で相関研究を行った。結果，3p24.2-26上の*TIMP4*に明らかに有意な変異は認められなかったが，*TIMP2*のpromotor-418にて家族性もやもや病ではG/C　heterozygous genotypeを9名（G/G：2名），対照（G/G：8，G/C：1，C/C：1）と比してOR＝18.00と高値であることを指摘した。だが，孤発例では相関は認められなかった。またExon3＋853に有意な相関（OR＝3.2）を認めたと報告しているが，わが国における家族例では多型が確認されなかった[52]。

筆者らは常染色体優性遺伝形式を持つ疾患では，3世代以上の家系がたどれることに注目し，3世代以上のもやもや病家系を集め，発症年齢別および遺伝様式別に分類し，15家系について解析を行った。結果，発症者の子供の43.7%が発症していること，および父—息子形式の遺伝が存在することより，X染色体による遺伝や，常染色体劣性遺伝よりむしろ，不完全な浸透率を有する常染色体優性遺伝の可能性が示唆された[12]。

3世代家系をもとに，全ゲノムに対して常染色体優性遺伝形式を仮定したparametric linkage analysisを施行した。結果17q25-qterに厚生労働省の厳格な診断基準である狭義分類にて，HLOD：6.57，また筆者らが狭義分類を拡大し設定した広義分類にてHLOD：8.07と，もやもや病の遺伝子座が全ゲノム解析で初めて明らかにされた（狭義分類および広義分類についてはTable 4）。同領域内でその後2家系を追加し，解析を行い，より狭められた現在の候補領域とそこに存在する遺伝子を

Fig. 1 に示す。今回の筆者らの解析では，17 q 25.3 の遺伝子座以外への連鎖は認められなかったが，もやもや病では disease heterogeneity や genetic heterogeneity が存在するためと考えられる[32]。

おわりに

もやもや病に関連する遺伝子は，今までの研究において，さまざまな遺伝子座や，単一遺伝子，ゲノム刷り込み遺伝子などの解析が行われたが現在に至るまで同定には至っていない。

筆者らは，東アジアという地域特異性が高いこと，家系内での浸透率が高いこと，罹患率が低いことなどより，単一遺伝子の rare variants が原因である可能性が高いと考えている。

現在，Fig. 2 に示す領域の全ゲノムの配列決定を行い，原因遺伝子の同定を目指している。

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〈お知らせ〉

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共　　催　日本脳神経外科漢方医学会／株式会社ツムラ東京支店

特別講演
『認知症に挑む―東西医学の統合から―』　荒井啓行（東北大学加齢医学研究所）

教育講演
『十全大補湯のグリオーマに対する抗腫瘍効果　―動物実験とレビュー』　高野晋吾（筑波大学大学院脳神経外科）
『脳神経外科医のための漢方入門セミナー*』　山口　巌（東京・山口診療所院長）
※漢方診断における腹診の実技をSP（模擬患者）とともに解説いただく予定です。

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ORIGINAL PAPER

Tissue Plasminogen Activator Enhances the Hypoxia/reoxygenation-induced Impairment of the Blood–brain Barrier in a Primary Culture of Rat Brain Endothelial Cells

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Abstract Hemorrhagic transformation is a major complication associated with tissue plasminogen activator (tPA) therapy for ischemic stroke. We studied the effect of tPA on the blood–brain barrier (BBB) function with our in vitro monolayer model generated using rat brain microvascular endothelial cells subjected either to normoxia or to hypoxia/reoxygenation (H/R) with or without the administration of tPA. The barrier function was evaluated by the transendothelial electrical resistance (TEER), the permeability of sodium fluorescein and Evans' blue-albumin (EBA), and the uptake of lucifer yellow (LY). The permeability of sodium fluorescein and EBA was used as an index of paracellular and transcellular transport, respectively. The administration of tPA increased the permeability of EBA and the uptake of LY under normoxia. It enhanced the increase in the permeability of both sodium fluorescein and EBA, the decrease in the TEER, and the disruption in the expression of ZO-1 under H/R conditions. Administration of tPA could cause an increase in the transcellular transport under normoxia, and both the transcellular and paracellular transport of the BBB under H/R conditions in vitro. Even in humans, tPA may lead to an opening of the BBB under non-ischemic conditions and have an additional effect on the ischemia-induced BBB disruption.

Keywords Tissue plasminogen activator · Brain capillary endothelial cells (rat) · Blood–brain barrier · Tight junction · Transendothelial permeability · Transendothelial electrical resistance · Hypoxia/reoxygenation (in vitro)

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Introduction

Tissue plasminogen activator (tPA) is a serine protease that activates plasminogen into plasmin, leading to the degradation of fibrin clots (Vassalli et al. 1991). Thrombolytic therapy with tPA is widely accepted to be effective for the treatment of acute thromboembolic stroke (NINDS 1995), however, symptomatic brain hemorrhaging remains a major complication

associated with such a treatment (NINDS 1997). Moreover, tPA has been reported to potentiate neuronal death both in vitro and in vivo (Liu et al. 2004). Therefore, it is considered very important to investigate the effect of tPA on the blood–brain barrier (BBB) function. Recently, it has been suggested that tPA promotes leakage of the BBB (Yepes et al. 2003), which is in agreement with the ability of tPA to generate hemorrhage (NINDS 1997). In contrast, other in vivo studies have shown that tPA injection does not compromise the BBB integrity in the acute stage of cerebral ischemia (Benchenane et al. 2005).

The major aim of the present study was to investigate the effect of tPA on BBB function. First, we established a pathophysiological in vitro BBB model by exposing the endothelial cells to normoxic or hypoxia/reoxygenation (H/R) conditions. Second, we investigated the effect of tPA on the transcellular and paracellular transport using the same BBB model subjected to H/R conditions.

