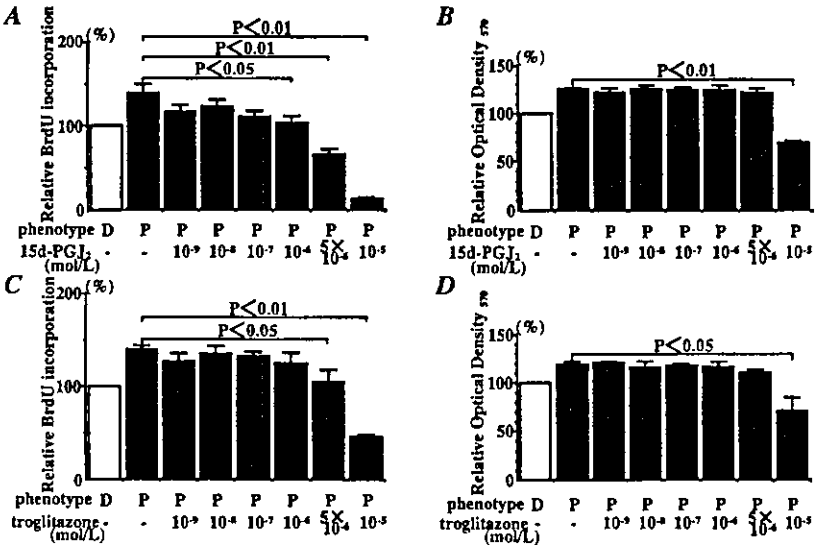

Figure 1. The effects of 15d-PGJ_2 or troglitazone on DNA synthesis (A or C) and viability (B or D) of VSMCs. VSMCs in the quiescent state were stimulated with growth factors and 5% fetal bovine serum (P) in the presence or absence of 15d-PGJ_2 or troglitazone as indicated or kept as the differentiated phenotype (D) for 24 hours in A and C and for 48 hours in B and D. Measurement of the incorporation of BrdU (A and C) and the assay of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (B and D) were performed as described in Methods. The value in the differentiated VSMCs was set as 100% in each experiment. The results are the mean±SE of 2 independent experiments, each carried out in duplicate.

or troglitazone (10^{-8} to 10^{-6} mol/L) for 48 hours. Lysates from these cells were then subjected to Western blotting. Expression levels of smooth muscle–specific proteins were quantified and normalized to the β-actin expression level. As shown in Figure 2A and B, administering 10^{-8} mol/L of 15d-PGJ_2 significantly induced the expression of SM-MHC and SM-α-actin in proliferative VSMCs. This induction did not occur at higher concentrations of 15d-PGJ_2. Similarly, as shown in Figure 2C and D, troglitazone significantly induced the expression of SM-MHC and SM-α-actin at a concentration of 10^{-7} mol/L but not at a concentration of 10^{-6} mol/L. Administering dimethyl sulfoxide, which dissolves 15d-PGJ_2 and troglitazone in their stock solutions, into proliferative VSMCs did not affect the expression of SM-MHC at any concentrations lower than 0.1% (Figure 2E).

15d-PGJ_2 Activates PPARγ-Dependent Pathways and Induces the Expression of SM-MHC in Proliferative VSMCs

To determine whether 15d-PGJ_2 actually activates PPARγ, we examined the activity of a PPRE-containing promoter. We transfected a luciferase expression vector driven by 3 copies of the PPRE (pPPREluc). As shown in Figure 3A, 10^{-8} mol/L of 15d-PGJ_2 resulted in marked activation of PPRE promoter activity. Administration of prostaglandin $F_{2\alpha}$ (10^{-8} mol/L; Wako Pure Chemical Industries), which causes inhibitory phosphorylation of PPARγ by stimulating mitogen-activated protein kinase,[21] largely blocked this activation. Next, we examined DNA-protein interaction between the consensus PPRE and endogenous PPARγ by EMSAs. Proliferative VSMCs were stimulated with 15d-PGJ_2 to activate PPARγ-dependent pathways. Nuclear extracts prepared from these

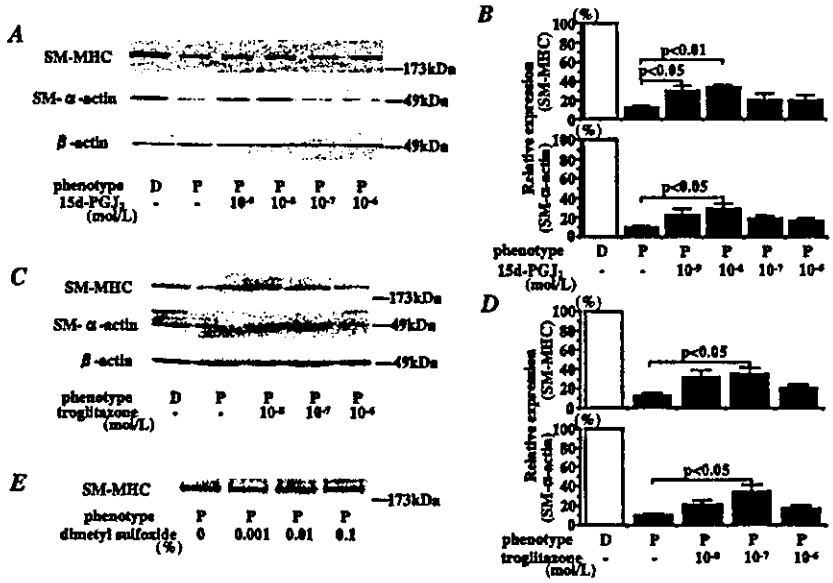

Figure 2. 15d-PGJ_2 and troglitazone induce the expression of smooth muscle–specific contractile proteins in proliferative VSMCs. VSMCs were cultured in differentiation (D) or proliferation (P) medium in the presence of the indicated concentrations of 15d-PGJ_2 (A and B), troglitazone (C and D), dimethyl sulfoxide (E), or saline for 48 hours. Whole-cell lysates from these cells were subjected to Western blotting with an anti–SM-MHC antibody, anti–SM-α-actin antibody, or anti–β-actin antibody. The signals of immunoblots were quantified as described in Methods. The relative expression levels of these proteins normalized to β-actin expression were determined, and that in the differentiated phenotype was set as 100% in each experiment. A, C, and E are representative photographs; B and D, the data of quantitative analysis, are shown as the mean±SE from 3 separate experiments.

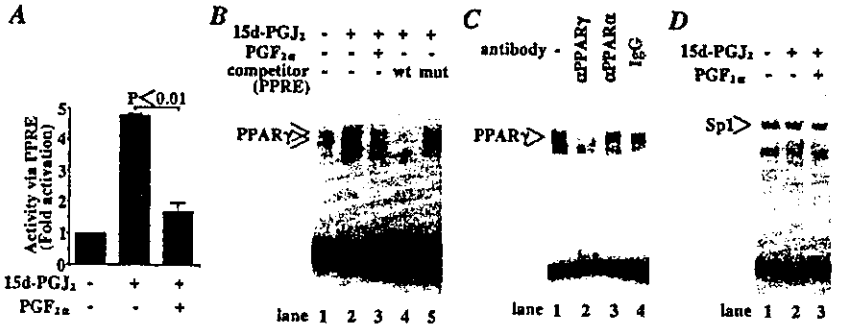

Figure 3. 15d-PGJ_2 activates PPARγ-dependent pathways in proliferative VSMCs. A, Proliferative VSMCs were transfected with 2.0 μg of pPPRELuc and 0.1 μg of pRSVCAT and stimulated with 15d-PGJ_2 (10^{-6} mol/L), prostaglandin $F_{2\alpha}$ (10^{-6} mol/L), or saline as indicated for 48 hours. The results are expressed as the fold activation of the normalized luc activities (luc/CAT) relative to those by saline stimulation. The data shown are the mean±SE of 2 independent experiments, each carried out in duplicate. In B, C, and D, proliferative VSMCs were stimulated with saline or 10^{-6} mol/L of 15d-PGJ_2 in the presence or absence of prostaglandin $F_{2\alpha}$ for 48 hours. Nuclear extracts from these cells were probed with a radiolabeled oligonucleotide containing consensus PPRE in B and C or Sp1-binding site in D. The arrows indicate the sequence-specific complex of the probe with nuclear factors. In B, unlabeled competitor DNAs were present in a 100-fold molar excess as indicated: wt, wild-type PPRE; mut, PPRE with a mutation in the PPAR binding site. In C, equal amounts of the anti-PPARγ antibody, the anti-PPARα antibody, or normal rabbit IgG were added to the mixture containing the probe and nuclear extracts from 15d-PGJ_2-stimulated VSMCs.

cells were probed with a radiolabeled oligonucleotide containing the consensus PPRE (Figure 3B and 3C). Competition EMSAs in lanes 4 and 5 of Figure 3B revealed that the retarded bands represented PPRE sequence–specific binding. As shown in Figure 3C, compared with the administration of normal rabbit IgG (lane 4), administering anti-PPARγ antibody markedly diminished the activity of these retarded bands. However, the anti-PPARα antibody (lane 3) only slightly diminished the activity. These findings indicated that the retarded bands mainly consisted of PPARγ and might contain a small amount of PPARα. Compatible with the results of transient transfection experiments, the activities of these bands were markedly increased by 15d-PGJ_2 stimulation (compare lanes 1 and 2 in Figure 3B). Further administration of prostaglandin $F_{2\alpha}$ almost completely blocked the 15d-PGJ_2–stimulated increase in these activities (lane 3 in Figure 3B). These findings suggest that stimulation of proliferative VSMCs with 15d-PGJ_2 predominantly activates the DNA binding of PPARγ rather than that of PPARα. In contrast, neither 15d-PGJ_2 nor prostaglandin $F_{2\alpha}$ affected the DNA binding activity of Sp1 (Figure 3D).

To examine the effect of 15d-PGJ_2 and prostaglandin $F_{2\alpha}$ on the endogenous expression of SM-MHC, we carried out immunocytochemical staining. As shown in Figure 4, differentiated VSMCs showed abundant expression of SM-MHC in the cytoplasm, whereas little expression was observed in proliferative VSMCs. Activation of PPARγ by stimulating proliferative VSMCs with 15d-PGJ_2 resulted in the induction of SM-MHC. This induction was abolished by further administration of prostaglandin $F_{2\alpha}$.

GATA-6 Is Required for PPARγ-Stimulated SM-MHC Transcription in Proliferative VSMCs

We examined the roles of GATA-6 and PPARγ-dependent pathways in SM-MHC transcription. NIH3T3 cells, which express neither PPARα nor PPARγ,[22] were transfected with pPPRELuc or pSM-MHC CAT together with the expression plasmid encoding human PPARγ (phPPARγ) and/or that encoding human GATA-6 (phGATA-6), and these were stimulated with 15d-PGJ_2 for 48 hours. As expected, the exogenous PPARγ activated the PPRE activity in NIH3T3 cells in the presence of 15d-PGJ_2 (Figure 5A). Interestingly, although expression of either PPARγ or GATA-6 mildly activated the 1346-bp rat SM-MHC promoter, expression of both PPARγ and GATA-6 synergistically activated this promoter (Figure 5B), suggesting that PPARγ signaling converges with GATA-6–dependent transcriptional mechanisms.

