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Research Report

Neural differentiation potential of peripheral blood- and bone-marrow-derived precursor cells

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ABSTRACT

Transplantation of mesenchymal stem cells (MSCs) prepared from adult bone marrow (BMSCs) has been reported to ameliorate functional deficits in several CNS diseases in experimental animal models. Bone marrow was enriched in MSCs by selecting for plastic-adherent cells that were grown to confluency in appropriate culture conditions as flattened fibroblast-like cells. Despite the fact that the stem/precursor cells in peripheral blood are widely used for reconstruction in the hematopoietic system, it is not fully understood whether peripheral blood-derived plastic-adherent precursor/stem cells (PMSCs) can differentiate into a neural lineage. To compare the potential of PMSCs and BMSCs for neural differentiation in vitro, BMSCs and PMSCs were prepared from the adult rat and expanded in culture. Although the growth rate of PMSCs was less than BMSCs, immunocytochemical and RT-PCR analyses indicated that both MSC types were successfully induced to nestin-positive neurospheres in the presence of EGF and bFGF. After withdrawal of the mitogens, these cells could differentiate into neurofilament-positive neurons or GFAP-positive glia. Thus, our findings suggest the potential use of PMSCs for a cell therapy in CNS diseases.

1. Introduction

Mesenchymal stem cells (MSCs) are thought to represent a very small proportion of cells in the mononuclear population of bone marrow. As originally described by Freidenstein (1976), these cells will grow to confluency in appropriate culture conditions as flattened fibroblast-like cells (Majumdar et al., 1998), and have been suggested to differentiate into bone, cartilage (Kobune et al., 2003), cardiac myocytes (Toma et al., 2002) and neurons and glia (Prockop, 1997; Woodbury et al., 2000; Kobune et al., 2003; Iihoshi et al., 2004; Honma et al., 2006; Nomura et al., 2005) both in vitro and in vivo. MSCs prepared from human bone marrow (BMSCs) have been used in clinical studies for metachromatic leukodystrophy (Koc et al., 2002), Hurler syndrome (Koc et al., 2002), myeloablative therapy for breast cancer (Koc et al., 2000), graft-versus-host disease (Aggarwal and Pittenger, 2005), and stroke (Bang et al., 2005).

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Human mesenchymal precursor cells found in the blood of normal subjects proliferated in culture with an adherent-spread morphology, and displayed cytoskeletal, cytoplasmic and surface markers ($CD34^-$, $CD45^-$, and $CD105^+$) of mesenchymal precursors (Zvaifler et al., 2000). These cells had a capacity for differentiation into fibroblast, osteoblast, and adipocyte lineages. A canine $CD34^-$ fibroblast-like cell in the peripheral blood showed mesenchymal stem cell characteristics (Huss et al., 2000). Because peripheral blood is readily accessible, stem cells isolated from blood may be a good candidate for a cell therapy. For example, the hematopoietic stem/precursor cells in peripheral blood are widely used for reconstruction in the hematopoietic system (Brown et al., 1997; Auner et al., 2005).

Thus, although the potential of mesenchymal precursor cells in peripheral blood (PMSCs) has received much attention, it is not known whether peripheral blood-derived plastic-adherent precursor cells (PMSCs) can differentiate into a neural lineage. Here, we compared the growth properties and differentiating potential to neural lineages of rat PMSCs with those of rat BMSCs.

2. Results

2.1. *Characteristics of BMSCs and PMSCs*

After removing nonadherent cells by replacing the medium (day 2 in culture), a small portion of attached nucleated cells was visualized in the BMSC culture dish. By day 14 in culture, the attached BMSCs had developed into an adherent layer containing abundant dispersed fibroblast-like cells, and each colony was predominantly formed by several fibroblast-like cells (Fig. 1A). By day 28 in culture, the BMSCs had proliferated and tended to form a near continuous layer comprising mainly fibroblast-like cells (Fig. 1B).

In the cultures of PMSCs derived from peripheral blood, fibroblast-like cells with thin elongated processes around a central nucleus made their appearance at 2 weeks after culture initiation (Fig. 1C). By day 28 in culture, the cells also continued proliferating and formed a layer of flattened cells (Fig. 1D), with morphological features resembling those of BMSCs.

Figs. 1E and F are flow cytometric data of the expression of surface antigens on BMSCs and PMSCs, respectively. These results show that both BMSCs and PMSCs express a similar pattern of surface antigens: $CD45^-$, $CD73^+$, $CD90^+$, and $CD106^-$.

2.2. *Growth rate*

The number of BMSCs and PMSCs was counted at weekly intervals in order to characterize the proliferation rate (Fig. 2). BMSCs slowly proliferated in the initial 2 weeks, and entered a rapid growth phase for the next 4 weeks. Proliferation of BMSCs became slower after 6 weeks, but cell number was maintained for the next 2 weeks. The number of BMSCs increased more than 4 logs for cultures maintained for 8 weeks. In contrast, PMSCs displayed slow but constant growth over 8 weeks in culture, and expanded over 6-fold.

2.3. *Transformation of MCSs to neurospheres*

BMSCs transformed to nestin-positive neurospheres using an induction protocol (Fig. 3A) described in Experimental

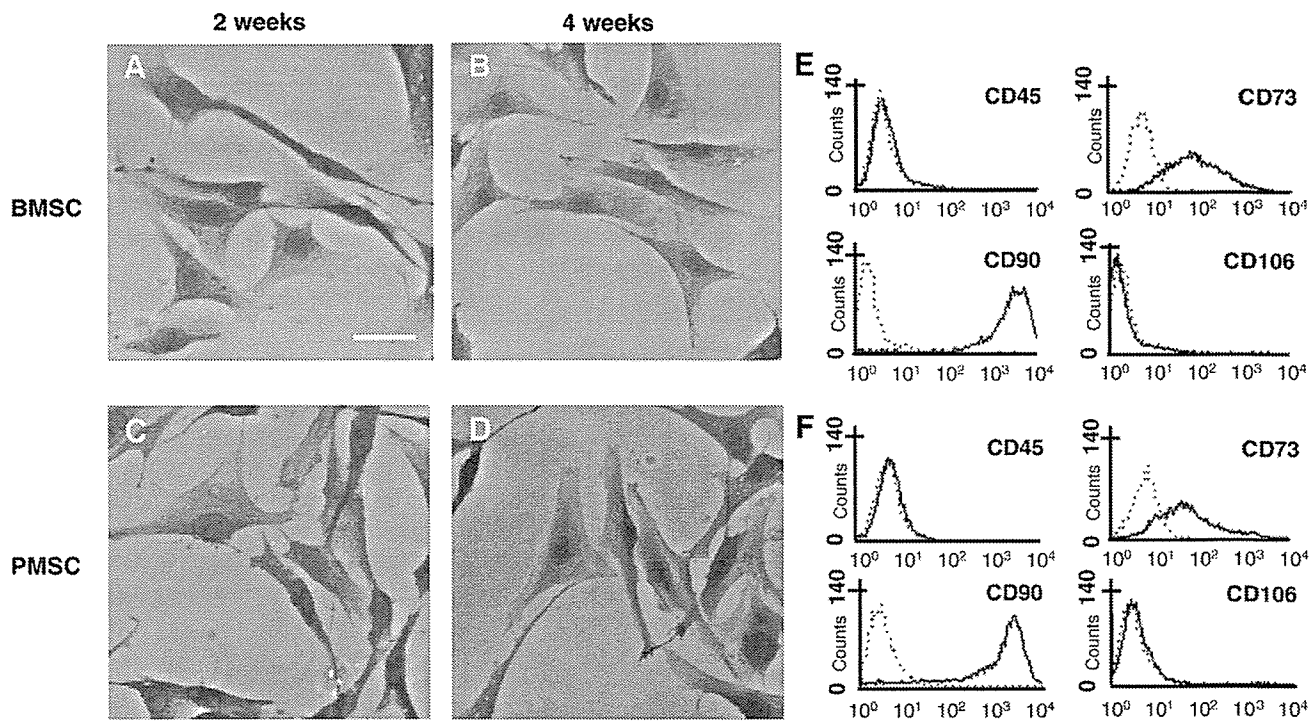

Fig. 1 – Phase-contrast photomicrograph of May-Giemsa stained BMSCs (A) and PMSCs (B) at 2 and 4 weeks in culture, respectively. Flow cytometric analysis of cultured BMSCs (E) and PMSCs (F) with CD45, CD73, CD90, and CD106 antibodies. Dotted lines in each panel indicate isotype-matched mouse IgG antibody control staining. Scale bar=10 μm.