Materials and Methods

All reagents were purchased from Sigma, USA, unless otherwise indicated. Wistar rats were obtained from Japan SLC Inc., Japan. All animals were treated in strict accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) and as approved by the Nagasaki University Animal Care Committee. Human recombinant tPA (Alteplase) was provided by Kyowa Hakko Kogyo Co, Japan.

Cell Cultures

Primary cultures of rat brain capillary endothelial cells (RBEC) were prepared from 3-week-old rats, as previously described (Deli et al. 1997; Hayashi et al. 2004). The meninges were carefully removed from the forebrains and the gray matter was minced into small pieces of approximately 1 mm^3 in ice-cold Dulbecco's modified Eagle's medium (DMEM), then dissociated by 25 up- and down-strokes with a 5-ml pipette in DMEM containing collagenase type 2 (1 mg/ml, Worthington, USA), 300 µl DNase (15 µg/ml), gentamicin (50 µg/ml), and then were digested in a shaker for 1.5 h at 37°C. The cell pellet was separated by centrifugation in 20% bovine serum albumin (BSA)-DMEM (1,000*g*, 20 min). The microvessels obtained in the pellet were further digested with collagenase-dispase (1 mg/ml, Roche, Switzerland) and DNase (6.7 µg/ml) in DMEM for 0.5 h at 37°C. Microvessel endothelial cell clusters were then separated on a 33% continuous Percoll (Pharmacia, Sweden) gradient, collected and washed twice in DMEM before plating on 35-mm plastic dishes coated with collagen type IV and fibronectin (both 0.1 mg/ml). The RBEC cultures were maintained in DMEM/Nutrient Mixture F-12 Ham (DMEM/F12) supplemented with 10% plasma-derived serum (Animal Technologies, USA), basic fibroblast growth factor (Roche, Switzerland, 1.5 ng/ml), heparin (100 µg/ml), insulin (5 µg/ml), transferrin (5 µg/ml), sodium selenite (5 ng/ml) (insulin-transferrin-sodium selenite media supplement), gentamicin (50 µg/ml), and puromycin (4 µg/ml) (Perriere et al. 2005) (RBEC medium I) at 37°C with a humidified atmosphere of 5% CO_2/95% air, for 2 days. On the third day, the cells received a new medium which contained all the components of RBEC medium I except puromycin (RBEC medium II).

BBB In vitro Monolayer Model

When the cultures reached 80% confluency (fourth day in vitro), the purified endothelial cells were passaged by a brief treatment with trypsin (0.05% wt/vol)-EDTA (0.02% wt/vol) solution. The endothelial cells (2.0×10^5 cells/cm^2) were seeded on the upper side of the polyester membrane of Transwell® inserts (diameter 12 mm, 0.40 µm pore size; Corning, Midland, MI) coated with collagen type IV and fibronectin. The day when the endothelial cells were plated was defined as Day 0 in vitro. From Day 1, the culture medium was supplemented with 500 nM hydrocortisone (Hoheisel et al. 1998). On Day 5, the experiments were performed. All experiments were repeated at least thrice, and the number of parallel inserts was 4.

H/R Studies

Normoxia

The cells were transferred into a serum-free medium containing 4.5 g/l glucose (control medium), DMEM/F12, with or without administration of tPA (20 µg/ml).

We used a dose of 20 μg/ml of tPA, based on the observation that such a concentration can be reached in blood (Godfrey et al. 1998). tPA was added in the luminal face at three different incubation times (3, 6, and 9 h).

H/R Conditions

H/R conditions consisted of 6 h hypoxia and 3 h reoxygenation with or without the administration of tPA. Hypoxia was generated using the AnaeroPack (Mitsubishi Gas Chemical). Briefly, cells were transferred to a serum- and glucose-free medium, Krebs-Ringer buffer (117 mM NaCl, 4.7 mM KCl, 1.2 mM $MgCl_2$, 1.2 mM NaH_2PO_4, 25 mM $NaHCO_3$, 2.5 mM $CaCl_2$, PH 7.4), which was previously bubbled with nitrogen gas for 30 min. Reoxygenation was initiated by adding serum-free medium, DMEM/F12. In all experiments, the pH of the medium remained stable during H/R conditions.

Transendothelial Electrical Resistance

The electrical resistance across the membrane was measured using an EVOM resistance meter (World Precision Instruments, Sarasota, FL). The extracellular matrix-treated Transwell® inserts were placed in a 12-well plates containing culture medium and then were used to measure the background resistance. The resistance measurements of these blank filters were then subtracted from those of filters with cells. The values are shown as $\Omega \times cm^2$ based on culture inserts.

Transendothelial Permeability

The flux of sodium fluorescein (Na–F) and Evan's blue-albumin (EBA) across the endothelial monolayer were determined as previously described (Kis et al. 2001). Cell culture inserts were transferred to 12-well plates containing 1.5 ml assay buffer (136 mM NaCl, 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$, 2.7 mM KCl, 1.5 mM KH_2PO_4, 10 mM NaH_2PO_4, 25 mM glucose, and 10 mM Hepes, pH 7.4) in the basolateral or lower compartments. In the inserts, the culture medium was replaced by 0.5 ml buffer containing 10 μg/ml Na–F (MW: 376 Da) and 165 μg/ml Evans' blue bound to 0.1% BSA (mw: 67 kDa). The inserts were transferred at 15, 30, and 60 min to a new well containing assay buffer. The emission of Na–F was measured at 535 nm (Wallac 1420 ARVO Multilabel Counter, Perkin Elmer; excitation: 485 nm), while the absorbency of Evans' blue is at 595 nm. The permeability of Na–F and EBA was used as an index of paracellular and transcellular transport, respectively. The apparent permeability coefficient, namely Papp (cm/s), derives from Fick' s Law (Youdim et al. 2003).