Next, to examine the role of GATA-6 in PPARγ-mediated activation of SM-MHC transcription in VSMCs, we transfected a luciferase expression vector driven by the 836-bp wild-type SM-MHC promoter (pwtSM-MHCluc) or the SM-MHC promoter with a mutation in the GATA-6 binding site (pmutSM-MHCluc). As shown in Figure 5C and 5D, the

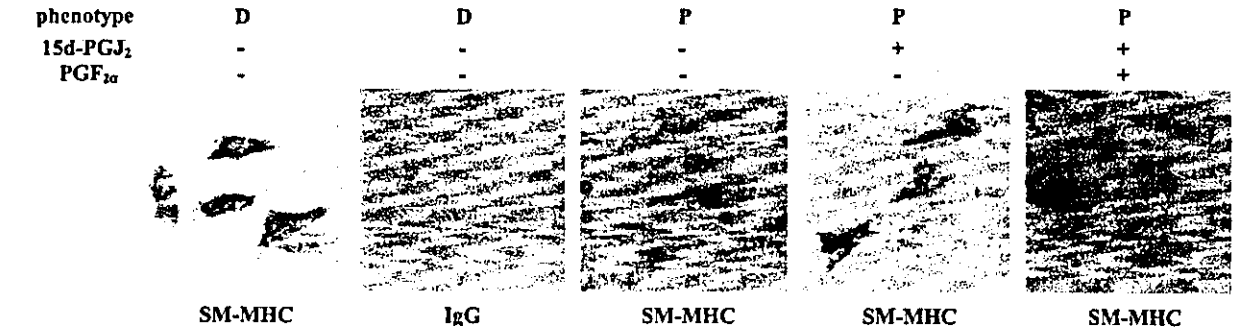

Figure 4. 15d-PGJ_2 induces the endogenous expression of SM-MHC in proliferative VSMCs. Differentiated (D) or proliferative (P) VSMCs were treated with 15d-PGJ_2 (10^{-6} mol/L), prostaglandin $F_{2\alpha}$ (10^{-6} mol/L), or saline as indicated for 48 hours and subjected to immunocytochemical staining with an anti–SM-MHC antibody or normal rabbit IgG as a negative control.

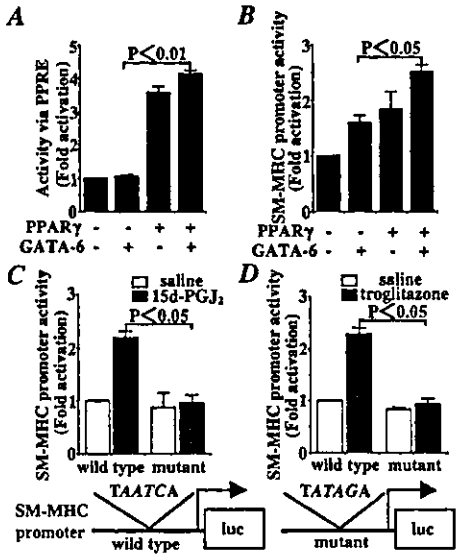

Figure 5. GATA-6 site within the SM-MHC promoter is required for PPARγ ligand-stimulated SM-MHC transcription. A and B, NIH3T3 cells were transfected with 0.3 μg of phPPARγ and/or 0.1 μg of phGATA-6 as indicated, in addition to 4.0 μg of pPPREluc and 0.2 μg of pRSVCAT (A), or 4.0 μg of pSM-MHC CAT and 0.1 μg of pRSVluc (B). Total amounts of transfected DNA were kept constant by cotransfecting pCMVβ-gal. Then these cells were stimulated with 10^{-6} mol/L of 15d-PGJ_2 for 48 hours. The results are expressed as the fold activation of the normalized reporter activities relative to those by pCMVβ-gal transfection. C and D, Proliferative VSMCs were transfected with 2.0 μg of pwtSM-MHCluc or pmutSM-MHCluc, and 0.2 μg of pRSVCAT. Then these cells were stimulated with 10^{-6} mol/L of 15d-PGJ_2 (C), 10^{-7} mol/L of troglitazone (D), or saline for 48 hours. The results are expressed as the fold activation of the normalized luc activities (luc/CAT) relative to those by saline stimulation. The data shown are the mean±SE of 2 independent experiments, each carried out in duplicate.

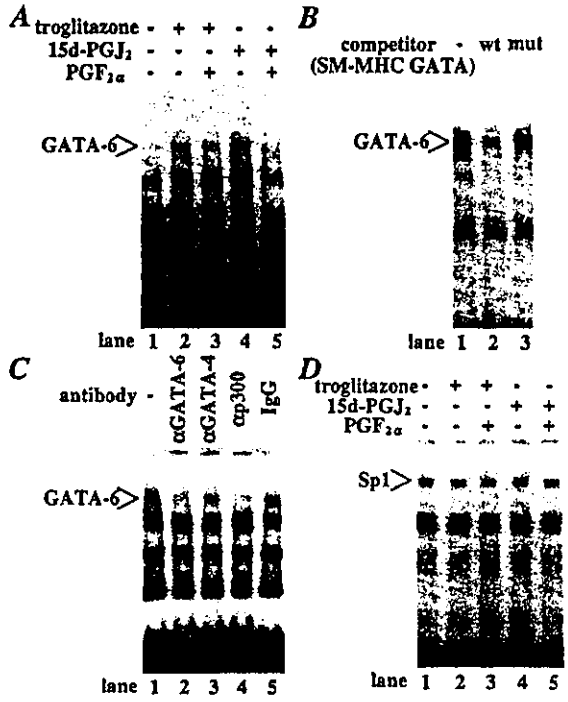

Figure 6. PPARγ ligands increase the DNA binding activity of endogenous GATA-6 in proliferative VSMCs. Proliferative VSMCs were stimulated with 10^{-7} mol/L of troglitazone, 10^{-6} mol/L of 15d-PGJ_2, or saline as a control in the presence or absence of 10^{-6} mol/L prostaglandin $F_{2\alpha}$ as indicated for 48 hours. Nuclear extracts from these cells were subjected to EMSA studies with a probe containing the GATA site of the SM-MHC promoter (A, B, and C) or the consensus Sp1 site (D). The arrows indicate the sequence-specific complex of the probe with nuclear factors. In B, unlabeled competitor DNAs were added to nuclear extracts from 15d-PGJ_2–stimulated VSMCs in a 100-fold molar excess: wt, wild type SM-MHC GATA; mut, SM-MHC GATA with a mutation in the GATA binding site. In C, equal amounts of the anti–GATA-6 antibody, the anti–GATA-4 antibody, the anti-p300 antibody, or normal rabbit IgG were added to the binding mixture containing the probe and nuclear extracts from 15d-PGJ_2-stimulated VSMCs.

administration of 15d-PGJ_2 and troglitazone resulted in the activation of the wild-type SM-MHC promoter. Mutating the GATA-6 binding site abolished the activation induced by 15d-PGJ_2 and troglitazone.

PPARγ Ligands Increase the DNA Binding Activity of GATA-6 in Proliferative VSMCs

To determine whether PPARγ ligands modulate the DNA binding activity of GATA-6 in proliferative VSMCs, we performed EMSAs by using an oligonucleotide containing the SM-MHC GATA site as a probe. As shown in lanes 2 and 3 of Figure 6B, competition EMSAs revealed that a retarded band represented GATA sequence–specific binding. As shown in Figure 6C, the activity of this band was diminished by the administration of anti–GATA-6 antibody (lane 2) but not by the anti–GATA-4 antibody (lane 3) or normal rabbit IgG (lane 5). The activity of this band was modestly diminished by the anti-p300 antibody (lane 4). These data indicate that the retarded band contains a significant amount of GATA-6 and p300. As shown in Figure 6A, the intensity of the specific band was increased in nuclear extracts from VSMCs stimulated with troglitazone (lane 2) or 15d-PGJ_2 (lane 4) compared with those from saline-stimulated cells (lane 1). Increases in the DNA binding activity of GATA-6 by troglitazone or 15d-PGJ_2 were reduced by prostaglandin $F_{2\alpha}$, a PPARγ-inhibitory compound (lanes 3 and 5). In contrast, troglitazone, 15d-PGJ_2, and prostaglandin $F_{2\alpha}$ did not alter the DNA binding activity of Sp1 (Figure 6D).

Discussion

Using 2 different and structurally unrelated PPARγ ligands, troglitazone and 15d-PGJ_2, we have demonstrated that PPARγ activation in proliferative VSMCs induced the endogenous expression of SM-MHC and SM-α-actin, highly specific differentiation markers. We confirmed the activation of PPARγ-dependent pathways by demonstrating the induction of PPARγ-specific DNA binding as well as PPRE-dependent transcription. Furthermore, exogenous expression of PPARγ in NIH3T3 cells, which express neither PPARα nor PPARγ, activated the GATA-6–dependent SM-MHC transcription in the presence of 15d-PGJ_2. Interestingly, induction of differentiated markers by 15d-PGJ_2 and troglitazone decreased at micromolar concentrations of these agents, whereas inhibition of DNA synthesis became evident

at these concentrations. At present, the precise mechanisms of this activity are unknown. However, several studies have shown that there is a very poor correlation between proliferation rates in intimal VSMCs and their modified differentiated phenotype in both humans and experimental animal models.[11] Thus, our data are compatible with these studies. It has been shown that micromolar concentrations of these agents possess PPARγ-independent activity and directly modify other signaling pathways, such as nuclear factor-κB, extracellular signal–regulated kinase, and phosphatidylinositol 3-kinase/protein kinase B.[23–27] Therefore, it is possible that these PPARγ-independent pathways are involved in the decreased expression of smooth muscle–specific contractile proteins at higher concentrations of these agents. Further studies are required on the regulated expression of smooth muscle–specific proteins by PPARγ-dependent and -independent pathways.

This study demonstrated that the 15d-PGJ_2–inducible expression of SM-MHC was mediated in part through a transcriptional mechanism and that the GATA-6 binding site within the SM-MHC promoter was required for the induction by PPARγ ligands. In addition, EMSA experiments showed that both troglitazone and 15d-PGJ_2 increased the DNA binding activity of GATA-6 in proliferative VSMCs. These findings demonstrate that GATA-6 is involved in the PPARγ-mediated transcriptional activation of the SM-MHC gene in VSMCs. However, neither troglitazone nor 15d-PGJ_2 affected the endogenous expression of GATA-6, PPARγ, or p300, an important cofactor of GATA-6 (data not shown). Thus, an increase in the DNA binding activity of GATA-6 might involve its posttranslational modification. It has been shown that other members of the GATA family of transcription factors, namely GATA-1, -2, -3, and -4, exist as phosphoproteins.[28,29] Interestingly, Altiok and colleagues[30] demonstrated that the activation of PPARγ decreased expression of the serine/threonine phosphatase PP2A and increased a phosphorylated form of transcription factor DP-1. Further studies are needed on the mechanisms by which PPARγ-dependent pathways increase the DNA binding activity of GATA-6.

Recently, we showed that a transcriptional coactivator, p300, interacted with GATA-6 and augmented the GATA-6–dependent transcription of the SM-MHC promoter.[16] A p300 protein may function as a bridge between GATA-6 and components of the basal transcription machinery. Interestingly, the PPAR/retinoid X receptor heterodimer also forms a complex with p300.[31] It would be interesting to study the relation between p300 and PPARγ-dependent signaling pathways during VSMC differentiation.

Although the data of our present study suggest a requirement of GATA-6 in PPARγ-induced activation of the 836-bp SM-MHC promoter, these data do not rule out other possible mechanisms. For example, we could not find PPRE, at least in the 2.4-kb SM-MHC promoter sequences, by our computer search. However, we cannot rule out the possibility that PPARγ directly binds and activates the SM-MHC promoter through PPRE, which might exist outside these sequences. In fact, smooth muscle–specific expression in vivo requires sequences outside the 2.4-kb SM-MHC promoter.[32] Another transcription factor that might be involved in the PPARγ-induced transcription of VSMC differentiation marker genes is serum response factor, a MADS box–containing transcription factor. This factor binds CArG sequences in the SM-MHC promoter and activates this promoter in a sequence-specific manner.[17,32] Interestingly, serum response factor is also reported to cooperate with p300 and GATA factors.[33] Roles of p300, GATA-6, and serum response factor in PPARγ-induced VSMC differentiation should be further investigated in the context of a longer SM-MHC promoter by a transgenic approach.