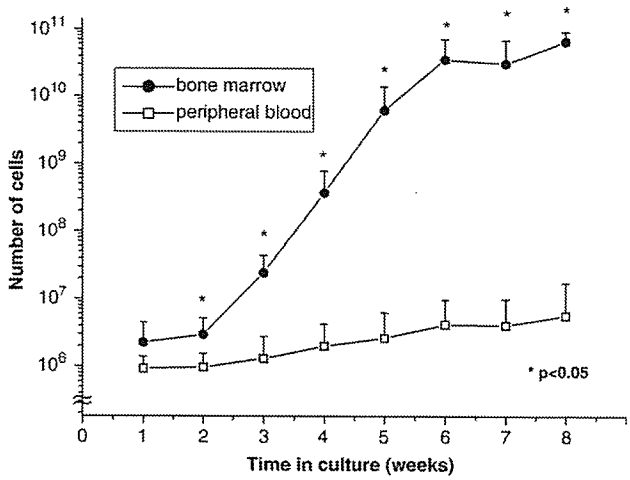

Fig. 2 – Culture expansion of BMSCs (black) and PMSCs (open square). The cell numbers of both MSCs were counted at each week. Error bars represent one SD from the mean. *$p<0.05$ ($n=16$).

procedures. BMSCs began forming floating cell masses and nestin positivity when they were inhibited from adhering to the culture dishes (non-treated dishes) and maintained in the appropriate medium and growth factors (see Experimental procedures). More than 90% of MSCs transformed to neurospheres. RT-PCR analysis for nestin mRNA expression in cDNA samples of cultured adherent BMSCs (Fig. 3E-a) and floating spheric cells (neurospheres) (Fig. 3E-b) are shown in Fig. 3E. The floating spheric cells displayed an amplification of a PCR fragment of the expected size for nestin (420–430 bp), but the cultured non-transformed BMSCs did not (Fig. 3F-a). PMSCs also showed similar transformation to nestin-positive neurospheres after induction (Fig. 3B), which was confirmed by RT-PCR (Fig. 3F-b).

2.4. *Differentiation from MSC-derived neurosphere to neural cells*

MSC-derived neurospheres differentiated into neuron- and glia-like cells in the appropriate culture condition. BMSC-derived neurospheres differentiated into adherent neural cells when they were mechanically dissociated, plated on plastic culture dish, and maintained in NPBM without growth factors. Adherent single layers contained abundant neuron- and glial-like cells. Immunocytochemical analysis indicated that the neuronal cells showed NF-M positivity (Fig. 4A), which was confirmed by RT-PCR. A sample of adherent cells displayed an amplification of a PCR fragment of the expected size for NF-M (330–340 bp) (Fig. 4E-b). In addition, GFAP-positive cell differentiation was also demonstrated with immunostaining

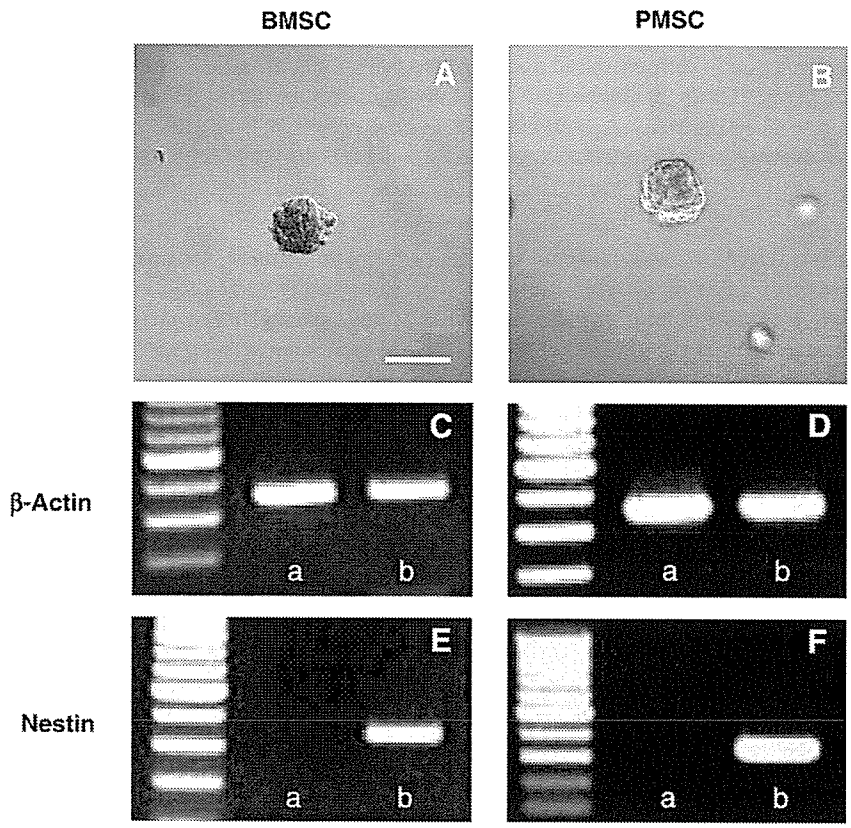

Fig. 3 – Transformation from MSCs to nestin-positive neurospheres. When BMSCs (A) and PMSCs (B) were placed in NPBM with growth factors and were inhibited to adhere on the culture dish, the cells formed neurospheres (scale bar=20 μm). RT-PCR analysis demonstrated that neurospheres transformed from BMSCs showed nestin positivity (E-b), which was negative before transformation (E-a). Nestin also became positive following transformation of PMSCs (F-b), which was negative in the primary PMSCs (E-a). Panels C and D showed control mRNA expression of β-actin of BMSCs and PMSCs, respectively.

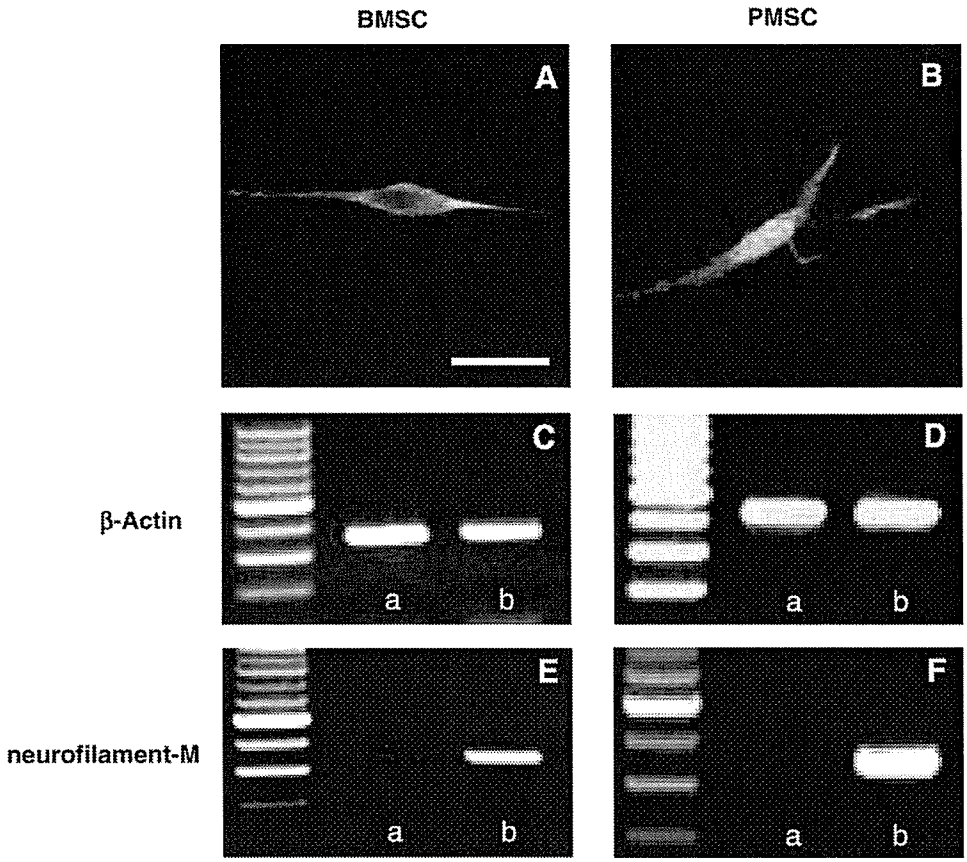

Fig. 4 – Neurofilament expression in differentiated neurosphere cells. Cells differentiated from neurospheres which had been transformed from BMSCs (A) or PMSCs (B) showed NF-M positivity in culture. RT-PCR analysis demonstrated that BMSCs (A) and PMSCs (B) differentiated from neurospheres showed NF-M positivity (E-b; F-b), which was negative in neurospheres (E-a; F-a). Panels C and D showed control mRNA expression of β-actin of BMSCs and PMSCs, respectively. Scale bar=10 μm.

(Fig. 5A) and RT-PCR analysis (Fig. 5E-b). The expected size for an amplified PCR fragment of GFAP is 430–440 bp. PMSC-derived neurosphere showed similar differentiating potential to NF-M-positive neurons (Fig. 4B) and GFAP-positive glia (Fig. 5B), which were confirmed by RT-PCR in Figs. 4F-b and 5F-b, respectively.

3. Discussion

The present study demonstrates that fibroblast-like adherent cells with phenotypic characteristics resembling those of mesenchymal stem cells prepared from the bone marrow can be cultured from rat peripheral blood. These cells showed proliferation and differentiation into neural lineages in vitro, confirmed by immunocytochemistry and RT-PCR.