Assessment of Cell Viability

The endothelial cells (2.5×10^4 cells/cm^2) were seeded on the bottom side of a 24-well plate coated with collagen type IV and fibronectin. On Day 5, the experiments were performed. The effect of tPA on the viability of the endothelial cells was assessed using a Vi-CELL™ Cell Viability Analyzer (Beckman Coulter, Inc., Miami, FL). Viable cells were counted by trypan blue exclusion. The relative viable cell density was calculated by the formula, 100 × (viable cell density)/(maximum viable density)%.

Endocytosis

The endothelial cells (2.5×10^4 cells/cm^2) were seeded on the bottom side of a 24-well plate coated with collagen type IV and fibronectin. On Day 5, the experiments were performed. The uptake of lucifer yellow (LY) by the RBECs was determined by the methods described elsewhere with a slight modification in the technique (Niwa et al. 2004). We chose the concentration of LY to be 100 μg/ml. After incubation of the cells for 2 h, the cells were washed four times with cold phosphate-buffered saline (PBS) and lysed with 2 ml of 0.2% Triton X-100 in PBS. The cell lysates were centrifuged to remove cell debris, and the fluorescence intensity of the supernatant was then measured (Wallac 1420 ARVO Multilabel Counter, Perkin Elmer; excitation: 485 nm, emission: 535 nm).

ZO-1 Immunocytochemistry

The cells were fixed in 3% paraformaldehyde in PBS for 10 min, as previously described (Honda et al. 2006). Non-specific reactions were blocked by 3% BSA in PBS for 30 min and then the cells were incubated with primary antibody (ZO-1; Zymed,

San Francisco, CA) overnight at 4°C. The cells were rinsed with PBS and incubated for 1 h at room temperature with the appropriate secondary antibodies labeled with Alexa Fluor 488 (green) (Molecular Probes, Eugene, OR). All samples were examined using a laser-scanning confocal microscope (LSM 5 PASCAL, Carl Zeiss) with excitation at 488 nm and a detection ranging from 500 to 535 nm.

Statistical Analysis

Data were all expressed as the mean ± SD. The effects of tPA on the permeability of Na–F, EBA, the cell viability, the uptake of LY, and the transendothelial electrical resistance (TEER) were analyzed by analysis of variance (ANOVA) for the two-way layout data; the linear models including the terms of two main factors and their interaction were used, and if any factors or interactions were found to be significant, then the respective two groups were compared by *t*-tests with the Bonferroni adjustment. We used TTEST and ANOVA of SAS® system for the calculations. We considered a *P*-value of <0.05 to indicate statistical significance.

Results

The TEER of this monolayer model displayed more than 100 $\Omega \times cm^2$. With respect to the permeability of Na–F and EBA under normoxia, no significant difference according to the incubation time was observed in controls (Fig. 1a, b). The administration of tPA (20 μg/ml, incubation at 37°C) had no effect on the permeability of Na–F, while it showed a significant effect on the permeability of EBA (0.23 ± 0.04 vs. $0.40 \pm 0.07 \times 10^{-6}$ cm/s at 3 h, 0.20 ± 0.04 vs. $0.53 \pm 0.05 \times 10^{-6}$ cm/s at 6 h, and 0.23 ± 0.06 vs. $0.48 \pm 0.09 \times 10^{-6}$ cm/s at 9 h; $P < 0.0001$) (Fig. 1a, b).

No effect of the tPA administration was observed at three different incubation times (3, 6, and 9 h) in regard to the cell viability (Fig. 2). The administration of tPA increased the uptake of LY under normoxia, and the effect was significant at an incubation time of 6 h (0.49 ± 0.16 vs. 1.13 ± 0.67 μg/mg protein; $P = 0.0074$) and 9 h (0.37 ± 0.26 vs. 1.09 ± 0.50; $P = 0.0004$) (Fig. 3).

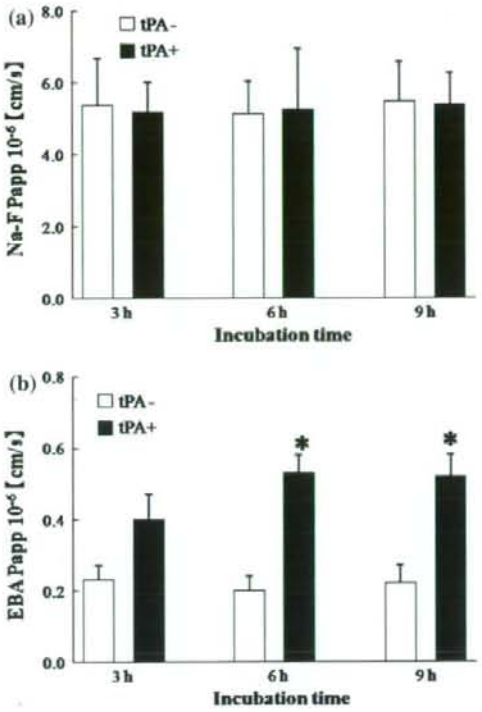

Fig. 1 Under normoxia, the administration of tPA had no effect on the permeability of sodium fluorescein (Na–F) (**a**), while it showed a significant effect on the permeability of EBA (**b**). The values are the mean ± SD, $n = 12$, * $P < 0.05$ vs. control

Since the two-way ANOVA demonstrated a significant effect of interaction between conditions (normoxia and H/R conditions) and treatments (vehicle and tPA administration) ($F = 31.4$ and 24.9 for Na–F and EBA permeability, respectively; $P \leq 0.0001$ for both) as well as the significant effects of conditions ($F = 285.3$ and 94.9 for Na–F and EBA, respectively; $P \leq 0.0001$ for both) and treatments ($F = 36.3$ and 242.8 for Na–F and EBA, respectively; $P \leq 0.0001$ for both), we analyzed the effects of tPA under normoxia and H/R conditions separately. Under normoxia (incubation time 9 h), the administration of tPA had no significant effect on the permeability of Na–F, while it significantly increased the permeability of EBA (0.23 ± 0.06 vs. $0.48 \pm 0.09 \times 10^{-6}$ cm/s; $P < 0.0001$) (Fig. 4a, b). On the other hand, under H/R conditions, the administration of tPA significantly increased the permeability

