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掲載論文

＜班研究紹介シリーズ＞

脳卒中に対する再生医療的技術を用いた治療法の開発に関する研究

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「脳卒中，脳血管性痴呆症に対する再生医療技術を用いた治療法の開発に関する研究」班

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緒　言

現在わが国においては，他の諸国においては類を見ないほどの急速な高齢化社会を迎えており，脳梗塞後遺症や血管性痴呆症に起因する要介護者の急激な増加は，日本の社会構造を根底から揺るがす極めて深刻な社会問題となっている．これらの疾患に対し様々な研究が積み重ねられてきたが，脳梗塞に対する確立された治療法は発症後数時間以内の血栓溶解療法以外になく，脳血管性痴呆に対しては全く有効な治療手段がないのが現状である．

近年，今までの医療では治療できなかった難治性の虚血性循環器疾患に対して，再生医療的手法を用いた治療法が開始され，虚血性心疾患や末梢動脈閉塞症に対しては著しい治療効果が報告されている．しかし，脳障害に対する治療法はほとんど進歩が見られていないのが現状であり，基礎研究レベルにおいても，ES 細胞あるいは胎児由来の神経幹細胞を用いた研究が盛んに行われてきたが，それらの単なる移植では神経細胞として脳内で生着，成熟し機能することが困難であることが明らかになりつつある．

我々は胎生期や成体における神経発生や神経再生において，神経幹細胞の分化や成熟，機能は，常に血管新生とともにプログラムされていることに着目し研究を行ってきたが，本研究班では①脳梗塞治療を目的とした，脳血管再生および神経細胞再生を含む脳組織再生，機能再生による治療法の確立に向けた研究，および②脳梗塞予防を目的とした，脳血管再生による治療法の確立に向けた研究，を行っており，本総説においてその研究経過を概説します．

A. 脳梗塞治療を目的とした，脳血管再生および神経細胞再生を含む脳組織再生，機能再生による治療法の確立に向けた研究

1. 再現性の非常に高いマウス脳梗塞モデルの作成：松山，田口

脳梗塞に対する新しい治療法の効果検定には，再現性の高い動物モデルが必要不可欠であるが，既存のげっ歯類脳梗塞動物モデルはその脳梗塞発症部位およびサイズの再現性が低く，かつ脳梗塞後の生存率に大きな問題を抱えていた．そのため，治療効果の判定には一過性脳虚血モデルが広く用いられてきたが，一過

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性脳虚血後に生じる神経細胞の脱落は，脳梗塞患者における神経組織壊死と病態的に大きく解離しているため，脳梗塞治療効果の検定には適当でない場合が多く，過去に一過性脳虚血モデルで有効とされた薬剤の臨床試験では，ほとんどのものが無効あるいは有害であった．そのため当研究班ではまず，再現性および生存率の高いげっ歯類脳梗塞モデルの確立に向けた検討を行った．様々な手法と種々のマウスおよびラットのstrainの検討を行った結果，SCID（Sever Combined Immunodeficiency）マウスの中大脳動脈のM1 distal部位を直視下で結紮することにより，中大脳動脈灌流領域の皮質部位に常に一定の脳梗塞を生じる非常に再現性の高い脳梗塞動物モデルの作成に成功した[1]．この脳梗塞モデルにおいては形態学的および組織学的にも常に同一の脳梗塞が作成されるだけでなく，神経学的にも均一な症状を呈することより，今後の新しい脳卒中治療法の開発に非常に有用な実験モデルとして，広く使用可能であると考えている．

2．血管血球系幹細胞投与による脳血管再生，神経再生の誘導：松山，明神，田口

我々が新しく開発した非常に再現性の高い脳梗塞モデルを用いて，脳梗塞後の骨髄系単核球細胞投与の治療効果およびその機序に関する検討を行った．

左中大脳動脈閉塞48時間後の脳梗塞マウスを用いて，骨髄系単核球細胞の分画であるCD34陽性細胞を尾静脈より単回投与し，骨髄系単核球細胞投与による①血管再生の促進効果，②内因性の神経再生促進効果，③脳組織再生効果および④神経機能再生効果に関して検討を行った．その結果，①脳梗塞後の骨髄系単核球細胞投与は梗塞周囲における主に内因性の血管再生を促進し，血流の再建を誘導する，②脳梗塞後の血管再生は内因性の神経再生を誘導するだけでなく，その生着に必須である，③骨髄系単核球細胞投与による脳梗塞後の血管再生は，脳神経組織の再生を誘導する，④脳梗塞後の血管再生による脳組織再生は脳機能の再生をもたらす，ことを世界に先駆けて明らかにしてきた[1]．

また骨髄単核球の末梢血中への動員を目的としてG-CSFの投与を行ったが，治療効果とは逆にG-CSFの投与により脳萎縮の進行が観察された．さらに，細胞処理過程の簡略化を目的として，骨髄細胞の投与実験を行ったが，G-CSFと同様に脳萎縮の進行が観察された．これらの結果は，①G-CSFで動員される顆粒球には負の作用が有ること，および②骨髄細胞中の骨髄単核球細胞分画を精製する必要があること，を示唆しており，これまでの報告における顆粒球細胞の脳梗塞巣に対する障害作用[2]とも合致すると考えている．

一方，脳梗塞後の血管構築に関する詳細な検討のため，放射光X線を用いた研究を行った．正常血管解剖としては脳表に分布する動脈から大脳灰白質を栄養する直径10μm程度のPerforating Arteryが明瞭に描出されたが[3]，脳梗塞後の未治療群では脳梗塞大脳半球は対側の大脳半球と比較して脳実質の萎縮が目立ち，梗塞巣周囲では脳表動脈は描出されるもののPerforatorは消失していた．骨髄単核球投与群では脳実質の萎縮はあまり認められず，対側と比較してやや拡張や蛇行があるもののPerforatorが撮像されており，機能血管が残存していた．また，梗塞巣周囲における正常ではあまり血管が存在しない分水嶺や脳室周囲深部白質においてPerforatorよりもさらに微細な血管の増生が観察されたが，これらは移植により誘導された再生血管であり，これら再生血管が梗塞巣への側副路となり，梗塞巣へ血液を供給していると考えている．