Mesenchymal precursor cell populations obtained from rat peripheral blood and bone marrow of the rat metaphysis easily expanded in vitro and exhibited a fibroblast-like morphology. Flow cytometry analysis to study the surface protein expression on undifferentiated BMSCs and PMSCs indicated that the myeloid progenitor antigen CD45 was not expressed by these cells. On the other hand, PMSCs and BMSCs expressed CD73 (SH3), which has been used to characterize mesenchymal stem cells (Majumdar et al., 1998; Kobune et al., 2003; Honma et al., 2006). In addition, nestin expression by PMSCs and BMSCs and their ability to grow in suspension in defined culture conditions brought them nearer to a neurosphere phenotype. When nestin-positive neurospheres were dissociated and plated onto an adherent surface without growth factors, neuronal and glial differentiation was observed. We are not certain from our results whether strictly regulated neuronal and glial cells were present in the PMSC population, or culture conditions determined cell fate into neurons or glia.

A distinct difference between PMSCs and BMSCs in the present study is their growth rate in culture. The BMSCs showed a sigmoidal growth curve and extensive expansion over 8 weeks. In contrast, the PMSCs had a more linear growth curve and were much less proliferative. A possible explanation is that samples of BMSCs contain more primitive progenitor cells than samples of PMSCs. Although the CD34 antigen has been used to identify hematopoietic precursor cells, $CD34^+$ cells were present in the circulation in only one-tenth the concentration of bone marrow and had a different spectrum of antigen expression. Indeed, CD33 (myeloid antigen) and CD45 (leukocyte common antigen) were highly expressed in $CD34^+$ cells in the peripheral circulation, but not in bone marrow (Bender et al., 1991).

Another hypothesis is that PMSCs require specific culture medium or growth factors which are different from those of

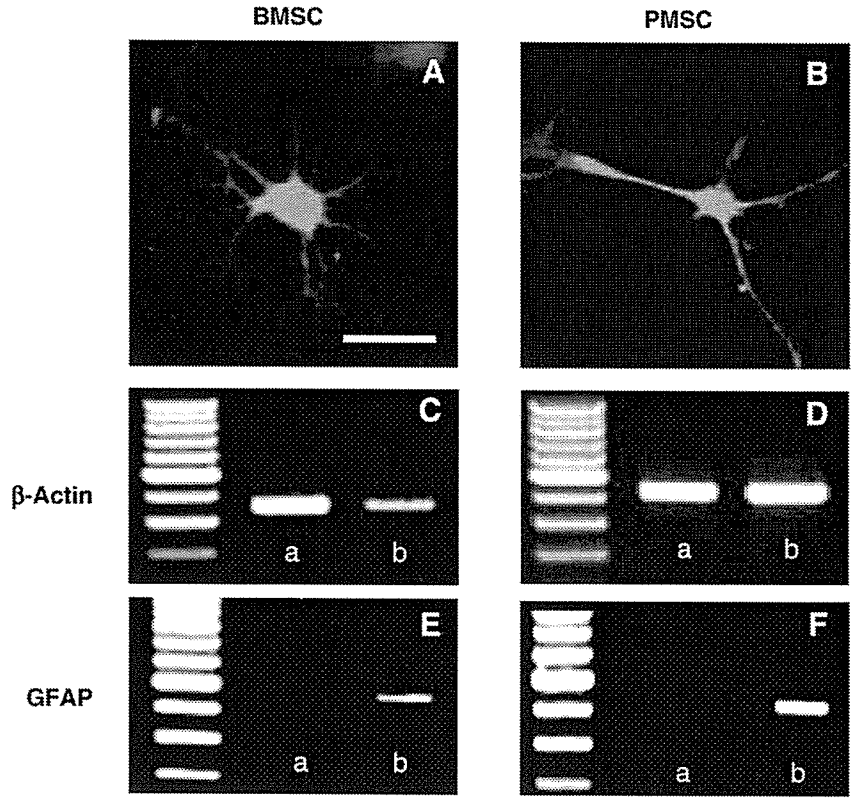

Fig. 5 – GFAP expression in differentiated neurosphere cells. Immunocytochemical analysis indicated that BMSCs (A) and PMSCs (B) differentiated from neurospheres showed GFAP positivity in culture. RT-PCR analysis demonstrated that cells differentiated from neurospheres which had been transformed from BMSCs showed the GFAP positivity (E-b), which was negative in neurospheres (E-a). GFAP also became positive following differentiation in the PMSCs group (F-b), which was negative before induction (F-a). Panels C and D demonstrated the mRNA expression of β-actin of BMSCs and PMSCs for control, respectively. Scale bar=10 μm.

BMSCs. To maintain viability and retain the ability to proliferate, stem cells need cell-to-cell contact and a microenvironment which provides necessary growth factors in adequate quantity, which may be supplied in an autocrine or paracrine fashion (Huss et al., 2000). The PMSCs in mobilized peripheral blood required BMSC conditioned medium which showed high proliferative potential with high express of Oct4, a transcriptional binding factor (Tondreau et al., 2005). Moreover, mesenchymal precursor cells in the peripheral blood of normal individuals proliferated logarithmically in 20% fetal calf serum without growth factor (Zvaifler et al., 2000). Thus, PMSCs may require a novel culture medium. The difference between cultures of BMSCs and PMSCs will be important in future considerations of PMSCs for clinical use, where a large number of cells will be necessary.

The morphological and antigenic characteristics of MSCs are different among species (Kobune et al., 2003; Honma et al., 2006; Rochefort et al., 2005), which may indicate that the appropriate culture medium or growth factors of human PMSCs is different from rat PMSCs. Nevertheless, in the present study, we showed that the rat PMSCs proliferated, highly transformed to nestin-positive neural stem cells (neurospheres), and differentiated into neuronal or glial cells in vitro.

Thus, autologous peripheral blood could be an important source of cells for a cell therapy, since they are easy to isolate and expand for autotransplantation with little risk of rejection. However, further investigations are needed to better characterize PMSCs and as well to further define their transplantability and differentiation potential in animal models.

4. Experimental procedures

4.1. *Cell preparation*

The use of animals in this study was approved by the animal care and use committee of Sapporo Medical University and all procedures were carried out in accordance with institutional guidelines. Bone marrow was obtained from femoral bone in adult female Sprague-Dawley rats weighing 200–250 g. Rats were anesthetized with ketamine (50 mg/kg) and xylazine (10 mg/kg) i.p. A small hole (2×3 mm) in the femoral bone was made with an air drill following skin incision (1 cm). Bone marrow (0.5 ml) was aspirated, diluted to 25 ml with Dulbecco's modified Eagle's medium (DMEM) (SIGMA, St. Louis, MO) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (GibcoBRL, Grand Island, NY), 2 mM L-glutamine (Gibco BRL), 100 U/ml penicillin, 0.1 mg/ml streptomycin (Gibco BRL), was plated on 50-cm^2 Tissue Culture Dish (IWAKI, Tokyo, Japan), and incubated in a humidified atmosphere of 5% CO_2 at

37 °C for 3 days. BMSCs, when selected by plastic adhesion, require the elimination of nonadherent cells by replacing the medium 48 h after cell seeding. When cultures almost reached confluence, the adherent cells were detached with trypsin–EDTA solution (SIGMA) and subcultured at 1×10^4 cells/ml.

Peripheral blood was obtained from adult Sprague–Dawley rats weighting 200–250 g. Rats were deeply anesthetized with ketamine (50 mg/kg) and xylazine (10 mg/kg) i.p. Peripheral blood (about 8 ml) was aspirated from vena cava superior with a 18-gauge needle. Peripheral blood was diluted 1:3 in Puregene RBC Lysis Solution (Gentra systems, Minneapolis, MN) and was incubated in a 50-ml conical centrifuge tube for 5 min at room temperature. The tube was centrifuged at 3500 rpm for 2 min and the supernatant was discarded. The cell pellet was suspended in DMEM supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin and was plated on 50-cm^2 plastic tissue culture dishes and incubated in a humidified atmosphere of 5% CO_2 at 37 °C. PMSCs, when selected by plastic adhesion, require the elimination of nonadherent cells by replacing the medium 48 h after cell seeding. When cultures almost reached confluence, the adherent cells were detached with trypsin–EDTA solution and subcultured at 1×10^4 cells/ml. The cell numbers of both BMSC and PMSC were counted in a cytometer every a week.

Some of cultured cells were rinsed in PBS for three times and fixed for 10 min with a fixative solution containing 4% paraformaldehyde in 0.14 M Sorensen's phosphate buffer, pH 7.4, at room temperature. The cells were counterstained with May-Giemsa (Honma et al., 2006), and phase-contrast microphotographs were obtained using a Zeiss microscope.