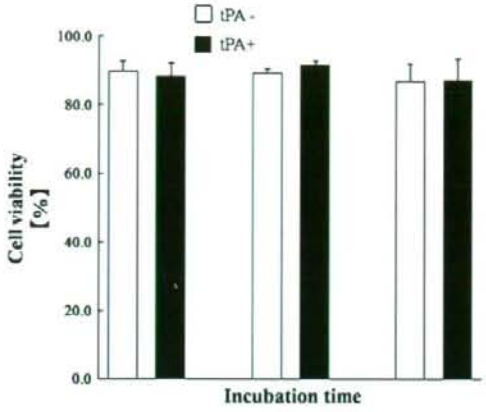

Fig. 2 No effect of the tPA administration was observed at three different incubation times (3, 6, and 9 h) in regard to the cell viability under normoxia. The values are the mean ± SD, $n = 5$

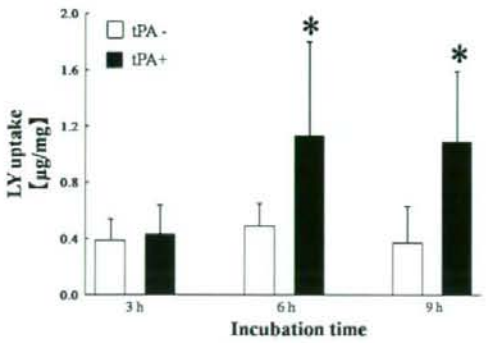

Fig. 3 The administration of tPA increased the uptake of LY under normoxia, and the effects were significant after an incubation time of 6 and 9 h. The values are the mean ± SD, $n = 12$, * $P < 0.05$ vs. control

of Na–F (5.70 ± 0.75 vs. 9.32 ± 0.95 × 10^{-6} cm/s; $P < 0.0001$) and EBA (0.34 ± 0.09 vs. 0.84 ± 0.09 × 10^{-6} cm/s; $P < 0.0001$) (Fig. 4a, b).

Furthermore, the permeability of Na–F was also significantly higher under H/R conditions than under normoxia both with (4.24 ± 0.69 vs. 9.32 ± 0.95 × 10^{-6} cm/s; $P < 0.0001$) and without (4.15 ± 0.70 vs. 6.70 ± 0.75 × 10^{-6} cm/s; $P < 0.0001$) the administration of tPA; similar results held for the permeability of EBA with (0.48 ± 0.09 vs. 0.84 ± 0.09 × 10^{-6} cm/s; $P = 0.0013$) and without (0.23 ± 0.06 vs. 0.34 ± 0.09 × 10^{-6} cm/s; $P \leq 0.0001$) the administration of tPA, respectively (Fig. 4a, b).

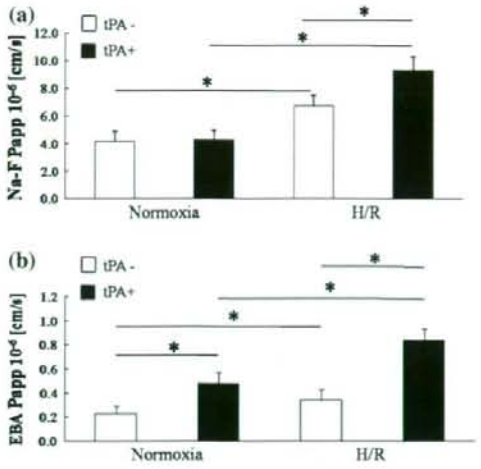

Fig. 4 (**a**) Under H/R conditions, the administration of tPA significantly increased the permeability of sodium fluorescein (Na–F), and the permeability of Na–F was significantly higher under H/R conditions than under normoxia both with and without the administration of tPA. (**b**) Under normoxia (incubation time 9 h), the administration of tPA significantly increased the permeability of EBA. Moreover, under H/R conditions, the administration of tPA significantly increased the permeability of EBA. The permeability of EBA was significantly higher under H/R conditions than under normoxia both with and without the administration of tPA. The values are the mean ± SD, $n = 12$. Significant differences between the normoxia and the H/R are indicated as * $P < 0.05$. Significant differences between the control and the tPA administration are indicated as * $P < 0.05$

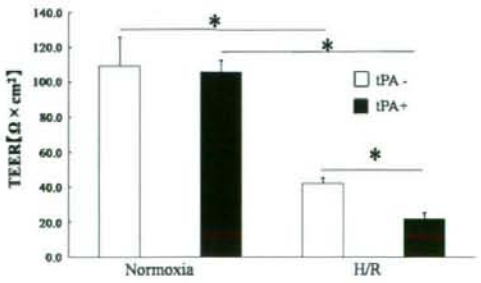

Fig. 5 The TEER was significantly lower under H/R conditions than under normoxia both with and without the administration of tPA. The administration of tPA significantly decreased the TEER under H/R conditions. The values are the mean ± SD, $n = 12$. Significant differences between normoxia and H/R are indicated as * $P < 0.05$. Significant differences between the control and the tPA administration are indicated as * $P < 0.05$