3．霊長類脳梗塞モデルにおける自己骨髄単核球投与：林，明神，田口

ヒト[4]やマウス[5]と同様にサルにおいても神経幹細胞が存在すること[6]，さらには脳梗塞後に神経幹細胞などによる修復過程[7]が存在することが知られているが，本検討では霊長類における脳梗塞後の骨髄採取およびその静脈内投与に関する安全性の検討を行った．

我々の予定している臨床試験では，対象疾患を心原性脳塞栓症患者としているため，当動物実験モデルにおいてもヒト病態に近い自己血栓によるカニクイザル塞栓モデルの作成を行った．全身麻酔下において血管撮像装置を使用し，超選択的カテーテル法により自己血栓を中大脳動脈M1遠位部より投与することにより，中大脳動脈領域の脳梗塞を作成し，脳梗塞の確認はMRIを用いて行った．脳梗塞作成後7日後に腸骨骨髄より骨髄液の採取（10ml）を採取し，静脈より自己骨髄由来単核球細胞の投与を行った．安全性の検査項目として①検血，②神経症状の悪化，③MRIによる出血および再梗塞の評価を行い，最長脳梗塞12カ月以降まで経過観察を行ったが，脳梗塞後の骨髄採取およびその静脈内投与によると考えられる有害事象は認められなかった．

4．脳梗塞患者における再生機転の病理的検討：植田

以上のような脳梗塞動物モデルで得られた知見が，

脳梗塞患者にても実際に起こっているかを検討するために，脳梗塞患者における病理標本を用いて脳梗塞後の血管新生及び神経再生に関する検討を行った．その結果，梗塞周囲領域における血管再生が観察されるとともに，Musashi-1 陽性の神経幹細胞と考えられる細胞群を同定した．これらの細胞群は脳梗塞発症後比較的早期から発現しているものの，30 日ごろにはかなり減少し，90 日にはほとんど見られなくなった．これらの所見は脳梗塞後の内因性の組織修復機構を用いた治療法の開発には，脳梗塞発症後 30 日以内をターゲットにすべきであることを示唆していると考えている．

5．成体由来の神経幹細胞の確立：出澤

血管再生を基盤とした内因性の神経再生誘導に関する治療法を，より効果的な治療法として発展させるために，神経幹細胞の樹立及びその投与による治療法の開発を行っている．本研究では骨髄間質細胞からの神経細胞への誘導を試み，最終分裂を終えた機能的な神経細胞を非常に高い効率で，しかも選択的に誘導する方法を開発した[8]．最初に，ヒトおよびラットの骨髄間質細胞において Notch 遺伝子を導入することによって神経幹細胞様に分化転換することを見いだしたが，これらの細胞は神経幹細胞に特異的なマーカー(GLAST，3-PGDH，nestin)を発現し promoter 解析においてもそれらの因子の活性の有意な上昇が観察された．神経幹細胞様に分化転換した細胞にサイトカイン刺激(bFGF，Forskolin，CNTF)を与えると非常に効率の高い選択的な神経誘導が引き起こされ，またこの最終産物にはグリア細胞が一切含まれておらず，神経細胞だけで最終産物が構成されていることを明らかにした．誘導された神経細胞はパッチクランプ実験において機能的な神経細胞であることが確認された．誘導された神経細胞にさらに GDNF を投与するとドーパミン作動性ニューロンが 40% 近くに増加し，これらの細胞をパーキンソンモデルラットの線状体に移植したところ，apomorphin 誘導の回転運動や paw reaching test，adjusting step test において顕著な症状改善を認めた．また，骨髄間質細胞由来の神経幹細胞の脳梗塞巣への移植を行った結果，量的には少ないものの移植神経幹細胞の生着，および神経機能の向上が観察された[9]．骨髄間質細胞由来神経幹細胞は移植治療において多くの利点を有し，血管再生などと組み合わせることにより生着率の向上を図ることにより，患者への適応の可能性が期待されると考えている．

B. 脳梗塞予防を目的とした脳血管再生による治療法の確立に向けた研究

1．末梢血中幹細胞の低下と脳神経機能の低下および虚血性疾患の発症に関する検討：田口，松山，林

血管内皮前駆細胞系の幹細胞を多く含む CD34，CD133 抗原陽性細胞数の減少が，脳梗塞巣の増加と有意に関連している一方，血管内皮前駆細胞と関連の薄い CD117 抗原および CD135 抗原細胞数との有意な関連は見られなかった[10]．さらに，脳主幹動脈閉塞あるいは高度狭窄のある患者の PET を使った検討では CD34，CD133 抗原陽性細胞数の減少が，慢性虚血部位の脳血流量の低下に有意に関連しており，側副血行路の形成低下や血管反応性の低下など，血流血管維持機構の低下と関連していると考えられた．CD34 抗原陽性細胞数減少患者においては，慢性虚血部位における酸素摂取率の反応性の上昇は認められず，その結果酸素代謝量の低下が認められた．さらに，脳梗塞患者において末梢血中の CD34 陽性細胞が減少している群においては，MMSE で評価される認知機能および CDR で評価される認知症の重症度が高いことを明らかにした．また，虚血性疾患のリスクが非常に高い患者においては，CD34 陽性細胞減少群においてその後の虚血性疾患の発症が高いことを明らかにした．これらの結果は，末梢血中血管血球系幹細胞の低下が血管内皮機能の低下と共に神経機能の低下にも関連していることを示しており“血管血球系幹細胞の補充”により虚血性疾患が予防できる可能性を示唆していると考えている．