4.2. *Phenotypic characterization*

Flow cytometric analysis of BMSCs and PMSCs was performed as previously described (Honma et al., 2006). Briefly, cell suspensions were washed twice with phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (BSA). For direct assays fifty thousand cells were incubated with FITC-conjugated CD45 (Leukocyte Common Antigen) (BD Bioscience Pharmingen, San Jose, CA), PE-conjugated CD 73 (Ecto-5′-nucleotidase) (BD Bioscience Pharmingen), PE-conjugated CD 90 (Thy-1) (eBioscience, San Diego, CA) and PE-conjugated CD106 (VCAM-1) (BD Bioscience Pharmingen) at 4 °C for 30 min, and then washed twice with PBS containing 0.1% BSA. The cells were analyzed by cytometric analysis using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA) with the use of CellQuest software.

4.3. *Induction of MSCs to floating spheric cells*

When inducing MSCs to floating spheric cells like neurospheres, MSCs were detached with trypsin–EDTA solution and were collected in a 50-ml tube in DMEM+10% FBS. After rinsing with DMEM, cells (1×10^6 cells/ml) were suspended in 20 ml Neural Progenitor basal medium (NPBM) (Cambrex, One Meadowlands Plaza, NJ) supplemented with 2 mM L-glutamine, 10 ng/ml epidermal growth factor (EGF), 10 ng/ml basic fibroblast growth factor (bFGF), 100 U/ml penicillin, 0.1 mg/ml streptomycin, were plated on 100 mm non-treated dish (IWAKI), and were incubated for 3 days. Growth factors (EGF and bFGF) were added every day.

4.4. *Differentiation of neurospheres to neural cells*

When inducing the floating spheric cells (neurospheres) to neural cells, floating spheric cells were collected by centrifuging at 1500 rpm for 5 min, suspended in NPBM supplemented with 2 mM L-glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin, mechanically dissociated, and were plated on plastic tissue culture dish for 1 week.

4.5. RT-PCR

Total RNA was extracted from each cell culture using RNeasy Mini Kit (QIAGEN, Hilden, Germany). We performed the reverse transcription with 100 ng RNA using the SuperScript II RNase H^- reverse transcriptase (Invitrogen, Carlsbad, CA). Briefly, we used a final volume of 20 μl containing 100 ng RNA, 4 μl First Strand Buffer, 10 mM dNTPs, 100 mM DTT, 0.5 μg Oligo$(dt)_{12-18}$ and 100 U of SuperScript II RNase H^- reverse transcriptase. We then carried out a PCR reaction using the Hot Star Taq Master Mix Kit (QIAGEN) in a final volume of 50 μl containing 25 μl Hot Star Taq Master Mix, and 10 mM upstream sense and downstream sense primers. Cyclical parameters were denatured at 94 °C for 30 s, annealed at 60 °C for 30 s, and finally elongated at 72 °C for 30 s. Thirty five cycles were performed for each primer set. We resolved the PCR products on 2% gel agarose. Primer sequence of amplified products was: mouse β-actin sense (5′-TGGAATCCTGTGGCATCCATGAAAC-3′), mouse β-actin antisense (5′-TAAAACGCAGCTCAGTAACAGTCCG-3′), rat nestin sense (5′-CTTAGTCTGGAGGTGGCTACATACA-3′), rat nestin antisense (5′-GAGGATAGCAGAAGAACTAGGCACT-3′), rat neurofilament M (NF-M) sense (5′-GGTCACTTCACATGCCA-TAGTCAA-3′), rat NF-M antisense (5′-GGCTCAGTTGGTA-CTTTGCGTAA-3′), rat glial fibrillary acid protein (GFAP) sense (5′-ATTCCGCGCCTCTCCCTGTCTC-3′), and rat GFAP antisense (5′-GCTTCATCCGCCTCCTGTCTGT-3′).

4.6. *Immunocytochemical analysis*

To identify the cell type derived from the BMSCs and PMSCs, immunocytochemical studies were performed with the use of antibodies to neurons (1:400 monoclonal mouse NF-M, SIGMA), and astrocytes (1:400 monoclonal mouse anti-GFAP, SIGMA). Cultured cells were rinsed in PBS for three times and fixed for 10 min with a fixative solution containing 4% paraformaldehyde in 0.14 M Sorensen's phosphate buffer, pH 7.4, at room temperature. After washing twice in PBS and incubating in PBS containing 0.1% Triton X-100 for 10 min at room temperature, fixed cells were incubated for 30 min in a blocking solution containing 0.1% Triton X-100, and 3% BSA before incubation with the primary antibody. Primary antibodies are labeled with Alexa Fluor 488 or Alexa Fluor 594 using Zenon mouse IgG Labeling Kits (Molecular Probes Inc., Eugene, OR) according to the manufacturer's instruction. After immunostaining, coverslips were mounted cell-side down on microscope slides using mounting medium (DAKO Corp., Carpinteria, CA). Confocal images were obtained using a Zeiss laser scanning confocal microscope with the use of Zeiss software.

4.7. *Data analysis*

All data are presented as mean values ± SD. Differences among groups were assessed by ANOVA with Scheffe's post hoc test to identify individual group differences. Differences were deemed statistically significant at $P<0.05$.

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The 5-Hydroxytryptamine$_{2A}$ Receptor Antagonist Sarpogrelate Hydrochloride Inhibits Acute Platelet Aggregation in Injured Endothelium

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In this study, the effect of sarpogrelate hydrochloride, a 5-hydroxytryptamine$_{2A}$ receptor antagonist, on platelet aggregation at the site of injured carotid artery endothelium was examined. The rat common carotid artery was clamped for 30 min to induce endothelial injury. Sarpogrelate hydrochloride was administered before and after the injury, and the effects were compared with those in rats receiving sham operation only and those receiving clipping injury but no sarpogrelate hydrochloride. The animals were killed 24 h after the procedure. The common carotid artery was examined by scanning electron microscopy and stained immunochemically for factor VIII. Sarpogrelate hydrochloride treatment was associated with reduced aggregation of platelets on electron microscopy and lower expression of factor VIII at the injured intima. Sarpogrelate hydrochloride has an inhibitory effect on platelet aggregation at the intima in the acute stage after injury, suggesting that this drug may be used to prevent early ischaemic complications after surgical or endovascular arterial intervention.

KEY WORDS: SARPOGRELATE HYDROCHLORIDE; INTIMAL INJURY; PLATELET AGGREGATION; FACTOR VIII; SCANNING ELECTRON MICROSCOPY

Introduction

The introduction of interventional treatment for cerebrovascular disease using endovascular techniques has offered new options in conditions that are difficult to treat by conventional surgical techniques, such as arterial steno-occlusive disease and cerebral aneurysms. Endovascular treatment involves the use of a variety of instruments inside the arterial lumen, including microcatheters, guidewires, stents and coils. These instruments may, however, occasionally injure the intima of the artery. Injury to the endothelium is a primary cause of ischaemic complications associated with endovascular treatment; it results in platelet aggregation in the acute stage and atherosclerotic changes in the chronic stage. Even minimal endothelial injury results in platelet aggregation at the site of injury.[1] It is therefore conceivable that anti-platelet treatment to inhibit platelet

aggregation after injury to the arterial endothelium may prevent ischaemic complications following endovascular interventions.

The endogenous monoamine serotonin (5-hydroxytryptamine, 5-HT) plays an important role in platelet aggregation. It is stored in dense granules and is released when platelets are activated at the site of vascular injury. Plasma serotonin levels have been reported to be substantially increased at the site of the injury.[2-4] Serotonin itself is a weak agonist, but its effect on the platelet functional response, which is mediated by the 5-HT_{2A} receptor, is amplified by the presence of low concentrations of agonists such as collagen and adrenaline, even when the level of each agonist alone is insufficient to induce platelet aggregation. This amplification effect is strongly inhibited by selective 5-HT_{2A} receptor antagonists.[5,6]

Sarpogrelate hydrochloride is a selective 5-HT_{2A} receptor antagonist that is widely used in Japan for the treatment of peripheral arterial disease. It strongly inhibits the effects of serotonin such as platelet aggregation, vasoconstriction and vascular smooth muscle proliferation.[7] Sarpogrelate inhibits human platelet aggregation induced by collagen and secondary aggregation by adenosine diphosphate or adrenaline by 50% (IC_{50}) at a concentration of 1 μM, and this effect was potentiated for aggregation induced by collagen plus serotonin, with an IC_{50} of 0.1 μM.[8] Furthermore, a major metabolite of sarpogrelate hydrochloride, M-1, was approximately 10 times more potent than the unaltered substance.[8]

Many studies have evaluated the inhibitory effects of selective 5-HT_{2A} receptor antagonists on platelet aggregation[9-12] but none has studied their potential effects at the site of vascular injury. This study investigated the effects of sarpogrelate hydrochloride on platelet aggregation using scanning electron microscopy and immunochemical staining in a rat model of acute arterial injury.

Materials and methods

Male Sprague–Dawley rats were used in this study. All animals were obtained from an official supplier, and had free access to food and water. The conditions they were kept in prior to the experiment and the experimental protocol were approved by the Animal Experiment Committee of Hokkaido University School of Medicine, Sapporo, Japan.