Thiazolidinediones are now widely used as insulin-sensitizing agents in patients with diabetes mellitus. These patients often have atherosclerosis complications. Given the inhibitory effects of thiazolidinediones on atherosclerosis in vivo, it will be of particular importance to elucidate the precise mechanisms by which PPARγ-dependent signaling pathways induce the differentiated phenotype in VSMCs.

Acknowledgments

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References

1. Bishop-Bailey D. Peroxisome proliferator-activated receptors in the cardiovascular system. *Br J Pharmacol.* 2000;129:823–834.
2. Rosen ED, Spiegelman BM. PPARγ: a nuclear regulator of metabolism, differentiation, and cell growth. *J Biol Chem.* 2001;276:37731–37734.
3. Lehmann JM, Moore LB, Smith-Oliver TA, Wilkison WO, Willson TM, Kliewer SA. An antidiabetic thiazolidinedione is a high affinity ligand for peroxisome proliferator-activated receptor-γ (PPAR-γ). *J Biol Chem.* 1995;270:12953–12956.
4. Forman BM, Tontonoz P, Chen J, Brun RP, Spiegelman BM, Evans RM. 15-Deoxy-delta 12,14-prostaglandin J2 is a ligand for the adipocyte determination factor PPAR gamma. *Cell.* 1995;83:803–812.
5. Kliewer SA, Umesono K, Noonan DJ, Heyman RA, Evans RM. Convergence of 9-*cis* retinoic acid and peroxisome proliferator signalling pathways through heterodimer formation of their receptors. *Nature.* 1992; 358:771–774.
6. Ricote M, Huang J, Fajas L, Li A, Welch J, Najib J, Witztum JL, Auwerx J, Palinski W, Glass CK. Expression of the peroxisome proliferator-activated receptor-γ (PPARγ) in human atherosclerosis and regulation in macrophages by colony stimulating factors and oxidized low density lipoprotein. *Proc Natl Acad Sci U S A.* 1998;95:7614–7619.
7. Law RE, Goetze S, Xi XP, Jackson S, Kawano Y, Demer L, Fishbein MC, Meehan WP, Hsueh WA. Expression and function of PPARγ in rat and human vascular smooth muscle cells. *Circulation.* 2000;101:1311–1318.
8. Li AC, Brown KK, Silvestre MJ, Willson TM, Palinski W, Glass CK. Peroxisome proliferator-activated receptor-γ ligands inhibit development of atherosclerosis in LDL receptor-deficient mice. *J Clin Invest.* 2000; 106:523–531.
9. Chen Z, Ishibashi S, Perrey S, Osuga J, Gotoda T, Kitamine T, Tamura Y, Okazaki H, Yahagi N, Iizuka Y, Shionoiri F, Ohashi K, Harada K, Shimano H, Nagai R, Yamada N. Troglitazone inhibits atherosclerosis in apolipoprotein E-knockout mice: pleiotropic effects on CD36 expression and HDL. *Arterioscler Thromb Vasc Biol.* 2001;21:372–377.
10. Law RE, Meehan WP, Xi XP, Graf K, Wuthrich DA, Coats W, Faxon D, Hsueh WA. Troglitazone inhibits vascular smooth muscle cell growth and intimal hyperplasia. *J Clin Invest.* 1996;98:1897–1905.
11. Owens GK. Regulation of differentiation of vascular smooth muscle cells. *Physiol Rev.* 1995;75:487–517.
12. Ross R. Atherosclerosis: an inflammatory disease. *N Engl J Med.* 1999; 340:115–126.
13. Suzuki E, Evans T, Lowry J, Truong L, Bell DW, Testa JR, Walsh K. The human GATA-6 gene: structure, chromosomal location, and regulation of

expression by tissue-specific and mitogen-responsive signals. *Genomics.* 1996;38:283–290.
14. Perlman H, Suzuki E, Simonson M, Smith RC, Walsh K. GATA-6 induces p21(Cip1) expression and G1 cell cycle arrest. *J Biol Chem.* 1998;273:13713–13718.
15. Mano T, Luo Z, Malendowicz SL, Evans T, Walsh K. Reversal of GATA-6 downregulation promotes smooth muscle differentiation and inhibits intimal hyperplasia in balloon-injured rat carotid artery. *Circ Res.* 1999;84:647–654.
16. Wada H, Hasegawa K, Morimoto T, Kakita T, Yanazume T, Sasayama S. A p300 protein as a coactivator of GATA-6 in the transcription of the smooth muscle-myosin heavy chain gene. *J Biol Chem.* 2000;275: 25330–25335.
17. Madsen CS, Hershey JC, Hautmann MB, White SL, Owens GK. Expression of the smooth muscle myosin heavy chain gene is regulated by a negative-acting GC-rich element located between two positive-acting serum response factor-binding elements. *J Biol Chem.* 1997;272: 6332–6340.
18. Wada H, Hasegawa K, Morimoto T, Kakita T, Yanazume T, Abe M, Sasayama S. Calcineurin-GATA-6 pathway is involved in smooth muscle-specific transcription. *J Cell Biol.* 2002;156:983–991.
19. Gurnell M, Wentworth JM, Agostini M, Adams M, Collingwood TN, Provenzano C, Browne PO, Rajanayagam O, Burris TP, Schwabe JW, Lazar MA, Chatterjee VK. A dominant-negative peroxisome proliferator-activated receptor-γ (PPARγ) mutant is a constitutive repressor and inhibits PPARγ-mediated adipogenesis. *J Biol Chem.* 2000;275: 5754–5759.
20. Iwakura A, Fujita M, Hasegawa K, Sawamura T, Nohara R, Sasayama S, Komeda M. Pericardial fluid from patients with unstable angina induces vascular endothelial cell apoptosis. *J Am Coll Cardiol.* 2000;35: 1785–1790.
21. Reginato MJ, Krakow SL, Bailey ST, Lazar MA. Prostaglandins promote and block adipogenesis through opposing effects on peroxisome proliferator-activated receptor-γ. *J Biol Chem.* 1998;273:1855–1858.
22. Tontonoz P, Hu E, Spiegelman BM. Stimulation of adipogenesis in fibroblasts by PPAR γ2, a lipid-activated transcription factor. *Cell.* 1994; 79:1147–1156.
23. Rossi A, Kapahi P, Natoli G, Takahashi T, Chen Y, Karin M, Santoro MG. Anti-inflammatory cyclopentenone prostaglandins are direct inhibitors of IκB kinase. *Nature.* 2000;403:103–108.
24. Straus DS, Pascual G, Li M, Welch JS, Ricote M, Hsiang CH, Sengchanthalangsy LL, Ghosh G, Glass CK. 15-Deoxy-delta 12,14-prostaglandin J2 inhibits multiple steps in the NF-κB signaling pathway. *Proc Natl Acad Sci U S A.* 2000;97:4844–4849.
25. Moore KJ, Rosen ED, Fitzgerald ML, Randow F, Andersson LP, Altshuler D, Milstone DS, Mortensen RM, Spiegelman BM, Freeman MW. The role of PPAR-γ in macrophage differentiation and cholesterol uptake. *Nat Med.* 2001;7:41–47.
26. Chawla A, Barak Y, Nagy L, Liao D, Tontonoz P, Evans RM. PPAR-γ dependent and independent effects on macrophage-gene expression in lipid metabolism and inflammation. *Nat Med.* 2001;7:48–52.
27. Takeda K, Ichiki T, Tokunou T, Iino N, Takeshita A. 15-Deoxy-delta 12,14-prostaglandin J2 and thiazolidinediones activate the MEK/ERK pathway through phosphatidylinositol 3-kinase in vascular smooth muscle cells. *J Biol Chem.* 2001;276:48950–48955.
28. Chen CH, Zhang DH, LaPorte JM, Ray A. Cyclic AMP activates p38 mitogen-activated protein kinase in Th2 cells: phosphorylation of GATA-3 and stimulation of Th2 cytokine gene expression. *J Immunol.* 2000;165:5597–5605.
29. Morimoto T, Hasegawa K, Kaburagi S, Kakita T, Wada H, Yanazume T, Sasayama S. Phosphorylation of GATA-4 is involved in α1-adrenergic agonist-responsive transcription of the endothelin-1 gene in cardiac myocytes. *J Biol Chem.* 2000;275:13721–13726.
30. Altiok S, Xu M, Spiegelman BM. PPARγ induces cell cycle withdrawal: inhibition of E2F/DP DNA-binding activity via down-regulation of PP2A. *Genes Dev.* 1997;11:1987–1998.
31. Kodera Y, Takeyama K, Murayama A, Suzawa M, Masuhiro Y, Kato S. Ligand type-specific interactions of peroxisome proliferator-activated receptory with transcriptional coactivators. *J Biol Chem.* 2000;275: 33201–33204.
32. Manabe I, Owens GK. CArG elements control smooth muscle subtype-specific expression of smooth muscle myosin in vivo. *J Clin Invest.* 2001;107:823–834.
33. Belaguli NS, Sepulveda JL, Nigam V, Charron F, Nemer M, Schwartz RJ. Cardiac tissue enriched factors serum response factor and GATA-4 are mutual coregulators. *Mol Cell Biol.* 2000;20:7550–7558.

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Effects of Dipyridamole and Aspirin on Shear-Induced Platelet Aggregation in Whole Blood and Platelet-Rich Plasma

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Key Words
Dipyridamole · Aspirin · Platelet · Shear stress

Abstract
Background: Shear-induced platelet aggregation (SIPA) is an important mechanism of thrombosis at arterial bifurcations or stenotic lesions. ***Methods:*** We investigated the in vitro effects of dipyridamole (DP) and acetylsalicyclic acid (ASA) on SIPA in whole blood and platelet-rich plasma (PRP). ***Results:*** In whole blood, DP 20 μ*M* significantly inhibited SIPA, while DP 5 μ*M* or ASA 5 or 20 μ*M* did not. SIPA in whole blood was, however, significantly inhibited by the combination of 5 or 20 μ*M* of DP and ASA. SIPA in PRP was not inhibited by any concentration of DP or ASA, alone or in combination. ***Conclusions:*** These results suggest that DP has an effect on red blood cells and that ASA enhances the inhibitory effect of DP on SIPA in whole blood. These effects may be related to the additive effect of combination therapy with DP and ASA on stroke prevention.

The European Stroke Prevention Study-2 (ESPS-2) [1] demonstrated the efficacy of dipyridamole (DP) and low-dose acetylsalicylic acid (ASA) in the secondary prevention of ischemic stroke and transient ischemic attacks (TIA). The results of the ESPS-2 demonstrated that DP (in an extended-release form, at a dose of 200 mg twice daily) and ASA 25 mg (twice daily) are equally effective for the secondary prevention of ischemic stroke and TIA (the relative risk reductions were 16 and 18%, respectively). Moreover, the ESPS-2 clearly showed the additive advantage of DP/ASA combination therapy (the relative risk reduction was 36%) [1]. Following the results of the ESPS-2, the US Food and Drug Administration approved a combination of DP and ASA (Aggrenox®; Boehringer Ingelheim Pharmaceuticals, Richfield, Conn., USA) for the secondary prevention of stroke. Each hard gelatin capsule contains 200 mg of DP in an extended-release form and 25 mg of aspirin as an immediate-release sugar-coated tablet [2]. This drug is also approved in Europe (Asasantine-ER®; Boehringer Ingelheim Pharmaceuticals, Ingelheim, Germany).