2．血管血球系幹細胞の生体外増殖：西村

ヒト臍帯血より CD133 陽性細胞を単離後，VEGF，SCF，TPO 等の最適化された growth factor を加えることにより，7 日間の無血清培養により 50～80 倍程度に増殖させることに成功した．これらの増殖細胞は血管内皮系コロニー形成能が保たれているとともに，虚血性心疾患モデルにおいても，心機能回復能を確認した．これらの手法は，患者由来の末梢血/骨髄由来血管血球系幹細胞でも可能であると考えており，臨床応用に向けた，展開が大きく開けたと考えている．

3．霊長類における慢性期脳虚血モデルの作成：飯田，林

げっ歯類においても慢性脳虚血モデルは存在しなかったが，飯田，林らは霊長類において慢性脳虚血モデルの作成に成功した．生体外増殖された血管血球系

幹細胞を慢性脳虚血患者に投与する前臨床試験には，本モデルでの検討は必須であると考えている．

総　括

我々は末梢動脈閉塞症患者に自己骨髄由来の血管血球系幹細胞を移植することにより虚血症状の改善することを示してきたが[11]，脳虚血動物モデルにおいても，血管血球系幹細胞の移植が，既存小血管の保護や微小血管網の再生，再構築を介して神経機能の改善をもたらすことを明らかにした．これらの知見は，急性期脳梗塞患者に対する血管血球系幹細胞移植の可能性を示唆するものであり，霊長類を用いた検討や脳梗塞患者における病理的所見を総合して，脳梗塞患者に対する新しい治療法の確立に向けたプロトコール作りを行っている．

また，脳梗塞予防に関する研究では末梢血中のCD34陽性細胞やCD133陽性細胞などの血管血球系幹細胞の減少が，脳梗塞の発症と強く関連しているだけではなく，Blood Brain Barrierを形成する血管内皮細胞の機能，神経組織の代謝およびその機能にまで影響を与えていることを我々は明らかにしており，血管血球系幹細胞の補充による新しい治療法の可能性を示唆していると考えている．

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Laboratory Investigation

A New Protocol for Quantifying CD34+ Cells

in Peripheral Blood of Patients with Cardiovascular Disease

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Increasing evidence points to a role for circulating CD34-positive (CD34+) cells in vascular maintenance and neovascularization. Although there are established methods for evaluating absolute numbers of CD34+ cells in bone marrow or mobilized peripheral blood, there is no convenient and highly reproducible method for quantifying low numbers of CD34+ cells in blood samples, such as those from the peripheral blood of patients with cardiovascular disease. With current commonly used methods, the mean percentage of CD34+ cells in leukocyte fractions from such patients was only 0.02%, and the cumulative intra-assay coefficient of variation was ~30%. With use of the protocol described herein, actual counts of CD34+ cells increased ~5-fold and cumulative intra-assay coefficients of variation were reduced to ~7%. The new method is useful to precisely measure low numbers of CD34+ cells in samples, and it has potential as a screening tool to evaluate cardiovascular risk in large patient populations. **(Tex Heart Inst J 2006;33:427-9)**

Key words: *Antigens, CD34/analysis; blood cell count; cardiovascular diseases; cell sorter, fluorescence-activated; CD34-positive cells; coefficients of variation; endothelium, vascular/cytology; stem cells*

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In vasculature maintenance and neovascularization, there is increasing evidence of a role for circulating endothelial progenitor cells (EPCs)—including the populations of CD34-positive (CD34+) cells that are present in peripheral blood.[1] As a source of numerous growth and angiogenesis factors at ischemic loci, CD34+ cells also contribute to vascular homeostasis.[2] Furthermore, initial clinical trials of cell transplantation in treating ischemia of the hind limb[3] and myocardium[4] have shown promising results. On the basis of these observations, circulating EPCs[5] and CD34+ cells[6] have been evaluated in patients with cardiovascular disease, and strong correlations of their levels with vascular function have been reported. However, procedures to evaluate EPCs and CD34+ cells are not simple[5]; because of low numbers of circulating CD34+ cells, routine FACS (fluorescence-activated cell sorter) analysis[7] of CD34+ cell counts in patients with cardiovascular disease is not feasible. In this report, we demonstrate a new method that facilitates determination of the absolute number of circulating CD34+ cells in patients with low levels of CD34+ cells.

Patients and Methods

This study was approved by the Human Assurance Committee of the National Cardiovascular Center and Osaka Minami Medical Center, and all subjects provided written informed consent. Results of experiments are reported as mean ± standard error.

Analysis of Peripheral Blood

Three milliliters of heparinized peripheral blood were obtained from 20 patients who had histories of cardiovascular disease: 14 had sustained myocardial infarction, and 9 had sustained cerebral infarction (3 had histories of both). Patients who had experienced vascular events within 30 days of measurement were excluded. The study group included 12 men and 8 women, with a mean age of 74 ± 1.7 years (range, 59–87 yr). Medicines taken by study subjects included anticoagulants (aspirin, 17); antihypertensive agents, including calcium-channel antagonists, angiotensin-converting enzyme (ACE) inhibitors, or both (14); and sulfonylureas for glycemic control (5). Patients who were taking HMG-CoA reductase inhibitors (statins) were excluded from the study.