EXPERIMENTAL PROTOCOL

One group of rats was injected intraperitoneally with 50 mg/kg sarpogrelate hydrochloride 1 h before the induction of anaesthesia. A further group, acting as the control group, received saline solution intraperitoneally. All rats were anaesthetized with an intraperitoneal injection of 50 mg/kg sodium pentobarbital and the right common carotid artery was exposed via a midline longitudinal linear skin incision (Fig. 1A). In the sarpogrelate hydrochloride and control groups, 10 Yasargil aneurysm clips (Aesclap Co., Tuttlingen, Germany) (Y-710, closing pressure, 120 g) were applied to the right common carotid artery for 30 min (Fig. 1B). All 10 clips were then removed and the circulation was re-established (Fig. 2). In a third group of rats (the sham operation group), clips were not applied. The skin incision was then closed in all rats and anaesthesia was permitted to wear off.

In the sarpogrelate hydrochloride group, 50 mg/kg sarpogrelate hydrochloride was again administered intraperitoneally 3 h after the first dose. The control group received saline solution intraperitoneally after the same time interval.

The animals were killed 24 h later using an overdose intraperitoneal injection of

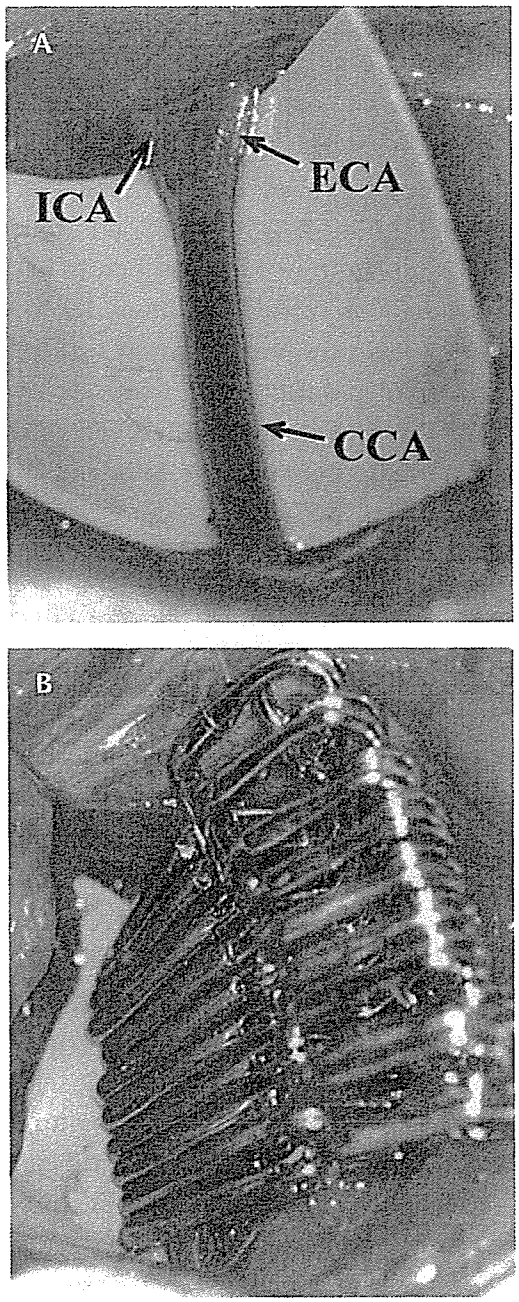

FIGURE 1: (A) Exposed right common carotid artery (CCA) and (B) right common carotid artery after application of 10 aneurysm clips in rats exposed to clipping injury alone (untreated) or 50 mg/kg sarpogrelate hydrochloride intraperitoneally before and after clipping injury, to investigate the effect of sarpogrelate hydrochloride on platelet aggregation. ICA, internal carotid artery; ECA, external carotid artery

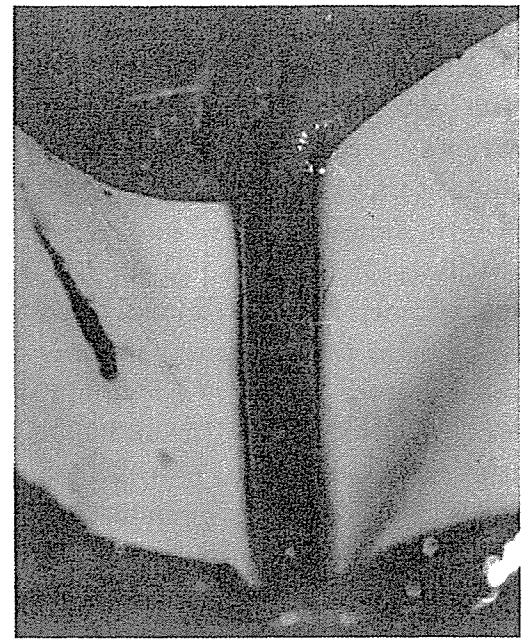

FIGURE 2: Right common carotid artery after removal of aneurysm clips and re-establishment of circulation in rats treated with clipping injury alone (untreated) or 50 mg/kg sarpogrelate hydrochloride intraperitoneally before and after clipping injury, to investigate the effect of sarpogrelate hydrochloride on platelet aggregation

pentobarbital, and the common carotid artery was isolated.

SCANNING ELECTRON MICROSCOPY

The isolated artery was fixed by irrigation of the left ventricle with 2% glutaraldehyde, and was then dissected. An arterial segment was post-fixed in osmium tetroxide for 60 min, rinsed in distilled water for 5 min and fixed in 50% alcohol for 15 min before being examined by scanning electron microscopy.

IMMUNOCHEMISTRY OF FACTOR VIII-RELATED ANTIGEN

A second arterial segment was subjected to immunochemistry using a factor VIII-related antigen. Fixed frozen sections were dipped in

cold acetone for 10 min and incubated in 5% normal goat serum for 10 min to block non-specific reactions. The sections were incubated with the primary antibody (polyclonal rabbit anti-factor-related antigen) (DakoCytomation, Kyoto, Japan) for 1 h, then incubated in 0.3% hydrogen peroxide in cold 100% methanol for 10 min to inhibit endogenous peroxidase activity. The sections were then incubated with rat avidin–biotin complex reagent (StreptABComplex/HRP, Dako) for 15 min and with 0.02% diaminobenzidine solution for 15 min, dehydrated, stained with Mayer's haematoxylin for nuclei and examined using light microscopy.

Results

Eighteen Sprague–Dawley rats were used in this study. Each weighed approximately 330 g (range, 320 – 340 g) and all were 7 – 8 weeks old. Six rats were injected intraperitoneally with 50 mg/kg sarpogrelate hydrochloride, six acted as the control group (receiving intraperitoneal saline solution) and six underwent sham operations.

On scanning electron microscopy, there was less platelet aggregation in the sarpogrelate hydrochloride-treated group compared with the untreated injured group. Four specimens from each of the three groups are shown in Fig. 3. The differences were difficult to quantify, but platelet aggregation is clearly suppressed in rats treated with sarpogrelate hydrochloride.

On immunochemical analysis for factor VIII-related antigen, normal endothelium and platelets aggregated on the injured endothelium were stained. In the sham operation group, the endothelium was positively stained (Fig. 4). In the untreated group, staining of aggregated platelets was seen on the denuded wall of the injured artery, but in the sarpogrelate hydrochloride group, platelet aggregation was rarely observed on the endothelium. Factor VIII-positive cells observed on the arterial surface of rats in the sham operation group were considered to be normal endothelial membrane (Fig. 4).

Discussion

Ischaemic complications are one of the main causes of morbidity associated with endovascular procedures. For example, in carotid stenosis, migration of soft atheroma stripped from the fragile intimal wall to the distal side of the internal carotid artery can occur as an ischaemic complication of angioplasty using a stent. Platelet aggregation to the injured endothelium is also presumed to be associated with endovascular treatment. Indeed, the process of platelet aggregation is integral to some types of endovascular treatment; in particular, the primary aim of embolization treatment of an aneurysm using coils is to induce thrombus formation around the coil and consequent obliteration. Moreover, as we have previously shown, in surgical bypass surgery, early occlusion of the anastomosed vessel is caused by thrombosis at the site of the anastomosis.[13]

Various treatments have been applied before and after endovascular treatment in an attempt to avoid ischaemic complications. Intravenous administration of an anticoagulant such as heparin is the standard treatment in the acute stage after placement of an intravascular foreign body such as a stent or coil, but currently no treatment avoids platelet aggregation in the acute stage after surgical or endovascular treatment for cerebral ischaemia.