The antiplatelet mechanisms of DP are not as well understood as those of ASA. Previous investigators have suggested multiple mechanisms [3]. DP inhibits cellular adenosine uptake, thereby increasing the antithrombotic effect of adenosine, which enhances intraplatelet cyclic adenosine monophosphate (cAMP) [3–6]. The second antiplatelet mechanism of DP is the inhibition of cyclic guanosine monophosphate (cGMP)-specific phosphodiesterase (PDE), which allows for the accumulation of cGMP in

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platelets [3, 7]. Thus, increased cAMP and cGMP in platelets inhibit platelet aggregation through different mechanisms [8, 9]. More recently, interactions of DP with vascular endothelial cells have been suggested. DP causes the release of the platelet antiaggregating and vasodilating prostacyclin, PGI_2, as well as nitric oxide (NO) from vascular endothelial cells [10–12].

Shear-induced platelet aggregation (SIPA), especially at high shear, which requires the interaction of von Willebrand factor (vWF) and glycoprotein (GP) Ib/IX and GP IIb/IIIa complexes, is an important mechanism of thrombosis at arterial bifurcations or stenotic lesions [13, 14]. The binding of vWF to its platelet membrane receptor GP Ib/IX under high shear force causes an increase in intracellular Ca^{2+} concentrations due to an influx of extracellular Ca^{2+}, which is followed by the activation of the GP IIb/IIIa receptor and binding of vWF to GP IIb/IIIa [14]. However, the intracellular signal transduction pathway under high shear force remains uncertain. Increased SIPA has been demonstrated in patients with atherothrombotic stroke and TIA [15, 16].

Several groups, including ours, have shown that ASA has no effect on SIPA both in vitro and ex vivo, suggesting a lesser importance of thromboxane A_2 (TxA_2) in the mechanism of SIPA [13, 15–17]. DP has multiple antiplatelet mechanisms, although it has never been reported whether DP inhibits SIPA. Thus, we studied the in vitro effect of DP with or without ASA on SIPA in whole blood and platelet-rich plasma (PRP), using a cone plate streaming chamber, as described in detail elsewhere [15].

Materials and Methods

Blood Sampling and Preparation of PRP

Blood was obtained from 8 healthy volunteers (4 males and 4 females; mean age 31.5 years, range 21–36 years) who were recruited from our department's staff. None of the subjects had ingested any medication in the previous 10 days, and all consented to participate in the study. Blood was collected from the antecubital vein into siliconized tubes and prevented from coagulating with 1:10 (v/v) of 100 μ*M* argatroban, a thrombin inhibitor (Tokyo Mitsubishi Pharmaceuticals, Tokyo, Japan). To prepare PRP, the blood was centrifuged at 3,000 rpm for 10 min at room temperature. All blood samples were used within 4 h after collection.

Preincubation with DP and/or ASA

DP (Boehringer Ingelheim Pharmaceuticals, Tokyo, Japan) and ASA (Yamazen Pharmaceutical, Osaka, Japan) were dissolved in ethanol. For this study, 0.398 ml of whole blood or PRP plus 0.1 ml of saline plus 0.002 ml of ethanol (control) or drug solutions were preincubated in polypropylene tubes at room temperature for 10 min.

Preincubation with L-Arginine

The antithrombotic activity of DP is in part linked to the NO-cGMP system [3, 10–12]. Platelets contain NO synthase, and NO not only from endothelial cells, but also from platelets stimulates guanylyl cyclase to produce cGMP, which inhibits platelet aggregation [3, 18–20]. To examine whether DP and platelet NO have a synergistic effect on SIPA, we studied the effect of *L*-arginine, a substrate of NO synthase. *L*-Arginine (Sigma, St. Louis, Mo., USA) at a final concentration of 100 μ*M* was preincubated in polypropylene tubes at room temperature for 10 min after the incubation of DP in whole blood.

Measurements of SIPA

An apparatus for determining SIPA was previously described in detail [15]. The cone plate streaming chamber was composed of a rotating cone with a diameter of 30 mm and an angle of 1°, and a detachable cylindrical cell with a base plate. The distance from the apex of the cone to the base plate was adjusted to 0.04 mm by a micrometer screw in conjunction with a cone rotation unit. The cone was rotated by a rotor, and a constant shear stress was obtained for the entire streaming sample in the cell without producing a turbulent flow. The cell was filled with 0.4 ml of the sample after the preincubation, and then the sample was sheared by the rotating cone at a high shear rate (108 dyn/cm^2) for 6 min. Within 20 s after shearing, platelet counts were determined using an automated analyzer (Sysmex 8000, TOA Medical Electronics, Kobe, Japan). The disappearance of single platelets was used as an indicator of the formation of platelet aggregates. Thus, the percent decrease in initial platelet counts was evaluated as the percent increase in aggregated platelets. Therefore, SIPA was calculated as follows:

SIPA (%) = [(platelet count before exposure to shear stress) – (platelet count after exposure to shear stress)] × 100/(platelet count before exposure to shear stress).

Statistical Analysis

Data are presented as mean values ± standard deviations (SD). One-way ANOVA followed by Bonferroni/Dunn tests was used to assess differences in SIPA (%). These calculations were performed with the StatView 5.0 program (SAS Institute, Cary, N.C., USA) on a Macintosh personal computer. Statistical significance was accepted at $p < 0.05$.

Results

Whole blood SIPA in control samples, samples preincubated with DP 5 μ*M*, and samples preincubated with DP 20 μ*M* were 73.1 ± 5.6, 61.7 ± 12.1 and 50.7 ± 13.6% (mean ± SD), respectively. DP appeared to inhibit SIPA in a dose-dependent manner, although statistical significance was observed only between controls and DP 20 μ*M* (fig. 1). In contrast, ASA 5 or 20 μ*M* did not inhibit SIPA in whole blood (fig. 1). SIPA in whole blood samples preincubated with the combination of 5 or 20 μ*M* of DP and ASA were 52.0 ± 18.1 and 41.7 ± 14.4%, respectively (fig. 1). In spite of no significant inhibitory effect of 5 μ*M* of DP or ASA alone, the combination of 5 μ*M* of DP

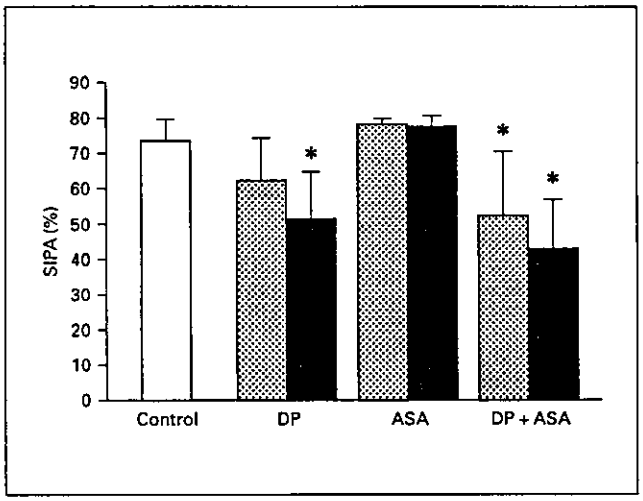

Fig. 1. Effect of DP and/or ASA on SIPA in whole blood. ▨ = SIPA with 5 μ*M* of DP and/or ASA; ■ = SIPA with 20 μ*M* of DP and/or ASA. Values are mean ± SD. * $p < 0.05$ (Bonferroni/Dunn test).

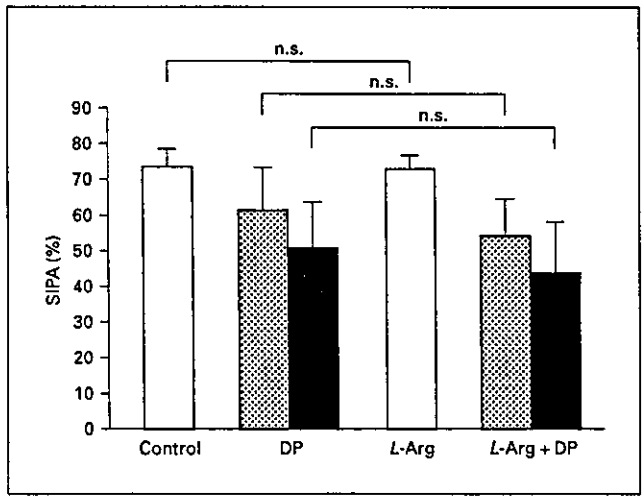

Fig. 3. Effect of DP and/or *L*-arginine on SIPA in whole blood. ▨ = SIPA with DP 5 μ*M* alone or DP 5 μ*M* plus *L*-arginine 100 μ*M*; ■ = SIPA with DP 20 μ*M* alone or DP 20 μ*M* plus *L*-arginine 100 μ*M*. L-Arg = *L*-Arginine; n.s. = not significant. Values are mean ± SD.

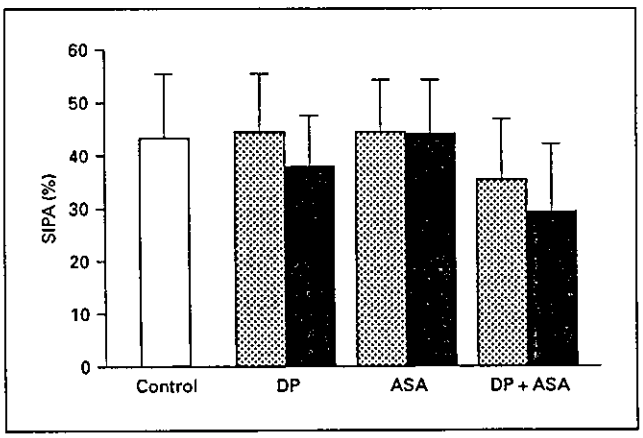

Fig. 2. Effect of DP and/or ASA on SIPA in PRP. ▨ = SIPA with 5 μ*M* of DP and/or ASA; ■ = SIPA with 20 μ*M* of DP and/or ASA. Values are mean ± SD.

and ASA significantly inhibited SIPA (fig. 1). Similarly, ASA 20 μ*M* enhanced the inhibitory effect of DP 20 μ*M* on SIPA, while there was no significant inhibitory effect of ASA 20 μ*M* on SIPA (fig. 1).

SIPA in PRP in control samples, samples preincubated with DP 5 μ*M*, and samples preincubated with DP 20 μ*M* were 43.7 ± 11.9, 44.5 ± 11.4 and 38.0 ± 9.3%, respectively, among which there were no statistical differences. ASA also did not inhibit SIPA in PRP (fig. 2). SIPA in PRP samples preincubated with the combination of 5 or 20 μ*M* of DP and ASA were 35.0 ± 12.1 and 28.9 ± 13.3%, respectively (fig. 2). DP 20 μ*M* had a tendency of inhibitory effect on SIPA in PRP, which was enhanced by ASA 20 μ*M*; however, there was no statistical difference between control and the combination of DP 20 μ*M* and ASA 20 μ*M* (fig. 2).

SIPA in control samples, samples preincubated with *L*-arginine, DP 5 μ*M* plus *L*-arginine and DP 20 μ*M* plus *L*-arginine was 73.1 ± 5.6, 73.0 ± 3.7, 54.3 ± 11.0 and 43.2 ± 15.1%, respectively. SIPA in samples preincubated with *L*-arginine alone did not differ from that in the controls. The extent of SIPA in samples preincubated with *L*-arginine plus DP 5 or 20 μ*M* appeared lower than in those preincubated with DP 5 or 20 μ*M* alone, whereas there were no statistical differences (fig. 3).

Discussion

In the ESPS-2, the efficacies of DP 200 mg and/or ASA 25 mg twice daily for secondary stroke prevention were studied. Plasma levels of DP and ASA 2 h after the last drug treatment at the dosage used in the ESPS-2 have been reported to be from 2.0 to 5.0 μ*M* and from 4.2 to

7.7 μ*M*, respectively, which did not differ between either drug alone or both drugs in combination [21]. Therefore, the 5 μ*M* dose used in our study was comparable to the plasma levels of these drugs, achieved in the ESPS-2. 20 μ*M* appeared to be the highest blood level estimated from the clinical trial.