First, we counted circulating CD34+ cells with ProCount™ (BD Bioscience; San Jose, Calif) and Stem-Kit™ (Beckman Coulter; Marseilles, France), according to the

manufacturers' protocols. (These protocols are based on International Society of Hematotherapy and Graft Engineering (ISHAGE) Guidelines[7] and are frequently used for quantification of CD34+ cells that have mobilized into peripheral blood.) Next, to increase the reproducibility of CD34+ cell counts, the Stem-Kit protocol was modified as follows: the blood sample volume, antibodies, and lysing solution were doubled. After adding 30 µL of internal control particles (stem count; Beckman Coulter), samples were centrifuged for 5 min at 450 G, and 3,860 µL of supernatant was removed carefully with a pipette. Samples were analyzed by Coulter CYTOMICS™ FC500 & XL-system II software (Beckman Coulter) for 6 min each (Fig. 1).

Results

Increase of CD34+ Cell Counts

The mean percentage of CD34+ cells in the leukocyte fraction obtained from mobilized peripheral blood has been reported to be about 0.2% to 0.5%.[7] First, we used the ProCount and Stem-Kit protocols to count circulating CD34+ cells that had been obtained from patients with cardiovascular disease (Table I). The mean percentages of CD34+ cells in the leukocyte fraction were 0.024% ± 0.003% (range, 0.012%–0.046%) for ProCount and 0.021% ± 0.001% (range, 0.011%–0.032%) for Stem-Kit. The actual CD34+ cell counts per analysis were only 16 ± 1 (range, 10–31) for ProCount and 34 ± 4 (range, 16–58) for Stem-Kit. Because the absolute cell count is a major factor in the reproducibility of the measurement,[5] our approach was to modify the original protocol in order to obtain higher numbers of actual CD34+ cells per count. However, simply increasing sample volume or measurement time does not improve reproducibility, because of these factors: adhesion of internal control particles to cells in the patient sample, and precipitation and aggregation of cells in the sample. Our approach, as outlined under Methods, was to seek a method that produces higher cell counts while maintaining short measurement times. Through the use of our method, the mean CD34+ cell count increased to a level of 174 ± 18 (range, 88–404) per analysis, and the mean percentage of CD34+ cells in the leukocyte fraction was 0.019 ± 0.002 (range, 0.014%–0.038%), which was consistent with measure-

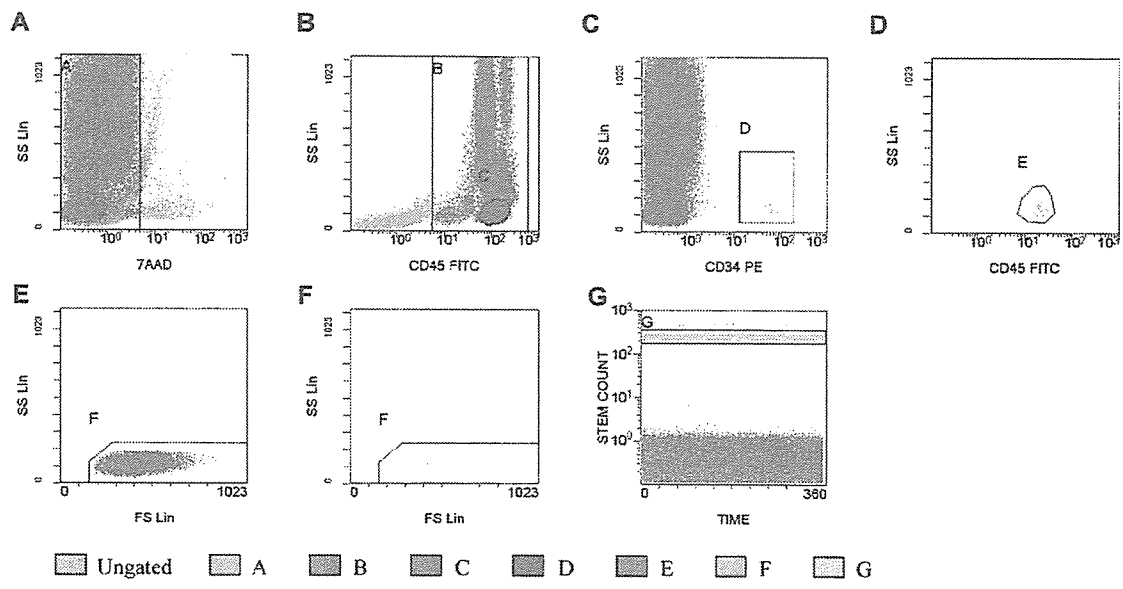

Fig. 1 *Quantification of circulating CD34+ cells by fluorescence-activated cell sorter analysis using our modified, improved protocol.* ***A)*** *All events: 7-aminoactinomycin-D viability dye-positive cells (dead cells) were excluded from region A.* ***B)*** *Events from region A: All CD45+ cells (leukocytes) were included in region B. Region C was adjusted to include only lymphocytes (bright CD45, low side-scatter).* ***C)*** *Events from regions A and B: Region D was adjusted to include CD34+ hematopoietic progenitor cells (HPC).* ***D*** *Events from regions A, B, and D: Region E was adjusted to include cells forming a cluster with characteristic CD34+ HPC (low side-scatter and low-to-intermediate CD45 staining). Brightly stained events were excluded from region E.* ***E)*** *Events from regions A and C: Region F was adjusted to include lymph/blast cells, excluding platelet aggregates if present.* ***F)*** *Events from A, B, D, and E: Lymph/blast region F identified a cluster of events that met all the fluorescence and light-scattering criteria of ISHAGE Guidelines for CD34+ HPC.* ***G)*** *All events: Region G was adjusted to enclose the internal control.*

7AAD = 7-aminoactinomycin-D; CD34 PE = cluster of differentiation 34 phycoerythrin; CD45 FITC = cluster of differentiation 45 fluorescein isothiocyanate; FS Lin = forward-scatter linear scale; ISHAGE = International Society of Hematotherapy and Graft Engineering; SS Lin = side-scatter linear scale

TABLE I. Reduction in the Coefficient of Variation Using Our Modified, Improved Protocol for Determining CD34+ Cell Levels in Peripheral Blood

	ProCount	Stem-Kit	Improved Protocol
Actual CD34+ cell counts	16 ± 1	34 ± 4	174 ± 18
Range	10–31	16–58	88–404
CD34+ cells in leukocytes (%)	0.024 ± 0.003	0.021 ± 0.001	0.019 ± 0.002
Range	0.012–0.046	0.011–0.032	0.014–0.038
Circulating CD34+ cells (cells/μL)	0.82 ± 0.05	0.81 ± 0.06	0.88 ± 0.06
Cumulative intra-assay coefficient of variation (%)	30.3	25.7	7.4

ments using the standard method (Table I). The supernatant that was removed during the procedure was also analyzed and was found not to contain either cells or internal control particles.