Experimental injury to the endothelium using aneurysm clips has been widely used as a standard technique to observe the acute and chronic changes after damage to an artery.[14] This procedure does not induce

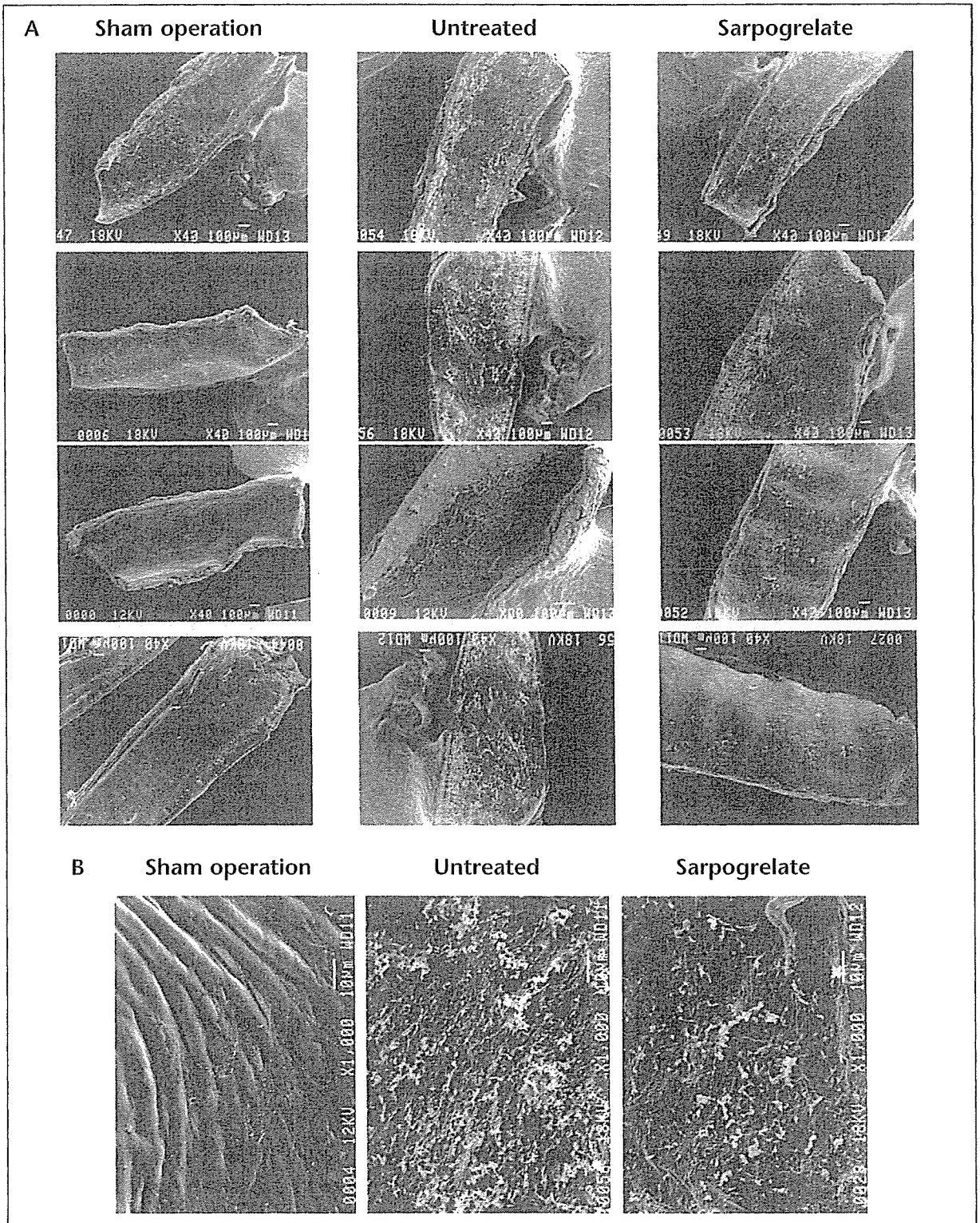

FIGURE 3: Scanning electron microscopy findings in the right common carotid artery in rats treated with sham operation, clipping injury alone (untreated) or 50 mg/kg sarpogrelate hydrochloride intraperitoneally before and after clipping injury, to investigate the effect of sarpogrelate hydrochloride on platelet aggregation. (A) Magnification × 26. (B) Magnification × 400

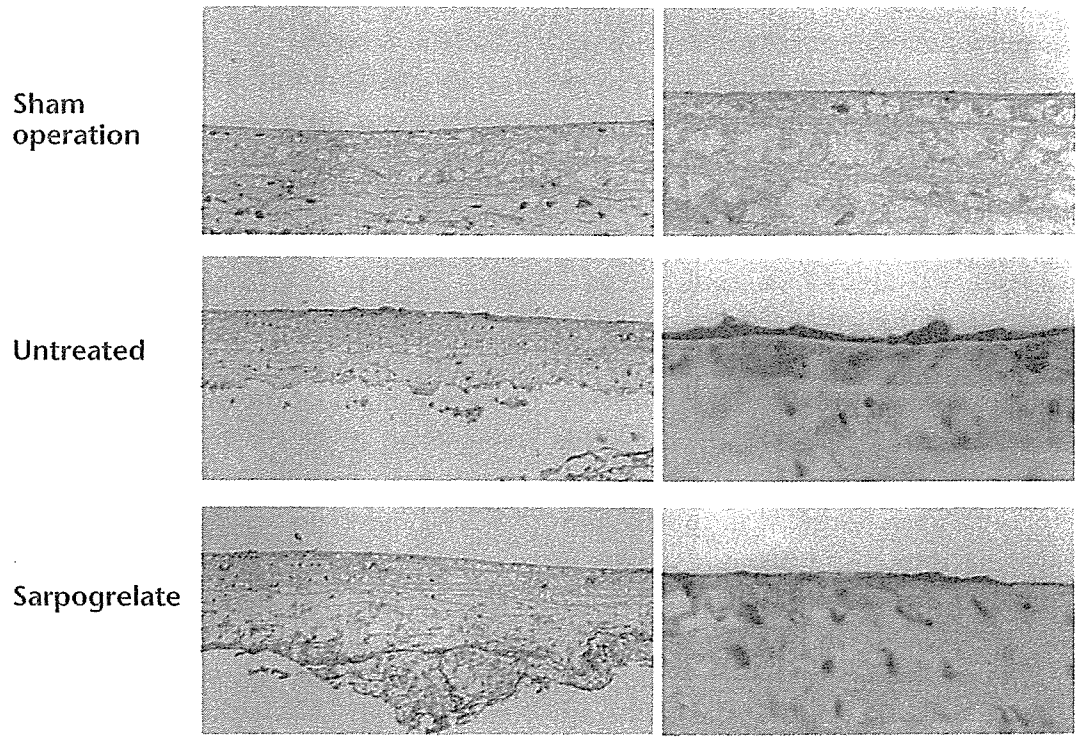

FIGURE 4: Immunochemical analysis for factor VIII-related antigen in the right common carotid artery in rats treated with sham operation, clipping injury alone (untreated) or 50 mg/kg sarpogrelate hydrochloride intraperitoneally before and after clipping injury, to investigate the effect of sarpogrelate hydrochloride on platelet aggregation. Left column shows lower magnification (× 110) and right column shows higher magnification (× 550)

substantial ischaemic or post-ischaemic hyperperfusion damage to the rat brain,[15] and several authors have investigated longitudinal change after arterial injury using this technique.[16 - 18] Experimental injury produces a number of changes, including acute denudation of the endothelium on the arterial lumen surface, platelet aggregation on the denuded intimal surface of the damaged and exposed smooth muscle in the acute stage, and endothelial regrowth from the denuded end to cover the damaged area in the chronic stage. This procedure does not necessarily induce atherosclerotic degeneration. From this it can be seen that control of platelet aggregation in the acute stage has a crucial role in avoiding ischaemic complications in clinical endovascular treatment.

Several anti-platelet drugs are available for clinical use, each acting via a different mechanism, and each having particular advantages and drawbacks. Aspirin and ticlopidine, the most frequently used anti-platelet drugs, take several days to achieve their anti-platelet effect.[19] Refractory bleeding may occur if these anti-platelet drugs have not been withdrawn prior to surgery, so they are frequently stopped several days before major surgery or endovascular intervention.

The biological actions of serotonin are mediated by a number of different receptors. To date, 14 distinct subtypes of serotonin

receptor have been identified by molecular techniques on the basis of their binding profile, the functional activity of ligands and sequence homology.[20] The 5-HT_{2A} receptor on platelets and vascular smooth muscle cells is mainly associated with vascular events; acceleration of serotonin-mediated platelet activation via 5-HT_{2A} receptors at the site of vascular injury leads to vascular occlusion.

Sarpogrelate hydrochloride is a novel 5-HT_{2A} receptor antagonist that has been approved for use in peripheral arterial disease in several Asian countries. One unique characteristic of sarpogrelate hydrochloride compared with other anti-platelet drugs is its swift inhibition of platelet aggregation. Sarpogrelate hydrochloride inhibits aggregation of platelets 1.5 h after treatment,[21] which is similar to the immediate response observed in the present study, and acts via direct antagonism of 5-HT_{2A} receptors on circulating platelets.[8] If a sufficiently large dose is given, this effect occurs without a time delay. Furthermore, as the effect of sarpogrelate hydrochloride on the platelet is reversible, it can be withdrawn only 1 or 2 days before surgery, which is an advantage compared with conventional anti-platelet drugs.[22]

In the present study, aggregation of platelets to the injured intima observed by electron microscopy was inhibited by 50 mg/kg sarpogrelate hydrochloride. This dosage has been shown to be effective in an experimental model of peripheral obstructive disease.[23] Expression of factor VIII at the injured intima was also suppressed by sarpogrelate hydrochloride. Our study suggests that serotonin may play an important role in platelet aggregation via 5-HT_{2A} receptors at the site of endothelial injury. These results indicate that sarpogrelate hydrochloride may be used to prevent early ischaemic complications after surgical or endovascular arterial interventions; however, further quantitative analysis is required to validate the effect.