DP is known to inhibit PDEs in platelets [3, 7]. PDEs have recently been classified into five major families, PDEs I–V. PDEs I, II and III use both cAMP and cGMP as substrates, while IV is cAMP-specific, and V is cGMP-specific [22–24]. Human platelets contain three species of PDE isozymes, I, III and V. DP inhibits the activity of cGMP-specific PDE (PDE V), thereby increasing the cGMP level in platelets [3, 7, 24]. Minami et al. [25] have demonstrated that cilostazol, a selective PDE III inhibitor, inhibits SIPA in vitro and ex vivo. Cilostazol raises the level of cAMP by the inhibition of PDE III and potentiates the effect of PGE_1 to increase the cAMP level [25]. In addition to cilostazol, stable PGI_2 analogues, forskolin and PGE_1, all of which increase cAMP by activating adenylate cyclase, have also been shown to inhibit SIPA [15]. Similarly, DP is expected to inhibit SIPA through the upregulation of cGMP in platelets, although it had never been studied.

We and the other investigators demonstrated previously that DP inhibits platelet aggregation induced by adenosine diphosphate (ADP) in whole blood [4, 26]. ADP is an essential cofactor for SIPA mediated by large vWF multimers [14, 17], and selective platelet ADP receptor blockers such as ticlopidine are known to inhibit SIPA [13, 15–17]. Therefore, there is another possibility that DP inhibits SIPA in part through the inhibition of ADP-mediated mechanisms in platelets.

Another important mechanism of DP for the inhibition of platelet aggregation is the blockade of cellular adenosine uptake [3–6]. Adenosine enhances intraplatelet cAMP, which inhibits platelet aggregation [3–6]. Early aggregometric studies of DP using PRP failed to show a clear antiplatelet effect except at high concentrations. Therefore, DP was regarded as a weak antiplatelet agent [4]. More recently, with the development of impedance aggregometry, several investigators have shown that DP inhibits platelet aggregation more significantly in whole blood than in PRP, indicating higher adenosine levels in whole blood than in PRP due to the blockade of adenosine reuptake of red blood cells by DP [4]. We have demonstrated in this study that the inhibitory effect of DP on SIPA was more prominent in whole blood than in PRP, indicating that the blockade of adenosine reuptake by DP is an important mechanism for the inhibition in SIPA.

Previous studies have shown that SIPA is not significantly inhibited by ASA in vitro and ex vivo [13, 15–17]. This study also demonstrated that ASA alone did not inhibit SIPA in either whole blood or PRP. ASA inhibits TxA_2 synthesis by the blockade of the enzyme cyclooxygenase, thereby inhibiting prostaglandin biosynthesis. Therefore, failure of ASA in the inhibition of SIPA suggests a lesser importance of TxA_2 in the mechanism of SIPA. However, our study demonstrated that ASA synergistically enhanced the inhibitory effect of DP on SIPA when combined with DP. Some previous tests of platelet function have also shown that the final effect was greater than the sum of the individual effects of DP and ASA [27, 28]. Actually, some clinical studies have demonstrated that the combination of DP and ASA has an additive or even synergistic advantage for the prevention of secondary thrombotic events [1, 29, 30]. It remains to be determined what mechanism is responsible for this synergistic effect of ASA. Nonsteroidal anti-inflammatory drugs have been reported to cause increases in extracellular adenosine levels [31] with anti-inflammatory consequences; therefore, ASA may increase the plasma adenosine level synergistically with DP. Moreover, ASA has recently been shown to increase leukocyte NO synthesis [32], and the combination of DP and ASA has been reported to increase the antiplatelet effect of leukocytes synergistically through an increase of NO [12]. Therefore, it is possible that the combination of DP and ASA may inhibit SIPA synergistically through the NO-cGMP system involving leukocytes in whole blood. In any case, this study suggested an additional advantage of the DP/ASA combination therapy in the synergistic inhibitory effect on SIPA.

Platelets as well as endothelial cells contain NO synthase, and NO released from platelets can stimulate guanylyl cyclase to produce cGMP which prevents aggregation [3, 18–20]. Radomski et al. [20] have suggested that the formation of NO from *L*-arginine in the platelet cytosol is dependent on the free Ca^{2+} concentration, indicating that the NO synthase in platelets is Ca^{2+}-dependent. Therefore, we presumed that NO generated from platelets by shear stress (which causes increases in intracellular Ca^{2+} concentrations due to an influx of extracellular Ca^{2+}) may affect platelet aggregation, and DP may enhance NO production from platelets [10, 11]. To examine whether DP and platelet NO have a synergistic inhibitory effect on SIPA, we studied the effect of *L*-arginine as a substrate of NO synthase on SIPA. Our results showed that *L*-arginine did not inhibit SIPA by itself or significantly enhance the inhibitory effect of DP on SIPA, which may reflect that

NO production in platelets is not sufficient to inhibit SIPA or enhance the inhibitory effect of DP on SIPA.

In conclusion, our results showed that DP alone significantly inhibited SIPA in whole blood, and ASA alone did not inhibit SIPA, although ASA enhanced the inhibitory effect of DP on SIPA in whole blood. The synergistic inhibition of SIPA by the combination of DP and ASA might be an additional explanation for the results of the ESPS-2, which have demonstrated an additive effect of DP and ASA in stroke prevention.

References

1 Diener HC, Cunha L, Forbes C, Sivenius J, Smets P, Lowenthal A: European Stroke Prevention Study 2. Dipyridamole and acetylsalicylic acid in the secondary prevention of stroke. J Neurol Sci 1996;143:1–13.

2 Henney JE: New drug for stroke: From the Food and Drug Administration. JAMA 2000; 283:46.

3 Vane JR, Meade TW: Second European Stroke Prevention Study (ESPS 2): Clinical and pharmacological implications. J Neurol Sci 1997; 145:123–125.

4 Gresele P, Zoja C, Deckmyn H, Arnout J, Vermylen J, Verstraete M: Dipyridamole inhibits platelet aggregation in whole blood. Thromb Haemost 1983;50:852–856.

5 Bullough DA, Zhang C, Montag A, Mullane KM, Young MA: Adenosine-mediated inhibition of platelet aggregation by acadesine. A novel antithrombotic mechanism in vitro and vivo. J Clin Invest 1994;94:1524–1532.

6 Menegatti M, Cristalli G, Gallo L, Mannucci PM, Pareti F: Effect of adenosine derivatives on in vitro thrombus formation induced by shear stress. Haematologica 1999;84:721–725.

7 Lugnier C, Schoeffter P, Le Bec A, Strowthou E, Stoclet JC, Strasbourg FR: Selective inhibition of cyclic nucleotide phosphodiesterase of human, bovine and rat aorta. Biochem Pharmacol 1986;35:1743–1751.

8 Alheid U, Reichwehr I, Förstermann U: Human endothelial cells inhibit platelet aggregation by separately stimulating platelet cyclic AMP and cyclic GMP. Eur J Pharmacol 1989; 164:103–110.

9 Qi R, Ozaki Y, Satoh K, Yang LB, Asazuma N, Yatomi Y, Kume S: Intracellular levels of cyclic AMP and cyclic GMP differentially modify platelet aggregate size in human platelets activated with epinephrine or ADP. J Cardiovasc Pharmacol 1996;28:215–222.

10 Sakuma I, Akaishi Y, Fukao M, Makita Y, Makita M-A, Kobayashi T, Matsuno K, Miyazaki T, Yasuda H: Dipyridamole potentiates the anti-aggregating effect of endothelium-derived relaxing factor. Thromb Res 1990; 12(suppl):87–90.

11 Bult H, Fret HRL, Jordaens FH, Herman AG: Dipyridamole potentiates the anti-aggregating and vasodilator activity of nitric oxide. Eur J Pharmacol 1991;199:1–8.

12 De La Cruz JP, Blanco E, Sanchez De La Cuesta F: Effect of dipyridamole and aspirin on the platelet-neutrophil interaction via the nitric oxide pathway. Eur J Pharmacol 2000;397:35–41.

13 Moake JL, Turner NA, Stathopoulos NA, Nolasco L, Hellums JD: Shear-induced platelet aggregation can be mediated by vWF released from platelets, as well as by exogenous large or unusually large vWF multimers, requires adenosine diphosphate, and is resistant to aspirin. Blood 1988;71:1366–1374.

14 Ikeda Y, Handa M, Kawano K, Kamata T, Murata M, Araki Y, Anbo H, Kawai Y, Watanabe K, Itagaki I, Sakai K, Ruggeri ZM: The role of von Willebrand factor and fibrinogen in platelet aggregation under varying shear stress. J Clin Invest 1991;87:1234–1240.

15 Uchiyama S, Yamazaki M, Maruyama S, Handa M, Ikeda Y, Fukuyama M, Itagaki I: Shear-induced platelet aggregation and its inhibition by antiplatelet agents in cerebral ischemia. Clin Hemorheol 1993;13:623–636.

16 Uchiyama S, Yamazaki M, Maruyama S, Handa M, Ikeda Y, Fukuyama M, Itagaki I: Shear-induced platelet aggregation in cerebral ischemia. Stroke 1994;25:1547–1551.

17 Tanigawa T, Nishikawa M, Kitai T, Ueda Y, Okinaka T, Makino K, Ito M, Isaka N, Ikeda Y, Shiku H, Nakano T: Increased platelet aggregation in response to shear stress in acute myocardial infarction and its inhibition by combined therapy with aspirin and cilostazol after coronary intervention. Am J Cardiol 2000;85: 1054–1059.

18 Sase K, Michel T: Expression of constitutive endothelial nitric oxide synthase in human blood platelets. Life Sci 1995;57:2049–2055.

19 Zhou Q, Hellermann GR, Solomonson LP: Nitric oxide release from resting human platelets. Thromb Res 1995;77:87–96.

20 Radomski MW, Palmer RMJ, Moncada S: An *L*-arginine/nitric oxide pathway present in human platelets regulates aggregation. Proc Natl Acad Sci USA 1990;87:5193–5197.

21 Müller TH, Su CAPF, Weisenberger H, Brickl R, Nehmiz G, Eisert WG: Dipyridamole alone or combined with low-dose acetylsalicylic acid inhibits platelet aggregation in human whole blood ex vivo. Br J Clin Pharmacol 1990;30: 179–186.

22 Nicholson CD, Challiss RAJ, Shahid M: Differential modulation of tissue function and therapeutic potential of selective inhibitors of cyclic nucleotide phosphodiesterase isozymes. Trends Pharmacol Sci 1991;12:19–27.

23 Maurice DH, Haslam RJ: Molecular basis of the synergistic inhibition of platelet function by nitrovasodilators and activators of adenylate cyclase: Inhibition of cyclic AMP breakdown by cyclic GMP. Mol Pharmacol 1990;37:671–681.

24 Yu SM, Tsai SY, Kuo SC, Ou JT: Inhibition of platelet function by A02131-1, a novel inhibitor of cGMP-specific phosphodiesterase, in vitro and in vivo. Blood 1996;87:3758–3767.

25 Minami N, Suzuki Y, Yamamoto M, Kihira H, Imai E, Wada H, Kimura Y, Ikeda Y, Shiku H, Nishikawa M: Inhibition of shear stress-induced platelet aggregation by cilostazol, a specific inhibitor of cGMP-inhibited phosphodiesterase, in vitro and ex vivo. Life Sci 1997; 61:383–389.

26 Nakamura T, Uchiyama S, Yamazaki M, Iwata M: In vitro effects of dipyridamole and aspirin on whole blood platelet aggregation and ATP release (in Japanese with English abstract). Jpn J Stroke 2000;22:343–347.