Improvement in the Cumulative Intra-Assay Coefficient of Variation

In mobilized peripheral blood, the coefficients of variation have been reported to be about 8% and 4% using ProCount and Stem-Kit, respectively, on the basis of the manufacturers' published information. However, in non-mobilized peripheral blood of patients with cardiovascular disease, the mean percentage of $CD34^+$ cells in the leukocyte fraction was less than 10%, compared with mobilized blood, and the calculated cumulative intra-assay coefficients of variation were 30.3% and 25.7%, as evaluated by ProCount and Stem-Kit, respectively. Our method increased the absolute number of $CD34^+$ cells by about 5-fold during the same measurement period, and it resulted in a reduced (7.4%) cumulative intra-assay coefficient of variation (Table I).

Discussion

Although our modified method for quantifying $CD34^+$ cells in blood was similar to established methods for the calculation of mean circulating $CD34^+$ cell counts, our method substantially improved reproducibility of the measurement.

The coefficient of variation of $CD34^+$ cell counts is inversely proportional to the square root of the number of $CD34^+$ cells detected in the sample. A minimum of 100 $CD34^+$ cells is required to ensure a coefficient of variation in the range of 10%.[8] Our modified protocol yielded more than 100 $CD34^+$ cells in each count (confirmed by duplicate counting), and the coefficient of variation was reduced to 7%. Simply increasing the sample volume or lengthening the time for measurement of cell numbers does not necessarily improve reproducibility of the counts.[8] Our results indicate that absolute numbers of circulating $CD34^+$ cells in peripheral blood of patients who have low levels of such cells can now be quantified precisely using a modification of the ISHAGE protocol. This easy method enables precise measurement of the $CD34^+$ cell population of stem cells in peripheral blood and can be broadly applied to screening patients for cardiovascular risk.

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Moderate Atheroma of the Aortic Arch and the Risk of Stroke

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Key Words
Aorta · Cardiovascular diseases · Cerebral infarction · Echocardiography

Abstract
Background and Purpose: Severe atheroma ≥4 or 5 mm of the aortic arch is a risk factor for stroke. We investigated the most predictive characteristics of arch atheroma, including maximal plaque thickness, for subsequent cardiovascular events, and also examined whether moderate atheroma <4 mm is a risk of cerebral emboli. ***Methods:*** The maximal plaque thickness (MPT) and plaque morphologies of the aortic arch were evaluated by transesophageal echocardiography in 236 patients with ischemic stroke. We assessed the relationship between the incidence of cardiovascular events, recurrent stroke or myocardial infarction, and the characteristics of the atheroma. We also investigated the thickness of atheroma in patients with known causes of stroke (n = 148) and in patients with undetermined causes (n = 19). ***Results:*** Cardiovascular events occurred in 47 patients in the follow-up period with a mean of 3.5 years. MPT was a significant risk factor of the cardiovascular events, although plaque morphologies were not. For the receiver operator characteristics curve analysis, the suitable cutoff point of MPT associated with the cardiovascular events was 3.5 mm. Patients with MPT ≥3.5 mm had a higher risk of cardiovascular events than did those with MPT <3.5 mm. In addition, aortic atheroma with MPT ≥3.5 mm was more frequently observed in patients with undetermined causes of stroke than those with known causes at 68 vs. 39% (p = 0.024). ***Conclusions:*** MPT ≥3.5 mm is the best predictor of subsequent cardiovascular events and a possible cause of embolic stroke.

Aortic arch atheroma in patients with stroke, which is generally diagnosed using transesophageal echocardiography (TEE), is one of embolic sources. Several studies indicated that aortic arch atheroma is an important independent risk factor for stroke or vascular events. In these studies, thick atheroma, the presence of ulcerative or mobile plaques along the aortic arch or plaque extension to the branches was found to be associated with stroke [1–9]. However, the definition of thick atheroma related to ischemic stroke varies among these studies. Amarenco et al. [1] reported in a prospective case control study that the odds ratio for cerebral infarction increased with plaque thickness, especially for a thickness of 4 mm or more. Tunick et al. [4] reported in a prospective case control study that protruding ≥5 mm or mobile atheroma in the aortic arch was a predictor of vascular events. Toyoda et al. [8] defined aortic atheroma >3 mm with an irregular surface or broad acoustic shadow to be an echographi-

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cally significant lesion and showed that it has high sensitivity and specificity for permitting detection compared with histopathologically complicated lesions. However, most of these studies defined severe atheroma as a maximal intima-media thickness ≥ 4 mm.

Patients with embolic stroke may have only aortic atheroma less than 4 mm in thickness and no other embolic sources such as atrial fibrillation, right-left shunt or stenosis of the extracranial or intracranial artery. Even moderate atheroma less than 4 mm in thickness may be a possible risk of stroke. None of the previous studies evaluated the detailed thickness of aortic arch atheroma as a risk of stroke. The aims of our study were to determine the characteristics of arch atheroma including the plaque thickness, which have the most predictive value for the occurrence of ischemic stroke and other vascular events in patients with ischemic stroke, and to evaluate the clinical importance of these plaques as possible sources of cerebral emboli.