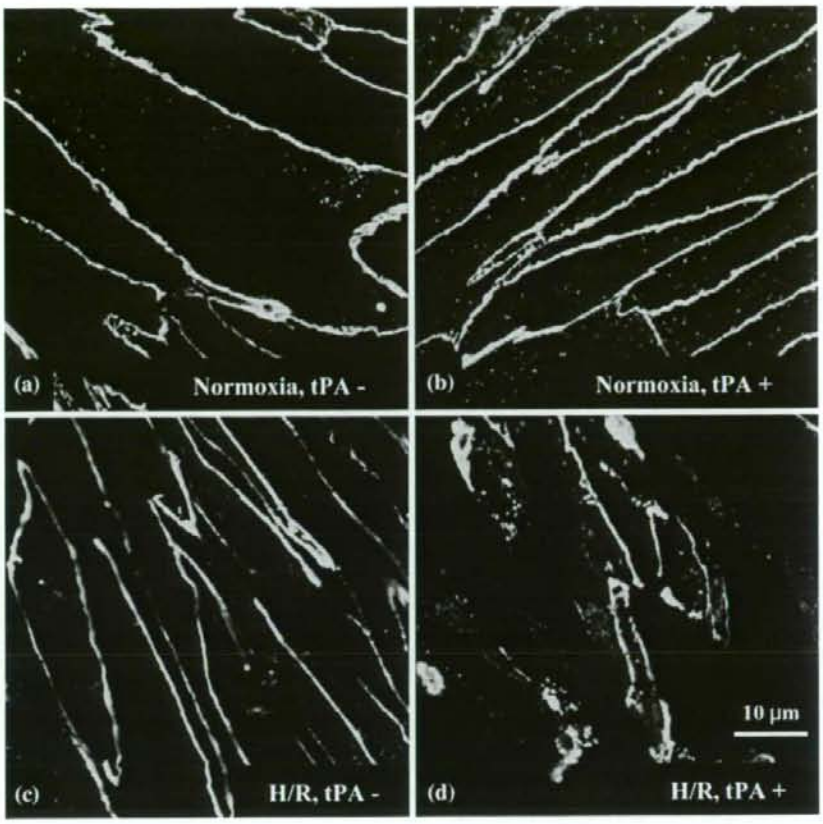

Fig. 6 The ZO-1 protein expression was analyzed immunocytochemically using anti-ZO-1 antibody. Under normoxia (incubation time 9 h), the administration of tPA showed no effects on the expression of ZO-1 (**a**, **b**). H/R decreased the expression of ZO-1 (**c**), and administration of tPA enhanced the disruption in the expression of ZO-1 during H/R conditions (**d**)

A similar separate analysis was carried out for TEER since the interaction between conditions and treatments was significant ($F = 9.7$, $P \leq 0.0033$) as well as the effects among such conditions ($F = 814.5$, $P \leq 0.0001$) or treatments ($F = 20.2$, $P \leq 0.0001$). The TEER was significantly lower under H/R conditions than normoxia for both with (22.00 ± 3.33 vs. $105.92 \pm 6.79\ \Omega \times cm^2$; $P < 0.0001$) and without (42.17 ± 3.43 vs. $109.58 \pm 16.38\ \Omega \times cm^2$; $P < 0.0001$) the administration of tPA. The administration of tPA significantly decreased the TEER under H/R conditions (42.17 ± 3.43 vs. $22.00 \pm 3.33\ \Omega \times cm^2$; $P < 0.0001$) (Fig. 5).

H/R decreased the expression of ZO-1. Moreover, tPA enhanced the disruption in the expression of ZO-1 under H/R conditions (Fig. 6).

Discussion

In the present study, we established a pathophysiological in vitro BBB model by exposing the endothelial cells to normoxia or H/R. Other in vitro studies have demonstrated that hypoxia with reoxygenation increases endothelial cell permeability (Utepbergenov et al. 1998; Dohgu et al. 2007; Nishioku et al. 2007).

In this study, the exposure times to hypoxia and reoxygenation were determined as 6 and 3 h, respectively, to cause dysfunction of the endothelial cells. The results showed that H/R conditions led to an increase in the permeability of both Na–F and EBA, and a decrease in the TEER. Moreover, H/R conditions reduced the expression of ZO-1. These findings indicate that H/R-induced paracellular hyperpermeability mediated by changes of TJ expression, and transcellular hyperpermeability. The exposure of brain-derived endothelial cells to hypoxic conditions has been reported to cause alterations in occludin, ZO-1, ZO-2, and claudin-5 localization (Mark and Davis 2002; Fischer et al. 2004; Koto et al. 2007).

Regarding the next concern, we demonstrated that in this in vitro monolayer model, tPA at a concentration of 20 μg/ml induced an increase in the permeability of EBA and an uptake of LY during normoxia, which suggested that tPA may increase the transcellular transport. These results seem to correlate with Yepes' in vivo observations that tPA led to an opening of the BBB by a mechanism involving low-density lipoprotein receptor-related protein in the absence of brain injury (Yepes et al. 2003). Deli et al. (2001) reported that tPA dose-dependently inhibited P-glycoprotein activity during normoxia. Benchenane et al. (2005) reported that oxygen and glucose deprivation (4 h) enhances an increase in the permeability of both sucrose and insulin. We showed that the co-application of tPA exacerbated the increase in the permeability of both Na–F and EBA. In addition, tPA enhanced the decrease in the TEER and the disruption in the expression of ZO-1 during this H/R conditions in our study. Our recent study reported that cellular communications play an important role in inducing and maintaining the barrier function of brain endothelial cells (Nakagawa et al. 2007). Cheng et al. (2006) showed that tPA induced NF-κB-dependent up-regulation of metalloproteinase-9 in ischemic brain endothelium in vivo and in vitro. Wang et al. (2006)reported that tPA induced metalloproteinase-9 dysregulation in rat cortical astrocytes. These results require further study using the in vitro co-culture model to assess the effect of tPA on the BBB. These findings raise the possibility that in humans, preventing the tPA-induced hyperpermeability of the BBB may be an adjunctive strategy to diminish the potentially deleterious effects of tPA.

In summary, tPA could therefore cause an increase in the transcellular transport under normoxia, and both the transcellular and paracellular transport of the BBB under H/R conditions in vitro. In humans, tPA may thus lead to an opening of the BBB under non-ischemic conditions, thereby having an additional effect on the ischemia-induced BBB disruption.