27 De La Cruz JP, Sanchez De La Cuesta F: Does the association dipyridamole-aspirin only act by a functional synergism? Gen Pharmacol 1991;22:271–274.

28 Violi F, Praticò D, Iuliano L, Balsano F: Dipyridamole potentiates the inhibition of platelet aggregation by aspirin (in human platelet rich plasma and whole blood). J Lipid Mediat 1991; 4:61–68.

29 Sanz G, Pajaron A, Alegria E, Coello I, Cardona M, Fournier JA, Gomez-Recio M, Ruano J, Hidalgo R, Medina A: Prevention of early aortocoronary bypass occlusion by low-dose aspirin and dipyridamole. Circulation 1990;82: 765–773.

30 DAMAD Study Group: Effect of aspirin alone and aspirin plus dipyridamole in early diabetic retinopathy. A multicenter randomized controlled clinical trial. Diabetes 1989;38:491–498.

31 Cronstein BN, Weissmann G: Targets for antiinflammatory drugs. Annu Rev Pharmacol Toxicol 1995;35:449–462.

32 Lopez-Farre A, Caramelo C, Esteban A, Alberola ML, Millas I, Monton M, Casado S: Effect of aspirin on platelet-neutrophil interactions. Role of nitric oxide and endothelin-1. Circulation 1995;91:2080–2088.

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THROMBOSIS RESEARCH

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Regular Article

Flow cytometric analysis of reticulated platelets in patients with ischemic stroke

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Abstract

Reticulated platelets are newly formed platelets containing a residual amount of RNA, and percentage of reticulated platelets (%RP) has been shown to reflect platelet turnover. Recently, a new flow cytometric approach for analyzing %RP in patients with thrombocytopenic disorders has been reported. We measured %RP by flow cytometry using the fluorescent dye thiazole orange (TO) to evaluate platelet kinetics in patients with different clinical categories of ischemic stroke. Patients with ischemic stroke were categorized into lacunar ($n=25$), atherothrombotic ($n=26$) and cardioembolic stroke ($n=17$). %RP was significantly higher in patients with cardioembolic stroke than in controls ($n=140$). Stepwise multiple regression analysis also showed cardioembolic stroke ($R^2=0.14$) to be significant independent predictors of %RP among stroke patients even after adjustment for other factors. We concluded that %RP is increased in patients with cardioembolic stroke, which may reflect increased platelet turnover as a consequence of platelet consumption during thrombogenesis.

Keywords: Reticulated platelet; Flow cytometry; Thiazole orange; Ischemic stroke

Platelets play an important role in the pathophysiology of ischemic stroke and transient ischemic attacks. Previous studies have demonstrated that platelet activities are enhanced in patients with stroke [1–4]. Some investigators have applied radionuclear techniques using indium-111 (^{111}In)-labelled platelets for the detection of intracardiac thrombus or platelet accumulation in carotid atherosclerosis of stroke patients [5–7]. Though these methods are useful for evaluating platelet kinetics, they are time-consuming, expose the patient to radioisotopes, and are difficult to perform in a routine clinical laboratory.

Recently, a new flow cytometric approach for evaluating platelet kinetics by examining the RNA content of circulating blood platelets has been reported [8–10]. The fluorescent dye thiazole orange (TO), which has a large fluorescence enhancement upon binding to RNA and is commonly used in flow cytometric analysis of reticulocytes, is also applied to the analysis of human platelets. Similar to red blood cell reticulocytes, reticulated platelets (RPs) are defined as the most recently released platelets with larger amounts of RNA. Therefore, numbers of RPs are increased in conditions characterized by greater platelet turnover in response to increased platelet destruction, as in the case of idiopathic thrombocytopenic purpura [8–12]. Thus, it is certain that the flow cytometric technique of measuring RPs is useful for the diagnosis of thrombocytopenic disorders [8–12].

More recently, this flow cytometric technique has also been applied in patients with preeclampsia [13], dialysis [14] and thrombosis related to chronic thrombocytosis [15]. A significant increase of RPs has demonstrated in these patients, suggesting that analysis of RPs may be useful to monitor the activation-dependent turnover of platelets. Flow cytometric analysis of RPs has the advantages of simplicity and rapidity to evaluate platelet kinetics. However, to our

Abbreviations: %RP, percentage of reticulated platelets; TO, thiazole orange; RPs, reticulated platelets; PBS, phosphate-buffered saline; EDTA, ethylenediamine tetraacetate; ATS, atherothrombotic stroke; CES, cardioembolic stroke; LAS, lacunar stroke.

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knowledge, there have been no reports of analysis of RPs in stroke patients.

In this study, we performed flow cytometric measurement of the percent of RPs (%RP) to analyze platelet kinetics in patients with various clinical categories of ischemic stroke.

1. Materials and methods

1.1. Control subjects and patients

Between June 1999 and March 2000, we collected blood samples from 77 consecutive patients with ischemic stroke, who visited the outpatient clinic or admitted to the Department of Neurology, Neurological Institute, Tokyo Women's Medical University. Informed consent was obtained from all subjects before blood collection. A total of nine patients were excluded because of stroke with unknown etiology ($n=7$), aortitis ($n=1$) and chemotherapy treatment ($n=1$). Finally, 68 consecutive patients with ischemic stroke (59 men, 9 women; mean age, 63.5; range 26 to 95 years) were included in our study. Control subjects were selected from consecutive patients who visited our outpatient clinic or admitted to our hospital between June 1999 and November 1999, and were undertaken brain MRI (1.5 T). Thus, 140 consecutive control subjects (78 men, 62 women; mean age, 67.9; range 45 to 85 years) were also included in our study. Out of these control subjects, 77 (55%) were not detected any neurological disorders in spite of the patient's complaint such as dizziness or numbness on their hands or feet. Diagnosis for the other control subjects included benign positional paroxysmal vertigo for 13 (9.3%), tension headache for 12 (8.6%), initial stage of Parkinson's disease for 7 (5%), vasovagal syncope for 6 (4.3%), peripheral type of hemifacial palsy for 6 (4.3%), very mild neuropathy for 3 (2.1%), transient global amnesia for 3 (2.1%) and other mild neurological disorders for 13 (9.3%). Out of 140 control subjects, 118 (84.3%) had one or more asymptomatic tiny infarctions or etat criblé on brain MRI.

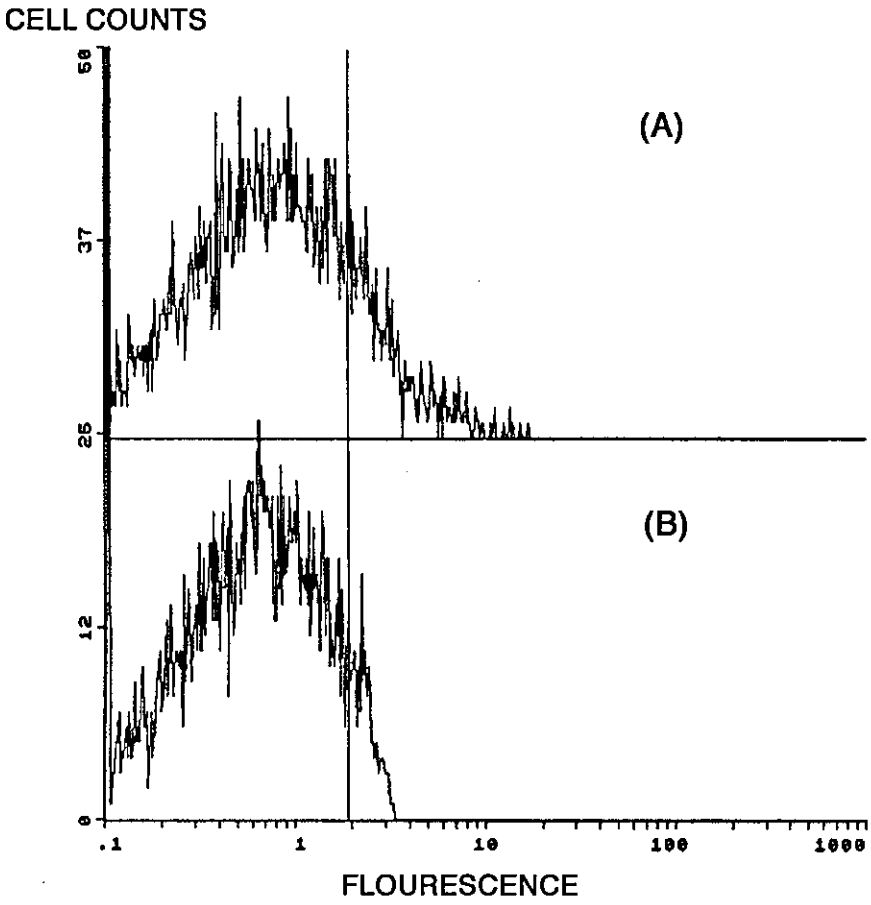

Fig. 1. Fluorescence histograms of platelets after staining with thiazole orange (TO). (A) Untreated with RNase. (B) Treated with RNase. Vertical lines in the histograms indicate the gate position for selection of highly fluorescent subset as determined in a TO unstained control sample (not shown). Each histogram was obtained by analyzing 5000 platelets. The percentage of TO-positive platelets was 23.5% in sample A and 12.7% in sample B. %RP was calculated by subtracting of the percentage of TO-positive platelets treated with RNase (B) from untreated platelets (A). Therefore, %RP of this subject was calculated to be 10.8%.

Patients with ischemic stroke were classified into atherothrombotic, cardioembolic and lacunar stroke by the clinical categories according to the Classification of Cerebrovascular Diseases III by the National Institute of Neurological Disorders and Stroke [16]. Briefly, atherothrombotic stroke was defined as appropriate clinical syndromes associated with cortical or subcortical lesions with diameters larger than 15 mm on CT and/or MRI, greater than 50% stenosis or obstruction of a responsible large artery. Cardioembolic stroke was defined as abrupt onset of appropriate cortical or hemispheric syndromes associated with cortical or subcortical lesions with diameters larger than 15 mm on CT and/or MRI and cardiac embolic sources including atrial fibrillation, left atrial or ventricular thrombus, dilated cardiomyopathy and mitral valve stenosis, and no relevant large artery stenosis. Lacunar stroke was defined as lacunar syndromes associated with responsible subcortical lesions less than 15 mm in diameter on CT and/or MRI, and without more than 50% of ipsilateral extra- and intra-cranial major vessel stenosis, and without potential cardioembolic sources. Patients classified into other clinical categories such as coagulopathies or specific vasculopathies other than atherosclerosis, or patients who did not meet any criteria of the above three clinical categories were excluded from this study.

The controls and stroke patients were regarded as being hypertensive if they were taking antihypertensive agents or if the systolic blood pressure was greater than 160 mm Hg or the diastolic blood pressure was greater than 95 mm Hg. A diagnosis of diabetic mellitus was made if they were treated with an oral hypoglycemic agent or insulin, or if their glycosylated hemoglobin (HbA1c) level was greater than 6.2%. They were considered hypercholesterolemic if they were taking any antihyperlipidemic agents and/or their serum cholesterol level was greater than 220 mg/dl. Chronic atrial fibrillation was diagnosed on the basis of ECG and coronary artery disease was diagnosed by the presence of history of angina pectoris and/or myocardial infarction.

Antiplatelet agents and anticoagulants were used as antithrombotic treatment for some patients with ischemic stroke. Antiplatelet agents included aspirin (40–300 mg/day) and ticlopidine (100–200 mg/day). Anticoagulants included warfarin (INR of 1.5 to 3.5), intravenous heparin (5000–20,000 IU/day) and argatroban (60 mg/day for the first 2 days and 10 mg twice daily for next 5 days).