Table 1. Clinical diagnoses of the study patients

Clinical diagnosis	
Cardiogenic	81 (34.3)
Atherothrombotic	45 (19.1)
Lacunar	22 (9.3)
TIA	54 (22.9)
Other causes of stroke	34 (14.4)
Dissection of cerebral artery	7
Coagulopathy	2
Complication of conventional angiography	1
Moyamoya disease	1
Unknown	23
No definitive embolic source in the heart, ipsilateral carotid artery or ipsilateral intracranial artery	19

Figures in parentheses are percentages.

Methods

Between January, 1997, and July, 1999, we performed TEE in 296 Japanese stroke patients, all of whom were admitted into our institute, for extracranial embolic sources such as intracardiac thrombus and complicated atherosclerotic lesions in the aortic arch. Sixty (20.3%) of them were excluded from the study, since they were immediately lost on follow-up (n = 49) or full data were not available (n = 11). The study group comprised 236 patients, 163 men and 73 women with 64.3 ± 12.0 (mean ± SD) years of age.

We performed contrast TEE using a commercially available Model SSD-2200 real-time two-dimensional echocardiography system (Aloka, Tokyo) equipped with a 5.0-MHz phased array omniplane transesophageal transducer variable from 3.5 to 7.5 MHz. We evaluated the maximal plaque thickness of the aortic arch in the short-axis view (MPT), the presence of any mobile, ulcerative or calcified plaque along the aortic arch, and right-left shunts using TEE. Ulcerative plaque was defined to be the presence of surface defects showing a depth and length of 2 mm or more. Calcified plaque was defined to be focal increase in echo density within the aortic plaque combined with a broad acoustic shadow. Regarding right-left shunts, we first inspected the left atrium for debris appearing in its inside during the Valsalva maneuver and after release of the maneuver without any contrast medium. Next, the contrast medium, the mixture of 9 ml saline and 1 ml air, was infused into the right antecubital vein during the Valsalva maneuver. When the right atrium was opacified by the contrast medium as seen on the monitor, we asked patients to release the Valsalva maneuver. When contrast medium different from the debris was found in the left atrium after the release of the Valsalva maneuver, we diagnosed it to be the sign of right-left shunt.

The information obtained at the time of baseline TEE for each patient included their age, gender, referring diagnosis, history of hypertension, diabetes, hyperlipidemia, heart diseases of potential embolic sources such as atrial fibrillation, congestive heart failure, cardiopathy or valvular diseases, and finally carotid stenosis and intracranial artery stenosis. Carotid stenosis was evaluated by ultrasound sonography using a Model Sonos 5500 duplex color-coded ultrasonographic device (Philips Medical Systems, Massachusetts) equipped with a 7.5-MHz transducer, and intracranial stenosis was evaluated by magnetic resonance angiography or digital subtraction angiography. The percentage stenosis of arterial diameter was calculated by dividing the narrowest linear diameter at the stenotic segment by the distal diameter at the normal-looking vessel. In all patients, the follow-up information comprised the occurrence of cardiovascular events such as recurrent ischemic stroke or myocardial infarction during the period after TEE, therapeutic variables including anticoagulation or antiplatelet agents, and the occurrence and cause of death. The follow-up information was obtained by reviewing the patients' medical records until December, 2002 (n = 206) or by telephone interview (n = 30). When patients could not come to our hospital for more than 3 months, such as because of their removal or discontinuation of visits to our outpatient clinic, we determined their status by telephone interview with them or their family to ensure the survival of the patients. The mean follow-up period was 3.5 years (42 ± 13 months).

We investigated the relationship between the characteristics of the aortic arch atheroma and the occurrence of cardiovascular events, using two-tailed t tests for the comparison of means. For the comparison of proportions, we used χ^2 tests, which were replaced by Fisher's exact tests when the expected cell count was <5. We also used Cox proportional hazards analysis to assess the contribution of clinical and echocardiographic variables to the development of cardiovascular events. Receiver operator characteristics (ROC) curve analysis was used to establish a suitable cutoff point of MPT which was related to the cardiovascular events, cumulative event-free rates for the time until a cardiovascular event occurred were estimated by the Kaplan-Meyer product limit method, and the 2 groups with MPT cutoff points were compared by the log-rank test.

Table 2. Univariate analyses comparing patients with and without cardiovascular events

	Total (n = 236)	Cardiovascular event (n = 47)	No event (n = 189)	p value
Age, years	64.3 ± 12.0	68.6 ± 11.0	63.2 ± 12.0	0.006
Gender (male/female)	163/73	28/19	135/54	0.12
Atrial fibrillation	89 (38)	26 (45)	63 (33)	0.15
Right-left shunt	60 (25)	9 (19)	51 (27)	0.25
Diabetes	69 (29)	13 (28)	56 (28)	0.79
Hypertension	156 (66)	35 (74)	121 (83)	0.18
Hyperlipidemia	106 (45)	25 (53)	81 (53)	0.21
Carotid stenosis ≥ 50%	37 (16)	11 (23)	26 (14)	0.10
Cranial artery stenosis ≥ 50%	47 (20)	12 (26)	35 (19)	0.28
Antiplatelet therapy	134 (57)	30 (64)	104 (55)	0.28
Anticoagulation therapy	100 (42)	18 (38)	82 (43)	0.53
Characteristics of aortic atheroma				
MPT, mm	3.2 ± 1.7	3.8 ± 1.8	3.0 ± 1.6	0.009
Ulcerative plaque	24 (10)	8 (17)	16 (8)	0.08
Calcified plaque	15 (6)	3 (6)	12 (6)	0.99
Mobile component	4 (2)	1 (2)	3 (2)	0.99

Figures in parentheses are percentages.