Conflicts of interest

No conflicts of interest were declared in relation to this article.

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Chapter 1 脳虚血の病態生理

1. 脳虚血を理解するために

A 3つの概念

虚血性ペナンブラ（ischemic penumbra）の概念を提唱したAstrupが述べているように，もし，臨床的な脳虚血が，全て心肺停止の場合のように完全な血流停止（CBFがゼロで，MTTが無限大）であれば，以下に述べるような複雑な概念は不要である[1,2]．血流の完全な停止の場合には，神経電気活動（neuronal electrical activity）の停止と代謝の停止による神経細胞のエネルギーとイオン環境の完全な崩壊の間には，臨床的に意味のある時間差は存在しない[1-4]．神経電気活動の停止と細胞エネルギー状態の崩壊は，5～10分程度時間内で前後して起こると考えられている．この場合にも，以下に述べるような3つの概念は時間的にはきわめて短時間であるが成立する．この10分以内の時間以後の現象は，不可逆性の病理学的変化が完成してゆくだけであり，ある意味，完全に予測可能（predictableである）．しかし，心肺停止のような場合を除くと，一般の脳卒中では，虚血領域が全脳に及び，かつ，完全な血流停止（global complete ischemia）になることはない．臨床的に問題となるような脳卒中では，虚血は残存血流の存在する局所的不完全脳虚血（local incomplete ischemia）である．このために，その経過を予想することは容易ではない．

これまでに，様々な予後予測因子（predictor）が明らかにされてきた．しかし，実際には，この複雑な自然現象の経過予想は，的中率の低いことでは，天気予報の比ではない．まして，人為的なinterventionによる血行再建術が，その経過に対してどのような変化をもたらすかは，さらに予測が困難なものであることは容易に想像される．しかし，これまでの基礎的研究，さらに，臨床的知識の蓄積により，この複雑きわまりなく予想困難（unpredictable）な急性期脳虚血の経過予測もある程度は可能となってきた．

急性期脳虚血を理解する上で重要な概念は，

1）Therapeutic time window（治療可能時間）

2）Penumbra（救済可能領域）

3) Reperfusion injury（再灌流障害）

という3つの概念で説明が可能である．

B Therapeutic time window（治療可能時間＝時間の窓）

急性期の血行再建は，いわゆるtherapeutic time window（治療可能時間）の考えが最も重要な理論的支柱となっている．脳の不可逆的な変化（脳梗塞）の完成は，脳の部位によっても異なるが，同一部位で同一の生理的なパラメーターを設定した場合，「虚血の持続時間」と「虚血の程度」の2つのパラメーターによって規定されるというのがこの考えである（図1-1）．この概念が霊長類の実験虚血脳において最初に提唱されたのは，Jonesらの論文であり，1981年に発表されている[5]．

図1-1に示された曲線は，脳のエネルギー代謝が障害され不可逆的な変化が完成する点をプロットしたものである．このラインの下の領域においては，脳組織は，不可逆的な脳梗塞になる．逆にこのラインの上では，脳組織は，可逆的な状態あるいは全く障害を受けていない状態である．

いい換えると，どんな変化をプロットするかによって，異なるtime windowが存在することになる．例えば，次に述べるようなischemic penumbraを電気生理活動の停止として，これをプロットすると別のtime windowが描けることになる．同じように，MRIの拡散強調画像法や灌流画像法での異常出現の限界値をプロットすると別のtime windowが描けることになる．これらの様々なtime windowの一致，不一致は，臨床における血行再建を考える上できわめて重要である．

一般に，therapeutic time windowという場合には，再灌流によって組織を救済できる時間を意味している．その意味では，再灌流の窓（reperfusion window）である．しかし，これに対して，細胞障害は，再灌流後も持続して起こることが知られている．いい換えると，再灌流の窓を

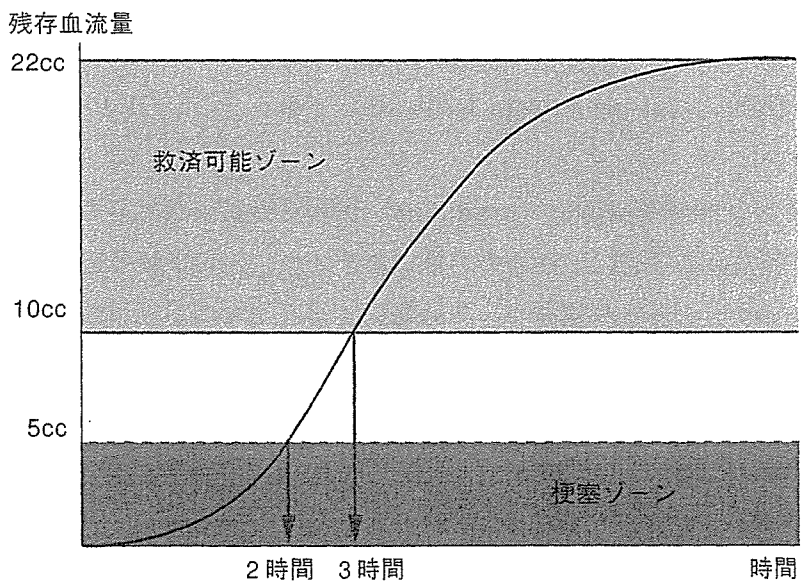

図1-1 Therapeutic time window

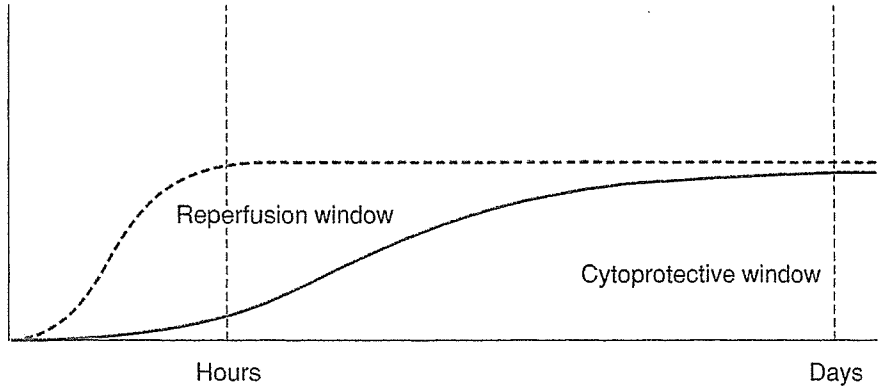

図1-2 2つの Therapeutic time window
数時間の幅の reperfusion window と数日に及ぶ cytoprotective window

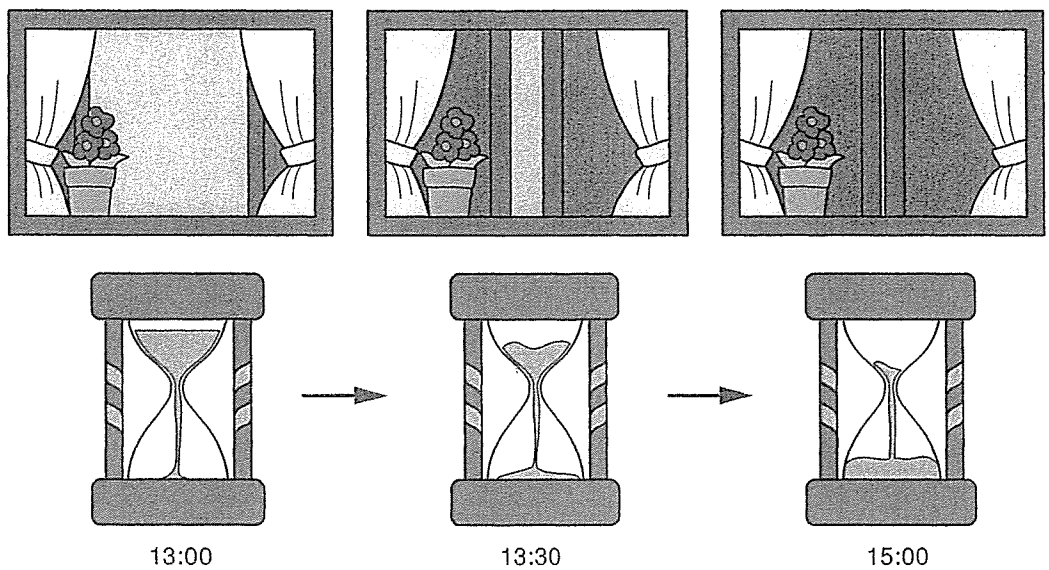

図1-3 時間の窓　Therapeutic time window
治療のための窓は時間と共に狭くなる

過ぎた後も，細胞障害を護る治療の窓は残されていると考えられる．これを cytoprotective window とよぶ場合もある．これは，実際には，各種薬物治療の有効な時間帯を意味している．臨床的には，この時間は，数日以上に及ぶものである．この窓の存在がなければ，今，一般に行われている内科治療は，そもそも意味がないことになってしまう．したがって，広い意味での therapeutic time window は reperfusion window と cytoprotective window の両方を含む．しかし，通常，therapeutic time window という場合には，reperfusion window を意味している（図1-2）[6-9]．