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Cerebral Hemodynamics and Oxygen Metabolism in Patients with Moyamoya Syndrome Associated with Atherosclerotic Steno-Occlusive Arterial Lesions

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Key Words

Moyamoya syndrome · Atherosclerosis · Cerebrovascular disease · Chronic cerebral ischemia · Cerebral blood flow · Intracranial arterial stenosis

Abstract

Background: Patients with major cerebral artery steno-occlusion and the formation of the moyamoya-like vessels associated with some other disorders have been distinguished from moyamoya disease and classified as moyamoya syndrome. The hemodynamic and metabolic backgrounds of the moyamoya syndrome associated with atherosclerosis have not yet been investigated. We aimed to elucidate the hemodynamic and metabolic characteristics associated with the development of basal moyamoya-like vessels in moyamoya syndrome with atherosclerosis. ***Methods:*** Twenty-one patients with chronic unilateral atherosclerotic steno-occlusive lesions of the internal carotid artery or middle cerebral artery (MCA) were enrolled in the study. Based on the angiographic findings, the patients were classified into 2 groups: the moyamoya syndrome group (n = 7) and the non-moyamoya-syndrome group (n = 14). We conducted angiographic evaluations of the extent of the development of basal moyamoya-like vessels in the moyamoya syndrome group. The cerebral blood flow, cerebral metabolic rate of oxygen, oxygen extraction fraction (OEF) and cerebral blood volume were measured using PET in the ipsilateral MCA area in the patients and in normal controls (n = 6). ***Results:*** The OEF in the ipsilateral MCA area, except in the basal ganglia, was significantly higher in the moyamoya syndrome group than in the non-moyamoya-syndrome group ($p < 0.001$). The extent of the development of basal moyamoya-like vessels was closely correlated with the elevation of the OEF ($r > 0.999$, $p < 0.001$). ***Conclusion:*** The basal moyamoya-like vessels are evidence of misery perfusion in patients with unilateral chronic atherosclerotic steno-occlusive lesions of major cerebral artery trunks.

Introduction

Moyamoya vessels in the basal ganglia region are one of the most characteristic findings on cerebral angiography in moyamoya disease [1]. Previous studies have sug-

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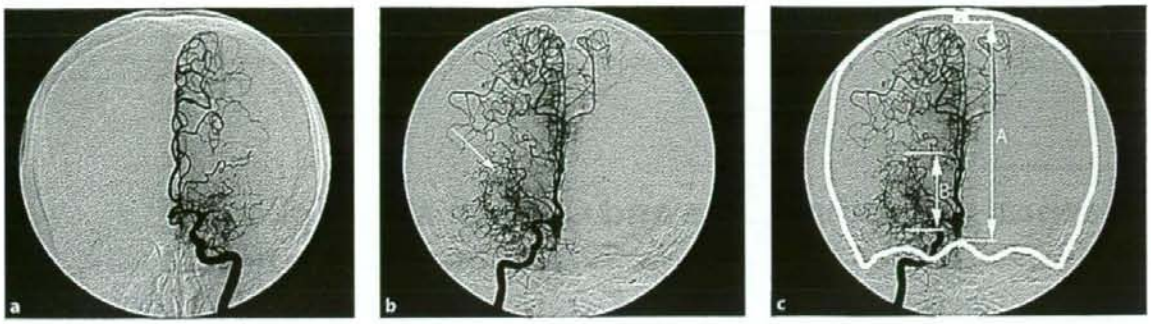

Fig. 1. Representative DSA images in the 2 patient groups. **a** Anteroposterior-view DSA image of patient No. 15 with left MCA occlusion. No apparent dilated angiographic basal moyamoya vessels are visualized (non-moyamoya-syndrome group). **b** The same view of patient No. 3 with right MCA occlusion. The arrow shows basal moyamoya-like vessels extending from the vicinity of the occlusive lesion to the ipsilateral basal ganglia (moyamoya syndrome group). **c** The extension index of the basal moyamoya-like vessels was calculated as the distance from the root to the top-end of the basal moyamoya-like vessels (B) divided by the distance from the base of the skull (top of the sella turcica) to the top of the skull (A).

gested that the development of moyamoya vessels in moyamoya disease is associated with the presence of severe hemodynamic impairment [2, 3]. On the other hand, patients with systemic disorders such as atherosclerosis also sometimes display angiographic features similar to those of moyamoya disease. Such symptoms are distinguished from moyamoya disease and have been classified as 'moyamoya syndrome' [4]. Only a few papers focusing on moyamoya syndrome associated with atherosclerosis have been published [5, 6], and the relationship between the development of moyamoya-like vascular abnormalities and cerebral hemodynamics has not yet been investigated. The aim of this study was to elucidate the hemodynamic and metabolic changes associated with the development of moyamoya-like vascular abnormalities in patients with moyamoya syndrome associated with unilateral atherosclerotic steno-occlusive disease of the internal carotid or middle cerebral arteries (MCA) in the chronic phase.