Following this, we also investigated the relationship between the characteristics of aortic arch atheroma in patients with a known cause of cerebral infarction and in those with an undetermined cause. A patient with cerebral infarction of known cause was defined to be one who was diagnosed to have lacunar infarction, cardioembolic infarction or atherothrombotic infarction on the basis of typical onset, topography, the size of the infarct, and known risk factors [10, 11]. Patients with transient ischemic attack (TIA) were excluded. A patient with cerebral infarction of undetermined cause was defined to be one who was not classified into lacunar infarction, cardioembolic infarction, atherothrombotic infarction or TIA, had no heart disease as a potential embolic source, including atrial fibrillation, patent foramen ovale, congestive heart failure, cardiopathy or valvular diseases, or had no ipsilateral carotid stenosis of 30% or more, ipsilateral intracranial artery stenosis or coagulopathy (table 1). Two-tailed t tests were used for the comparison of means and Fisher's exact test was for the comparison of proportions. Multiple logistic-regression analysis was performed to compare the prevalence of aortic arch atheroma in patients with cerebral infarction of known cause with that in patients with cerebral infarction of undetermined cause. The data were analyzed using StatView software, where a probability value <0.05 was considered to indicate statistical significance.

Results

There were no significant differences in age, gender, cardiovascular risk factors, echocardiographic variables, and therapeutic variables between the study group and the patients who were immediately lost on follow-up.

Table 3. Multivariate analysis used to predict cardiovascular events (n = 236)

Variable	HR	95% CI	p value
Age	1.04	1.00–1.07	0.03
Gender (female)	1.75	0.96–3.15	0.06
MPT	1.07	0.87–1.29	0.51
Ulcerative plaque	1.37	0.59–2.56	0.45
Carotid stenosis ≥ 50%	1.48	0.69–3.14	0.30

Clinical characteristics of the study group are shown in table 2. Cardiovascular events occurred in 47 patients at 5.2%/person-years for 44 with recurrent stroke and 3 with myocardial infarction. MPT was <3.0 mm in 107 patients, 3.0–3.4 mm in 32 patients, 3.5–3.9 mm in 26 patients, 4.0–4.4 mm in 27 patients, 4.5–4.9 mm in 15 patients, and ≥ 5.0 mm in 29 patients. Increased MPT was a significant risk factor for cardiovascular events (table 2). Ulcerative, calcified, and mobile plaques were found in 24 (10.2%), 15 (6.4%), and 4 (1.7%) patients, respectively, but such plaque morphologies were not related to the cardiovascular events. Risk factors for cardiovascular events with $p < 0.15$ on univariate analyses were entered into a multivariate Cox proportional hazard model. Age was the only independent factor which contributed to the cardiovascular events (table 3), and MPT and age showed

Table 4. Multivariate analysis used to predict cardiovascular events in elderly patients with 65 years of age or older (n = 130)

Variable	HR	95% CI	p value
MPT ≥3.5 mm	2.40	1.07–5.38	0.03
Age	1.08	1.01–1.15	0.03
Gender, female	1.98	0.98–4.01	0.06

Table 5. Univariate analyses comparing stroke patients with known causes and those with undetermined causes

	Known (n = 148)	Undetermined (n = 19)	p value
Age, years	65.0 ± 10.5	67.2 ± 11.6	0.38
Gender, male/female	98/50	14/5	0.61
MPT ≥3.5 mm	57 (39)	13 (68)	0.02
Ulcerative plaque	13 (9)	1 (5)	0.99
Calcified plaque	9 (6)	1 (5)	0.99
Mobile component	2 (14)	0 (0)	0.99

Figures in parentheses are percentages.

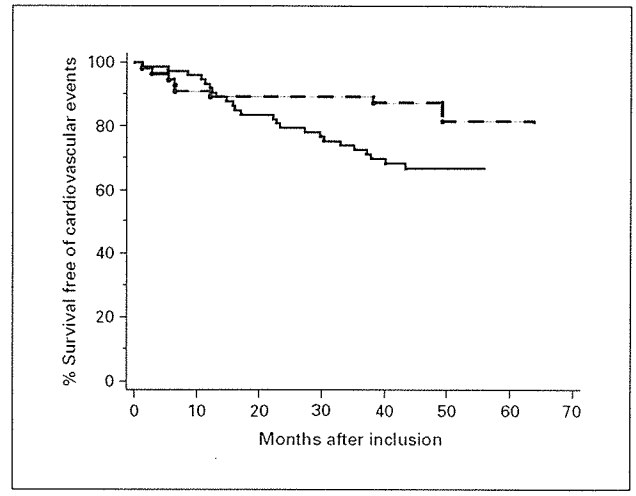

Fig. 1. Kaplan-Meyer curves showing that a significantly lower proportion of patients with MPT ≥3.5 mm (solid line) remained free of new cardiovascular events (p = 0.02, log-rank test) in elderly patients aged 65 years old or more (n = 130). The dashed line indicates MPT <3.5 mm.

a strong correlation by using quadratic regression analysis ($p < 0.001$). In the ROC curve analysis obtained using the cardiovascular event rates of each 0.5 mm MPT, the suitable cutoff point of MPT was 3.5 mm with a sensitivity of 57% and specificity of 63%. Patients with MPT ≥3.5 mm had a higher risk of cardiovascular events (hazard ratio [HR], 2.10; 95% confidence interval [CI], 1.18–3.74; p = 0.012). Next we focused on the subgroup of 130 patients with 65 years of age or older, in which 32 developed cardiovascular events (29 with recurrent ischemic stroke and 3 with myocardial infarction). In these elderly patients, MPT ≥3.5 mm was a significant risk factor for cardiovascular events even after adjusting for age and sex (HR, 2.40; 95% CI, 1.07–5.38; p = 0.034; table 4). Kaplan-Meyer curve analysis revealed a significant difference in the event-free survival between elderly patients with atheroma of MPT ≥3.5 mm and those without (fig. 1).