According to the time intervals between stroke onset and blood sampling, stroke patients were also classified into acute phase (days 1 to 7), subacute phase (days 8 to 30) and chronic phase (day 31 or more).

1.2. Sample collection and preparation

Reticulated platelets were measured according to the method reported previously [10,14]. Briefly, whole blood was drawn into a 1/10 volume of 3.8% trisodium citrate in a

Table 1
Background characteristics of the study populations

	Controls ($n=140$)	Ischemic stroke patients ($n=68$)			P
		Atherothrombotic ($n=26$)	Cardioembolic ($n=17$)	Lacunar ($n=25$)	
Demographic characteristics					
Age (years), mean ± S.D.	67.9 ± 8.2	62.6 ± 10.1	64.5 ± 15.8	63.5 ± 8.03	NS
Men/women	78:62	26:0	12:5	21:4	<0.0001
Risk factors					
Hypertension	73 (52)	19 (73)	4 (24)	19 (76)	0.0017
Diabetes	25 (18)	12 (46)	5 (29)	7 (28)	0.015
Hyperlipidemia	53 (38)	12 (46)	2 (12)	6 (24)	NS
Coronary artery disease	20 (14)	6 (23)	2 (12)	3 (12)	NS
Chronic atrial fibrillation	13 (9)	2 (8)	15 (88)	2 (8)	<0.0001
Treatment					
No antithrombotic drug	78 (55)	2 (8)	4 (24)	2 (8)	<0.0001
Antiplatelet agents	59 (42)	19 (73)	9 (53)	22 (88)	<0.0001
Anticoagulants	5 (3)	6 (23)	8 (47)	2 (8)	<0.0001
Periods from stroke onset to blood sampling					
Days 1 to 7 (acute)		3 (11)	4 (23)	3 (12)	NS
Days 8 to 30 (subacute)		7 (27)	3 (18)	2 (8)	NS
Day 31 or more (chronic)		16 (62)	10 (59)	20 (80)	NS
Hematological variables (mean ± S.D.)					
Platelet count ($\times 10^9$/l)	204 ± 46.2	232 ± 51.3	232 ± 65.1	198 ± 61.1	0.013
Hemoglobin (g/dl)	11.8 ± 1.38	12.4 ± 1.71	12.3 ± 1.53	12.4 ± 0.93	0.036
White cell count ($\times 10^9$/l)	4936 ± 1459	5540 ± 1333	6482 ± 2520 *	5016 ± 1361	0.0009

TIA indicates transient ischemic attack. NS indicates no significant. Values in parentheses are percentages.

* $p<0.05$ by Turkey–Kramer test following factorial ANOVA in the four groups, compared with controls and lacunar groups.

plastic tube. Hemoglobin, white cell and platelet count were determined using an automated analyzer (Sysmex 8000, TOA Medical Electronics, Kobe, Japan) within 1 h after blood sampling. Whole blood was centrifuged at 120 × *g* to separate platelet-rich plasma; platelets were washed and suspended in phosphate-buffered saline (PBS) (pH 7.2), containing 2 mmol/l ethylenediamine tetraacetate (EDTA), 0.02% sodium azide and paraformaldehyde at a final concentration of 1%. Samples were stored at 4 °C for at least 2 h. Following fixation, platelets were washed in PBS buffer, labeled with a phycoerythrin-conjugated monoclonal anti-glycoprotein Ib antibody (CD41) (PharMingen, San Diego, CA), and stained with TO (Polysciences, Warrington, PA) solution (0.1 μg/ml at final concentration) at room temperature for 1 h in a dark box.

1.3. Pretreatment with RNase

Previous reports have demonstrated that TO staining is only partially RNA specific, and other platelet components such as dense granular pool of nucleotides can cause a substantial amount of non-specific staining [10,17–19]. Therefore, in addition to TO-unstained platelets, we applied the method of using RNase-treated samples as a control for measurement of %RP [19]. Fixed platelets were divided into two aliquots; one was treated for 1 h at 37 °C with 1 mg/ml RNase (Sigma, St. Louis, MO) to eliminate RNA before incubation with TO, and the other aliquot remained untreated.

1.4. Flow cytometric analysis

Analysis of the samples was performed with a flow cytometer (Profile II, Coulter, Hialeah, Florida). Identification of TO positivity was made by measuring both forward light scatter and green (540 nm) fluorescence using logarithmic amplification. The platelet population in forward (versus side) scattering was confirmed as the population of CD41-positive events in the cytogram and is gated for this platelet population area. A positive range was set for platelets with fluorescence higher than 99% of the TO-unstained control sample. %RP was calculated by subtracting of the percentage of TO-positive platelets treated with RNase from untreated platelets (Fig. 1).

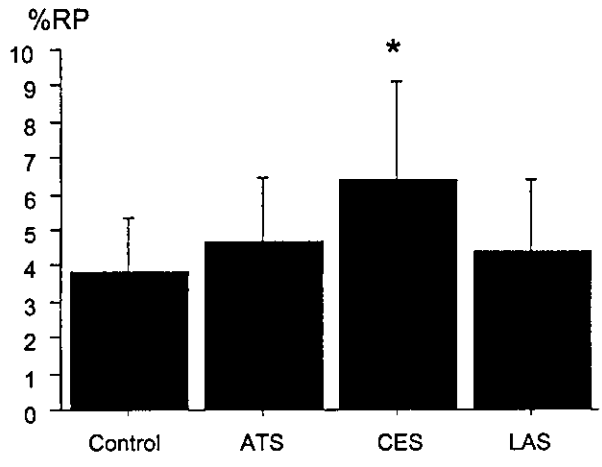

Fig. 2. %RP in patients with different subtypes of ischemic stroke and control subjects. ATS: atherothrombotic stroke; CES: cardioembolic stroke; LAS: lacunar stroke. Data are presented as mean values ± standard deviations. *$P<0.05$ as compared to controls, atherothrombotic and lacunar stroke patients.

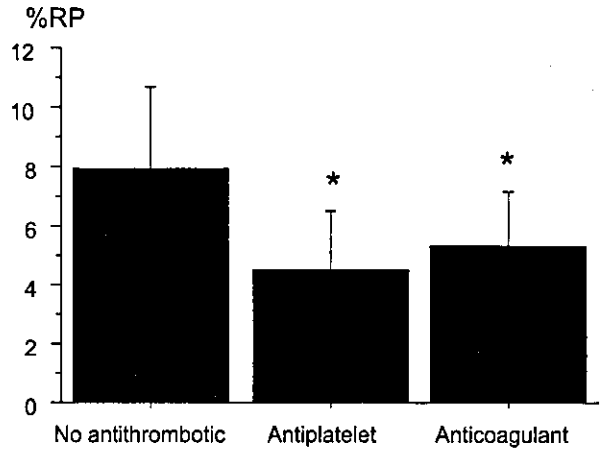

Fig. 3. %RP in stroke patients without antithrombotic treatment and with antiplatelet treatment or anticoagulant use. Data are presented as mean values ± standard deviations. *$P<0.05$ as compared to stroke patients with no antithrombotic treatment.

1.5. Statistics

Data are presented as mean values ± standard deviations (S.D.). Factorial ANOVA followed by Tukey–Kramer procedure was used to compare age and hematological variables among controls and stroke subgroups, and assess differences of %RP. χ^2 test was used for the comparison of background characteristics (gender, hypertension, diabetic mellitus, hyperlipidemia, coronary artery disease, chronic atrial fibrillation, antiplatelet drug or anticoagulant use, and periods from stroke onset to blood sampling). Stepwise multiple regression analysis was applied to assess the relative importance of the independent variables in %RP among control subjects and clinical categories of stroke; to compare patients with stroke (atherothrombotic, cardioembolic or lacunar stroke); and to test for correlation among age, gender, the presence of hypertension, diabetes mellitus, hyperlipidemia, coronary artery disease, or chronic atrial fibrillation, antiplatelet drug or anticoagulant use, periods from stroke onset to blood sampling, platelet count, hemoglobin, and white cell count. These calculations were performed with the StatView 5.0 programs on a Macintosh personal computer. Statistical significance was accepted at $p<0.05$.

2. Results

2.1. Background characteristics of stroke patients

Background characteristics of the study populations and hematological variables are summarized in Table 1. There were no significant differences in age between control subjects and three clinical categories of ischemic stroke, while men were more common in stroke patients compared to control subjects. Hypertension was more common in patients with atherothrombotic or lacunar stroke compared to control subjects or patients with cardioembolic stroke. Diabetes was more common in patients with atherothrombotic stroke. Patients with cardioembolic stroke had more chronic atrial fibrillation and were received more anticoagulant. Patients with atherothrombotic or lacunar stroke were given more antiplatelet agents. Patients with cardioembolic stroke had more white cell counts compared to those with lacunar stroke or control subjects.

2.2. Determinations of %RP

Fig. 2 illustrates results in patients with different subtypes of ischemic stroke and control subjects. %RP was $3.85 \pm 1.47\%$ in the 140 control subjects, $4.62 \pm 1.80\%$ in patients with atherothrombotic stroke, $6.36 \pm 2.71\%$ in patients with cardioembolic stroke, and $4.36 \pm 2.04\%$ in patients with lacunar stroke. %RP was significantly higher in patients with cardioembolic stroke when compared to controls. In addition, patients with cardioembolic stroke had a significantly higher %RP when compared to those with atherothrombotic stroke and lacunar stroke.

Table 2
Multiple stepwise regression analysis for determination of %RP

Variable	Coefficient	F	P
Cardioembolic stroke	1.90	15.3	<0.0001
Control subject	−0.62	4.7	<0.0001
Constant	4.46		
Age		2.25	NS
Gender		0.05	NS
Hypertension		0.34	NS
Diabetes		0.01	NS
Hyperlipidemia		0.11	NS
Coronary artery disease		0.5	NS
Chronic atrial fibrillation		0.43	NS
Antiplatelet agents use		2.6	NS
Anticoagulants use		0.09	NS
Days 1 to 7 (acute)		1.47	NS
Days 8 to 30 (subacute)		0.52	NS
Day 31 or more (chronic)		0.12	NS
Platelet count		0.1	NS
Hemoglobin		0.01	NS
White cell count		0.13	NS

$R^2 = 0.14$.
NS indicates not significant.

Among the stroke patients, %RP was $7.95 \pm 2.7\%$ in the 8 patients with no antithrombotic treatment, $4.52 \pm 1.95\%$ in the 50 patients with antiplatelet treatment, and $5.35 \pm 1.78\%$ in the 16 patients with anticoagulant use (Fig. 3). %RP was siginificantly lower in patients under antiplatelet treatments and anticoagulant use than in those with no antithrombotic treatments.

Stepwise multiple regression analysis showed that %RP significantly and independently ($R^2 = 0.14$) correlated with control subjects and cardioembolic stroke even after adjustment for other factors (Table 2).

3. Discussion

In this study, we used a rapid and accessible technique of evaluating platelet kinetics (%RP), and found a significant increase in %RP in patients with cardioembolic stroke. Previous studies using platelet scintigraphy have demonstrated increased platelet accumulation in carotid atherosclerosis or intracardiac thrombus in stroke patients [5–7]. Uchiyama et al. [4] have demonstrated, using platelets labeled with indium-111-tropolone, that platelet survival is significantly shortened in patients with atherothrombotic and cardioembolic stroke, and platelet destruction is increased in patients with cardioembolic stroke. These studies suggest that platelet turnover is accelerated as a consequence of increased platelet consumption by thrombus formation in the arterial lumen or cardiac chamber in these patients. The results of our present flow cytometric study are consistent with those reported in these previous studies.