Patients and Methods

Patients

All the patients were seen at the Osaka University Medical School Hospital between August 2000 and December 2002, or between April 2004 and July 2006. Twenty-one patients (10 males and 11 females; mean age ± SD = 66 ± 9.0 years) who met the following criteria were included in this study: (1) the presence of atherosclerotic arterial changes of the carotid or the major cerebral arteries (e.g. plaques or intima-media thickening as detected by carotid ultrasonography, or arterial wall irregularities as detected by magnetic resonance angiography or brain angiography); (2) the presence of unilateral chronic atherosclerotic steno-occlusive lesions of the internal carotid artery (ICA; >90% diameter reduction according to the North American Symptomatic Carotid Endarterectomy Trial criteria [7]) or MCA M1 trunk (>80% diameter reduction) accessed by digital subtraction angiography (DSA); (3) independence in their daily life (modified Rankin Scale <3); (4) a time interval from the onset of the last cerebrovascular symptoms to the examinations of greater than 1 month; (5) DSA and ^{15}O gas PET conducted within an interval of 3 months in the chronic phase. Patients with subcortical cerebral infarcts measuring more than 3 cm in diameter or cortical cerebral infarcts in the ipsilateral MCA area or in the contralateral cerebral hemisphere (on MRI images), a history of intracerebral hemorrhage, head surgery, transient ischemic attacks or stroke in the hemisphere contralateral to arterial disease, clinical symptoms of ischemia in the vertebrobasilar artery territory or infarcts in the cerebellum or brainstem on MRI were excluded from the study. In addition, patients with the following disorders were also excluded: autoimmune disease, meningitis, brain neoplasm, Down's syndrome, Recklinghausen's disease, head trauma or irradiation to head. Six healthy persons (2 males and 4 females; mean age ± SD = 33 ± 6.6 years) were also enrolled in the study as normal controls. All of the control subjects had undergone ^{15}O gas PET. A detailed explanation of the purpose of the study and of all the procedures used in the study was given to all of the subjects prior to their enrollment. Written informed consent was obtained from each of the subjects. The study was approved by the Ethical Committee of Osaka University Hospital for Clinical Research.

Evaluation of DSA Images

The DSA images were first read independently by a neuroradiologist and a neurologist blinded to all clinical information about the subjects, and the decision about the presence of the moyamoya-like vascular abnormality was made by joint agreement at a conference between the two. Based on the presence or

Table 1. Symptoms, atherosclerotic lesions, vascularity and PET parameters in the ipsilateral hemispheres

Patient No.	Age years	Sex	Side	Symptoms	Site of lesion/ lesion type	Total vascularity index	PET parameters in the ipsilateral cortical MCA area			
							CBF ml/min/100 g	$CMRO_2$ ml/min/100 g	OEF %	CBV ml/100 g
Moyamoya syndrome group										
1	64	M	rt	TIA	rt MCA/O	8	39.5	2.90	51.1	6.92
2	65	F	rt	CI	rt MCA/O, rt PCA/S, bil ACA/O	5	31.1	2.52	56.9	4.14
3	74	F	rt	TIA	rt MCA/O, rt PCA/S	4	42.6	2.77	50.6	6.02
4	74	M	lt	CI	lt MCA/S	6	21.4	2.44	53.7	5.31
5	61	M	lt	TIA	lt MCA/S, bil ACA/S	6	23.2	2.46	53.6	4.75
6	63	M	rt	TIA, CI	rt ICA/O, rt PCA/S	7	26.3	2.52	55.8	5.08
7	69	F	rt	CI	rt MCA/S, bil ACA/S	4	33.8	3.66	59.5	5.76
Non-moyamoya-syndrome group										
8	57	F	rt	TIA	rt ICA/O	8	34.9	2.48	45.6	3.92
9	61	F	lt	TIA	lt ICA/O	8	48.3	2.62	36.5	4.38
10	55	M	lt	TIA	lt MCA/S, lt VA/S	6	36.4	2.99	45.1	4.01
11	58	M	rt	none	rt MCAO, lt VA/S	8	24.3	2.11	50.5	3.57
12	65	F	lt	TIA	lt MCA/O	8	33.5	3.14	57.1	4.88
13	66	M	lt	none	lt MCA/O	8	27.4	2.64	47.7	4.15
14	62	M	lt	none	lt MCA/O	8	27.2	2.13	46.5	4.32
15	62	M	lt	TIA	lt MCA/O	5	33.8	2.69	41.6	4.54
16	63	F	rt	none	rt MCA/S	7	38.7	2.73	43.6	4.45
17	39	M	rt	TIA	rt MCA/O	7	50.7	4.00	42.2	5.44
18	58	M	rt	CI	rt ICA/O	7	33.4	2.69	44.1	5.32
19	70	M	rt	TIA, CI	rt ICA/S	8	37.1	2.38	40.1	4.60
20	65	M	rt	TIA, CI	rt ICA/O	8	42.9	2.99	42.2	4.14
21	78	M	lt	TIA	lt ICA/S	7	32.9	2.65	52.8	4.16

rt = Right; lt = left; bil = bilateral; TIA = transient ischemic attack; CI = cerebral infarction; ICA = internal carotid artery; ACA = anterior cerebral artery; PCA = posterior cerebral artery; VA = vertebral artery; CBF = cerebral blood flow; $CMRO_2$ = cerebral metabolic rate of oxygen; OEF = oxygen extraction fraction; CBV = cerebral blood volume; S = stenosis; O = occlusion.

absence of the basal moyamoya-like vessels, the patients were classified into 2 groups: the moyamoya syndrome group (n = 7; 4 males and 3 females; mean age ± SD = 67 ± 5.3 years) and the non-moyamoya-syndrome group (n = 14; 10 males and 4 females; mean age ± SD = 65 ± 11 years). Figure 1a, b shows representative DSA images from the 2 groups. In all groups of patients, no apparent moyamoya-like vessels were found in any region other than the basal area. The interobserver agreement for this grouping was reasonably good ($\kappa = 0.8$). The clinical features of the patients in each group are shown in table 1.