Time window という言葉は，臨床的に modulation 可能な「時間の窓」という直感的なイメージを与える適切な言葉である（図1-3）．この「窓」は，虚血の程度が大きいほど狭く，かつ，時間経過と共に閉じてゆく「時限付きの窓」である．開かれた「窓」からのみ治療者は入り込むことが可能である．その意味では，「窓」からの侵入のイメージよりは，「door，入り口」の方がふさわしいような気もする（Therapeutic time door）．

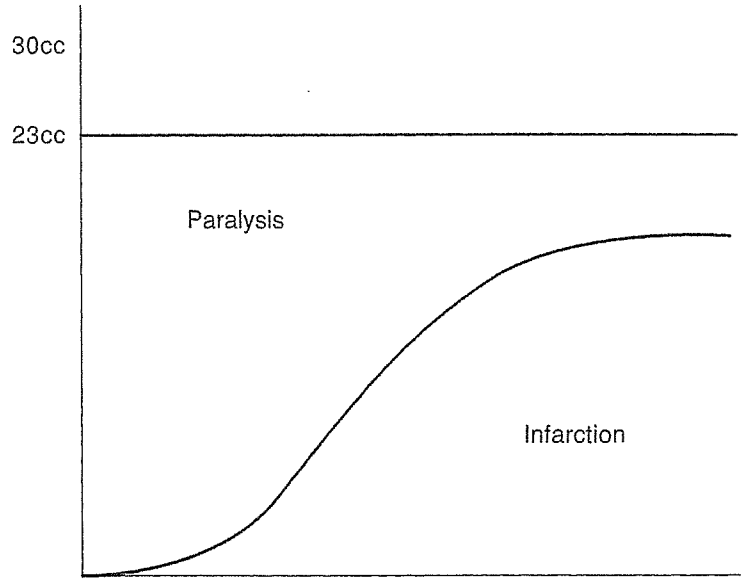

図 1-4 Jones の Therapeutic time window の概念
(Jones. J Neurosurgery. 1981 : 54, 773-83)

Therapeutic time window の概念の変遷について少し理解しておく必要がある．最初の論文は1981年に書かれおり，今日の理論的根拠になっている．Journal of Neurosurgery に発表された論文では，サルの覚醒モデルを用い，中大脳動脈に糸をかけ，閉塞させ，閉塞時間と血流，神経学的症状の推移をみた研究を行っている．対象が霊長類であり，覚醒モデルという点が，特筆される論文である．その論文に最後に図示されたのが，その後，有名になった therapeutic time window の図である．この図そのものは，必ずしも，データに基づいたものではなく，discussion のために追加されたものである（Chapter 1-3 章参照）（図 1-4）．サインカーブのように仮定された閾値のラインが正しいかどうか，特に血流の極端に低い部分では妥当なものかどうかは明らかではない[5]．

ヒトの脳においても，こうした therapeutic time window のパラダイムは成立すると考えられる．ヒトのデータは当然のことながら少ないが，Heiss らの PET によるデータでは，22ml/100 g/min 程度以下になると，急性の虚血性脳障害が発生するとされている[10]．

この急性の脳障害は，基本的にはグルコースと酸素の供給障害による糖代謝障害からスタートする悪循環（vicious cycle）である．その詳細は，本書においても松居が述べている（Chapter 1-2 章参照）．この結果，虚血部位は，時間が経過すると不可逆性変化を起こし，梗塞となる．この梗塞となる領域は，図 1-1 で示されたＳ字状のカーブの下の領域（area under the curve, AUC）である．

C Penumbra（救済可能領域）

一方，therapeutic time window（治療可能時間）だけでは，実際の脳虚血の複雑な病態は説

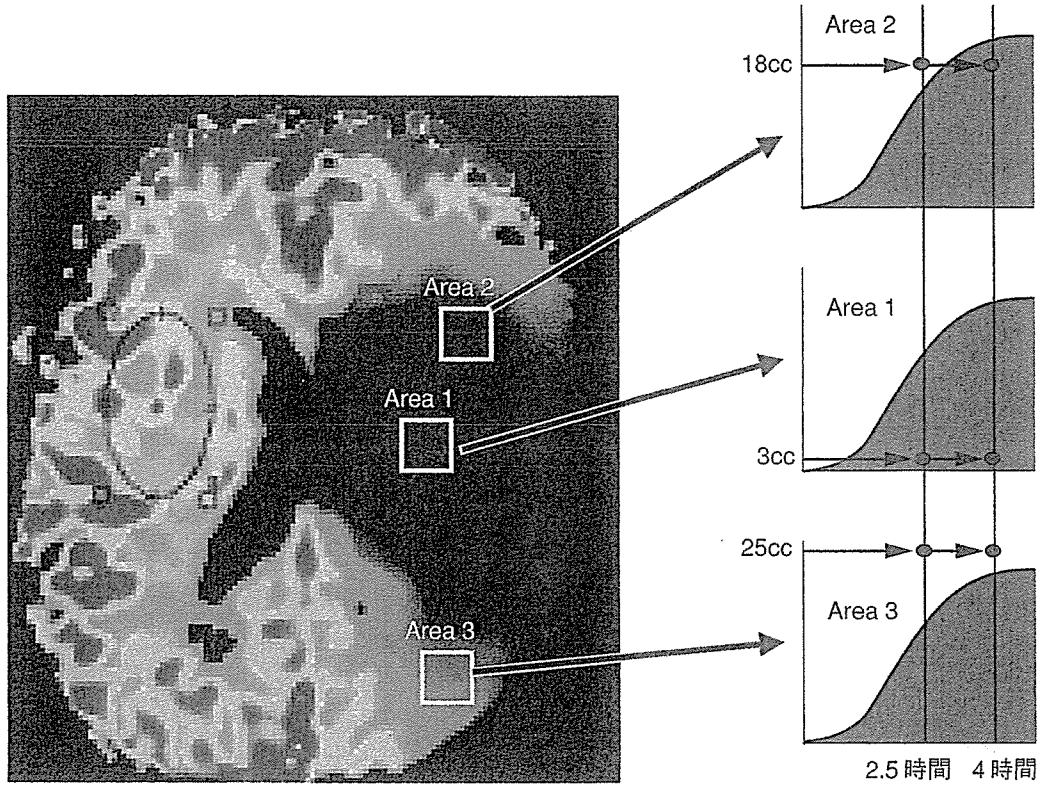

図1-5 脳虚血の部位による therapeutic time window の違い

虚血の中心 area 1 では残存血流が少なく（ここでは 3 cc），短時間で（ここでは救済領域から外れ，梗塞となる．虚血辺縁の area 2 では残存血流が一定程度あり（ここでは 18 cc）ある時間帯（ここでは 2.5 時間），梗塞にならない．しかし，ここも 4 時間後には，救済領域から外れ，梗塞となる．これに対して，十分な残存血流がある（ここでは 25 cc）area 3 では，時間が経過しても梗塞にならない．

明できない．当然のことながら，虚血の程度には，空間的な heterogenuity（不均一さ）が存在する．すなわち，虚血中心と周辺部では，虚血の程度が異なる．したがって，病変の中心と周辺では，虚血の持続時間は同等でも傷害の程度が異なってくる（図 1-5）．Therapeutic time window の考えによれば，虚血がその程度を変化することなく一定時間持続すると，この間に脳梗塞（不可逆性変化）の広さは，増大することになる（図 1-6）．これは，いい換えると時間経過と共に虚血中心の不可逆性変化が拡大し，生存可能な組織と置き換わってゆく過程と捉えることができる．この生存可能な領域が，penumbra（ペナンブラ）と命名され，時間をパラメーターとして経時的に変化する“救済可能領域”として位置づけられた[1-2]．この領域は，神経細胞としての生理的機能，ネットワーク機能は一過性に失われている．しかし，ATP の産生停止によるエネルギー障害やそれに続く膜機能の喪失には至っていない．一方，虚血中心では，グルコースと酸素供給の停止により，糖代謝は完全に障害される（図 1-7）．嫌気性代謝により乳酸が急激に増加し，神経細胞の marker である N-acetyl-aspartate（NAA）が消失することが磁気共鳴分光法（magnetic resonance spectroscopy）などで明らかにされている．一方，penumbra では，嫌気性代謝による乳酸の出現はあるが，その程度は低いし，NAA も保持されている（図