There were 148 patients with 65.0 ± 10.5 years of age composed of 98 men and 50 women with a known cause of stroke who were diagnosed to have cardioembolic infarction (81 patients), atherothrombotic infarction (45 patients) or lacunar infarction (22 patients), and 19 patients with 67.2 ± 11.6 years of age composed of 14 men and 5 women with undetermined causes of embolic stroke who had no heart diseases as a potential embolic source, ipsilateral carotid stenosis, ipsilateral intracranial artery stenosis or coagulopathy at the time of baseline TEE. In the group with undetermined causes, the MPT was <3.0 mm in 5 patients, 3.0–3.4 mm in 1, 3.5–3.9 mm in 5, 4.0–4.4 mm in 3, 4.5–5.0 mm in 2, and ≥5.0 mm in 3 patients. Aortic arch atheroma with MPT ≥3.5 mm was more frequently observed in patients with undetermined causes of stroke than in those with known causes at 68 versus 39%, and the difference between the two groups remained significant after adjusting for age and sex (p = 0.024; table 5). These two groups were not significantly different in terms of age and sex. When aortic arch atheroma was defined by another cutoff point, such as ≥3.0, ≥4.0, ≥4.5 or ≥5.0 mm, the significant difference between the two groups disappeared.

Discussion

Severe atheroma ≥4 or ≥5 mm along the aortic arch is associated with a high risk of subsequent vascular events, as shown in a study by the French Study of Aortic Plaques in Stroke Group [2], Tunick et al. [4], and several other studies. Our follow-up study showed that moderate atheroma between 3.5 and 4.0 mm also has an in-

creased risk of subsequent cardiovascular events. No other studies have examined the effect of critical thickness of aortic atheroma on the occurrence of cardiovascular events. The risk of aortic atheroma thickness <4 mm remains unclear. We used ROC analysis to determine the suitable cutoff point for aortic atheroma thickness which is associated with cardiovascular events, although the sensitivity and specificity of the ROC curve were not sufficiently high because of the contributions of many other risk factors. The plaque thickness was strongly associated with age. However, MPT ≥3.5 mm was an independent risk factor even after adjusting for age in patients 65 years of age or older. We believe that the cutoff point of 3.5 mm is an important index of extracranial atherosclerosis, which may be associated with subsequent cardiovascular events, especially in elderly patients with a history of ischemic stroke.

During the determination of aortic atheroma thickness, which may be critical for the association of cardiovascular events, race and ethnic differences should be taken into account. Most previous data regarding the relationship between aortic atheroma and stroke was derived from white populations. Gupta et al. [12] showed that white people tend to have increased complex plaques and plaques >4 mm thick along the thoracic aorta compared with African-Americans. Di Tullio et al. [13] reported on aortic atheroma morphology and the risk of stroke in a multiethnic population composed of whites, blacks, and Hispanics. However, few studies have described the differences in terms of aortic arch atherosclerosis between Asian and Caucasian populations. Angiographic and autopsy studies on stroke patients have shown that Japanese, Chinese, and Koreans tend to have more intracranial vascular lesions, whereas Caucasians tend to have more extracranial lesions [14–18]. Japanese may show less prevalence of severe aortic atheroma than do Caucasians, or smaller plaques may be more of a risk for vascular events in Japanese than in Caucasians.

In this cross-sectional study, we showed that aortic atheroma of MPT ≥3.5 mm is more frequently observed in patients with undetermined causes of stroke than those with known causes. Five patients with undetermined causes of strokes and MPT ≥3.5 mm developed recurrent ischemic strokes. Of them, 3 had recurrent embolic infarction, the embolic source of which was not identified other than aortic atheroma at the time of the second event. These results suggest that aortic atheroma of 3.5 mm or more may be a direct source of thromboemboli. Several studies using transcranial Doppler showed that embolic signals in the middle cerebral artery can be more frequently detected in patients with severe arch atheroma than in those without severe arch atheroma [19–21]. Severe atheroma along the aortic arch, therefore, can be a source of cerebral emboli. Moderate atheroma such as being 3.5–4.0 mm in thickness may also be a source of emboli. Ulcerated plaques were found in one-third of the aortic atheromas which were less than 4 mm in our study. A small unstable atheroma can cause dynamic changes such as plaque rupture and may cause cerebral embolism. Amarenco et al. [5] and others suggested that ulcerative plaques may be a cause of cerebral infarction. Mitusch et al. [3], Jones et al. [7] and others showed that mobile components are also a risk factor of cerebral infarction. A follow-up study by Cohen et al. [22] found that the risk of cerebral infarction associated with an aortic plaque thickness ≥4 mm is markedly increased by the absence of plaque calcification. However, we could not find any direct association between plaque morphology and stroke. A possible explanation is that the present study included fewer patients with ulcerative, mobile or calcified plaques than did previous studies and therefore failed to find any statistical differences in terms of such morphology.

The present study has several limitations. First, the study was performed in a retrospective manner, and most of the patients were originally recruited to assess embolic sources of the heart and thoracic aorta by TEE. The study population tended to have more patients with cardioembolic infarction and fewer patients with lacunar infarction than the general stroke population in our institution. Patients with known severe carotid artery stenosis may have been excluded from the study population. Thus, the subjects in the present study may not be representative of the general population of ischemic stroke. Second, the follow-up information obtained by telephone interview (13% of the study population) may not be highly accurate.

In conclusion, maximal plaque thickness ≥3.5 mm of the aortic arch is a predictor of subsequent cardiovascular events, especially in elderly patients with a history of ischemic stroke. Moderate atheroma with 3.5–4.0 mm in thickness may also be a possible cause of embolic stroke.