In addition, the degree of regional or total vascularity in the ipsilateral cerebral hemisphere, excluding that of the basal moyamoya-like vessels, was evaluated in each patient by angiography. We applied a scoring system to access the patency of the ipsilateral MCA and to evaluate the degree of development of the leptomeningeal collateral circulation from the ipsilateral anterior cerebral arteries (ACA) or posterior cerebral arteries (PCA). Vascular patency of the MCA was classified into 4 grades (MCA vascularity index): grade 0, occlusion of the MCA with almost no visualization of the distal branches; grade 1, visualization of a single M2 branch; grade 2, visualization of more than two M2 branches; grade 3, normal MCA. The development of collateral vessels from the ACA was classified into 5 grades (ACA vascularity index): grade 0, occlusion of the ACA with almost no visual-

ization of the distal branches; grade 1, poor visualization of the distal branches of the ACA; grade 2, almost normal ACA with no development of leptomeningeal collateral vessels; grade 3, leptomeningeal cortical branches found in one lobe; grade 4, leptomeningeal cortical branches found in two or more lobes. Development of collateral vessels from the PCA was classified into 6 grades (PCA vascularity index): grade 0, occlusion of the PCA with almost no visualization of the distal branches; grade 1, poor visualization of the distal branches of the PCA; grade 2, almost normal PCA with no development of leptomeningeal collateral vessels; grade 3, leptomeningeal cortical branches found in one lobe; grade 4, leptomeningeal cortical branches found in two lobes; grade 5, leptomeningeal cortical branches found in more than three lobes. These vascularity indices were summed up in each patient as the total vascularity index in the ipsilateral hemisphere (maximum score: 12).

The extent of development of the basal moyamoya-like vessels was evaluated in anteroposterior-view DSA images in a plane perpendicular to the orbitomeatal (OM) line. The extension index of the basal moyamoya-like vessels was calculated as the distance from the root to the top-end of the basal moyamoya-like vessels divided by the distance from the base of the skull (top of the sella turcica) to the top of the skull in the same plane (fig. 1c).

PET Imaging

The Headtome V/SET 2400W system (Shimadzu, Kyoto, Japan) was used for the PET imaging. Prior to the emission scan, a Ge-68/Ga-68 transmission scan was performed for 10 min for attenuation correction. All scans were performed at a resolution of 3.7 mm full width at half maximum in the transaxial direction and of 5 mm full width at half maximum in the axial direction. Images were reconstructed using an ordered subset expectation maximization algorithm (12 iterations with 4 ordered subsets). Each subject's head was fixed in place with a head holder and positioned using light beams to obtain transaxial slices parallel to the orbitomeatal line. Data were formatted as a 3D dataset with 63 slices (3.17 mm thick) in 128 × 128 matrices. The cerebral blood flow (CBF), cerebral metabolic rate of oxygen ($CMRO_2$), oxygen extraction fraction (OEF) and cerebral blood volume (CBV) were measured using the conventional ^{15}O gas steady-state method [8]. The $CMRO_2$ and OEF were corrected by the CBV [9].

MRI

All the patients underwent PET and MRI within an interval of 3 months. Scans were obtained on the following scanners: GENESIS SIGNA 1.5T (GE Yokogawa Medical Systems, Tokyo, Japan), SIGNA EXCITE 1.5T (GE Healthcare, Chalfont St. Giles, UK), and MAGNETOM VISION 1.5T (Siemens, Erlangen, Germany). The MR protocol included T_2-weighted 2D fast spin echo sequences: (scan parameters: axial plane, FOV 250 mm, matrix 256 × 256 or 512 × 512, slice thickness: 5 mm, interslice gap 1–1.5 mm, TE: 90–131 ms, TR: 4,500–5,000 ms).

Data Analysis

Measurement of these parameters was executed in the ipsilateral MCA area using the automated constant region of interest (ROI) analysis software, FineSRT [10], which can perform the analysis using a precise constant 1,394 ROI (both hemispheres), with excellent objectivity and reproducibility. The MCA area was divided into 2 subdivisions: the cortical MCA area (i.e. the ipsilateral cortical MCA branch territory in the frontal, parietal and temporal lobes) and the basal ganglia, except the caudate nucleus. Ipsilateral ROI included in each of the two MCA divisions were selected from the constant 1,394 ROI and averaged. Statistical ROI analysis was carried out in each of the two subdivisions of the MCA area. Differences in the mean values among the groups were analyzed using a one-way ANOVA followed by the least significant difference test for ROI analysis, and the Mann-Whitney U test for other comparisons. Correlational analysis was performed by determining Spearman's rank-order correlation coefficient. Multivariate analysis was carried out by multiple linear regression analysis. Statistical significance was defined by a p value of less than 0.05.

Results

There were no significant differences in the clinical characteristics (age, gender, handedness, history of hypertension, hyperlipidemia and/or diabetes mellitus) or physiological parameters measured on the day of the PET study (blood pressure, partial pressure of arterial CO_2 and O_2, arterial pH, arterial O_2 saturation, hemoglobin and hematocrit) between the moyamoya syndrome group and the non-moyamoya-syndrome group. In the 17 symptomatic patients, there was no significant difference in the interval from the initial clinical symptoms to the time of performance of the DSA between the moyamoya syndrome group (n = 7, 55 ± 36 months) and the non-moyamoya-syndrome group (n = 10, 35 ± 20 months). Figure 2 shows the CBF, $CMRO_2$, OEF, CBV and CBF/CBV in the ipsilateral MCA area as measured by PET. The CBF was significantly lower in the moyamoya syndrome group than in the non-moyamoya-syndrome group in the ipsilateral cortical MCA area, and lower in the moyamoya syndrome group than in the normal controls in the basal ganglia. There was no significant difference in the $CMRO_2$ between the moyamoya syndrome group and the non-moyamoya-syndrome group in the whole MCA area, whereas the $CMRO_2$ values in the whole MCA area were significantly decreased in both the patient groups as compared with the values in the normal controls. The OEF was significantly higher in the moyamoya syndrome group than in the non-moyamoya-syndrome group in the cortical MCA area, but not in the basal ganglia. However, no significant difference in the OEF was found in the whole MCA area between the non-moyamoya-syndrome group and the normal controls. Although intergroup differences in the CBV were not significant as evaluated by ANOVA, the CBV in the moyamoya syndrome group was significantly higher than in the non-moya-