Stepwise multiple regression analysis demonstrated that only cardioembolic stroke was a significant independent predictor of %RP among three categories of ischemic stroke in our study. It is generally accepted that the coagulation system is activated, and fibrin-rich thrombi are formed in the heart chamber in patients with cardioembolic stroke [4,20–22]. Simultaneously, thrombin generated by activation of coagulation system may induce platelet activation, resulting in increased platelet release reaction in patients with cardioembolic stroke [3,4,23]. On the other hand, other reports have shown that platelet aggregation is not enhanced, but may even be attenuated despite enhanced platelet release reaction [4]. These conflicting observations can be explained by the term "empty exhausted platelets" or "platelet refractoriness", which means a profound depression of platelet status after repetitive platelet activation [14]. Himmelfarb et al. [14] have demonstrated a marked increase in circulating reticulated platelets in dialysis patients with qualitative platelet dysfunction compared to normal controls. This suggests increased platelet turnover after repetitive platelet activation and concomitant "platelet refractoriness" induced by the interactions of platelets with dialysis membranes. Similarly, repetitive platelet activation that occurs in the heart chamber through thrombin generation may accelerate platelet turnover, followed by in-

creased RPs in patients with cardioembolic stroke. More recent study has suggested that platelets store biologically active thrombopoietin, which may be instrumental in enhancing thrombocytopoiesis in cases of massive activation-induced platelet destruction [24]. Therefore, we speculate that the increased %RP in cardioembolic stroke may reflect enhanced thrombocytopoiesis as a consequence of increased platelet turnover after repetitive platelet activation through a thrombin-mediated process.

Atherothrombotic stroke is also closely related to platelet activation in its etiology [1–4], and platelet accumulation in carotid lesions has been reported in previous studies using platelet scintigraphy [7]. In the present study, patients with atherothrombotic stroke had a tendency of increasing %RP compared to control subjects; however, stepwise multiple regression analysis did not show atherothrombotic stroke to be a significant independent predictor of %RP. A study using platelets labelled with indium-111-tropolone by Uchiyama et al. [4] have demonstrated that patients with cardioembolic stroke have not only shortened platelet survival but also increased platelet destruction, while those with atherothrombotic stroke have only shortened platelet survival. Our results indicate that the degree of platelet turnover following enhanced platelet activation in atherothrombotic patients is lower than that in cardioembolic patients, and these are compatible to the results of the platelet-labelling study. Therefore, we concluded that platelet activation is stronger and more substantial in patients with cardioembolic stroke than in those with atherothrombotic stroke.

It is controversial whether platelet aggregation would be enhanced in lacunar stroke [1–4]. Lacunar stroke contains several mechanisms as its etiology, e.g. lipohyalinosis in small vessels, microatheroma in perforating artery, and small emboli from major arteries or cardiac chamber. However, according to the classification of stroke subtypes, the major etiology of lacuner stroke was supposed to be lipohyalinosis, which is not likely to be associated with platelet activation but with hypertension [1–4]. We considered that %RP in patients with lacunar stroke did not significantly increase in our study because lacunar stroke is associated with much less platelet activation than the other stroke subtypes.

As we mentioned, increased %RP in cardioembolic stroke reflects increased platelet turnover as a consequence of platelet consumption following a profound depression of platelet functional status after repetitive platelet activation. It is intriguing whether this depressed functional status of platelets might cause hemorrhagic stroke after cardioembolic stroke. In our study, patients with cardioembolic stroke did not cause massive hemorrhagic transformations except one with mild hemorrhagic stroke. This patient with mild hemorrhagic stroke was sampled in acute phase (days 1 to 7) and had increased %RP (10.6%). However, two out of three other patients with cardioembolic stroke sampled in acute phase had also increased %RPs at similar level. Therefore, it is unclear whether increased %RP was related to hemorrhagic transformations in cardioembolic stroke. Besides, %RP in cardioembolic stroke can change according to the time intervals between stroke onset and blood sampling. We have examined the differences of %RP in patients with cardioembolic stroke among acute phase (days 1 to 7), subacute phase (days 8 to 30) and chronic phase (day 31 or more), and obtained the result of significant increase of %RP in patients sampled in acute phase than in those sampled in chronic phase ($9.08 \pm 2.8\%$ and $5.21 \pm 2.24\%$, respectively, $p < 0.05$). However, our study design is cross-sectional, and the numbers of these patients were too small (acute phase, $n = 4$; subacute phase, $n = 3$; chronic phase, $n = 10$) to conclude meaningful differences of %RP among three phases.

In the present study, %RP in stroke patients under antiplatelet treatments or anticoagulant use was lower than those without antithrombotic drug use. Recently, Rinder et al. [15] have demonstrated that %RP in patients with chronic thrombocytosis is successfully corrected by aspirin treatment. It is not clear whether aspirin or ticlopidine influence megakaryocytes or a feedback control system of platelet production in the bone marrow [25,26]. It can be inferred that aspirin or ticlopidine may have an effect on %RP by inhibiting platelet activation and consumption locally in the arteries or heart chamber. Also, warfarin may affect %RP by inhibiting platelet consumption following thrombin-mediated platelet activation. Otherwise, stepwise multiple regression analysis did not show antithrombotic drug use to be an independent negative predictor of %RP. Our study included the small number ($n = 8$) of patients without antithrombotic treatments and did not consider the basic conditions of these patients. Therefore, we could not conclude whether antithrombotic treatment have the influences on %RP or not. A prospective study is needed to determine more precisely the effects of antithrombotic agents on %RP by comparing %RP before and after treatments in a larger number of patients.

In summary, we have shown that %RP is increased in cardioembolic stroke, which may reflect increased platelet turnover as a consequence of platelet consumption during thrombogenesis presumably in the heart chamber. Measurements of %RP are useful for the evaluation of platelet kinetics in patients with ischemic stroke. A prospective study is needed to compare %RP before and after antithrombotic treatments, and to investigate sequential alterations of %RP after stroke onset and the predictive value of %RP for stroke recurrence.

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References

[1] Uchiyama S, Takeuchi M, Osawa M, Kobayashi I, Maruyama S, Aosaki M, et al. Platelet function tests in thrombotic cerebrovascular disorders. Stroke 1983;14:511–7.
[2] Uchiyama S, Yamazaki M, Maruyama S, Handa M, Ikeda Y, Fukuyama M, et al. Shear-induced platelet aggregation in cerebral ischemia. Stroke 1994;25:1547–51.
[3] Iwamoto T, Kubo H, Takasaki M. Platelet activation in the cerebral circulation in different subtypes of ischemic stroke and Binswanger's disease. Stroke 1995;26:52–6.
[4] Uchiyama S, Yamazaki M, Hara Y, Iwata M. Alterations of platelet, coagulation, and fibrinolysis markers in patients with acute ischemic stroke. Semin Thromb Hemost 1997;23:535–41.
[5] Powers WJ, Siegel BA, Davis HH, Mathias CJ, Clark HB, Welch MJ. Indium-111 platelet scintigraphy in cerebrovascular disease. Neurology 1982;32:938–43.
[6] Kessler C, Henningsen H, Reuther R, Kimmig B, Rosch M. Identification of intracardiac thrombi in stroke patients with Indium-111 platelet scintigraphy. Stroke 1987;18:63–7.
[7] Minar E, Ehringer H, Dudczak R, Schofl R, Jung M, Koppensteiner R, et al. Indium-111-labeled platelet scintigraphy in carotid atherosclerosis. Stroke 1989;20:27–33.
[8] Kienast J, Schmitz G. Flow cytometric analysis of thiazole orange uptake by platelets: a diagnostic aid in the evaluation of thrombocytopenic disorders. Blood 1990;75:116–21.
[9] Ault KA, Rinder HM, Mitchell J, Carmody MS, Vary CPH, Hillman RS. The significance of platelets with increased RNA content (reticulated platelets). A measure of the rate of thrombopoiesis. Am J Clin Pathol 1992;98:637–46.
[10] Richards EM, Baglin TP. Quantitation of reticulated platelets: methodology and clinical application. Br J Haematol 1995;91:445–51.
[11] Watanabe K, Takeuchi K, Kawai Y, Ikeda Y, Kubota F, Nakamoto H. Automated measurement of reticulated platelets in estimating thrombopoiesis. Eur J Haematol 1995;54:163–71.
[12] Fujii T, Shimomura T, Fujimoto T, Kimura A, Fujimura K. A new approach to detect reticulated platelets stained with thiazole orange in thrombocytopenic patients. Thromb Res 2000;97:431–40.
[13] Rinder HM, Bonan JL, Anandan S, Rinder CS, Rodrigues PA, Smith BR. Noninvasive measurement of platelet kinetics in normal and hypertensive pregnancies. J Obstet Gynaecol 1994;170:117–22.
[14] Himmelfarb J, Holbrook D, McMonagle E, Ault K. Increased reticulated platelets in dialysis patients. Kidney Int 1997;51:834–9.
[15] Rinder HM, Schuster JE, Rinder CS, Wang C, Schweidler HJ, Smith BR. Correlation of thrombosis with increased platelet turnover in thrombocytosis. Blood 1998;91:1288–94.
[16] National Institute of Neurological Disorders and Stroke. Classification of cerebrovascular diseases III. Stroke 1990;21:637–76.
[17] Balduini CL, Noris P, Spedini P, Belletti S, Zambelli A, DaPrada GA. Relationship between size and thiazole orange fluorescence of platelets in patients undergoing high-dose chemotherapy. Br J Haematol 1999;106:202–7.
[18] Robinson MSC, Mackie IJ, Khair K, Liesner R, Goodall AH, Savidge GF, et al. Flow cytometric analysis of reticulated platelets: evidence for a large proportion of non-specific labelling of dense granules by fluorescent dyes. Br J Haematol 1998;100:351–7.
[19] Schmitz G, Rothe G, Ruf A, Barlage S, Tschope D, Clemetson KJ, et al. European working group on clinical cell analysis: consensus protocol for the flow cytometric characterisation of platelet function. Thromb Haemost 1998;79:885–96.
[20] Fisher M, Francis R. Altered coagulation in cerebral ischemia: platelet, thrombin and plasmin activity. Arch Neurol 1990;47:1075–9.
[21] Takano K, Yamaguchi T, Kato H, Omae T. Activation of coagulation in acute cardioembolic stroke. Stroke 1991;22:12–6.
[22] Yamazaki M, Uchiyama S, Maruyama S. Alterations of haemostatic markers in various subtypes and phases of stroke. Blood Coagul Fibrinolysis 1993;4:707–12.
[23] Shah AB, Beamer N, Coull BM. Enhanced in vivo platelet activation in subtypes of ischemic stroke. Stroke 1985;16:643–7.
[24] Folman CC, Linthorst GE, van Mourik J, van Willigen G, de Jonge E, Levi M, et al. Platelet release thrombopoietin (Tpo) upon activation: another regulatory loop in thrombocytopoiesis? Thromb Haemost 2000;83:923–30.
[25] Cazenave JP, Gachet C. Anti-platelet drugs: do they affect megakaryocytes? Bailliere's Clin Haematol 1997;10:163–80.
[26] Erhart S, Beer JH, Reinhart WH. Influence of aspirin on platelet count and volume in humans. Acta Haematol 1998;101:140–4.

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◆総　説◆

脳血管障害における抗血栓療法

内　山　真　一　郎

Antithrombotic Therapy in Cerebrovascular Disease

Shinichiro UCHIYAMA

脳梗塞急性期に未分画ヘパリンの予後改善効果はないが，アテローム血栓性脳梗塞では低分子ヘパリノイドの転帰改善効果が期待できる．アテローム血栓性脳梗塞急性期に対するアルガトロバンとオザグレルの効果は同等である．脳梗塞急性期にアスピリンはわずかながら有意な転帰改善効果がある．脳梗塞やTIAの再発予防にはアスピリン，チクロピジン，クロピドグレル，少量アスピリン・ジピリダモール併用が有効であり，シロスタゾールの効果も示された．心房細動患者のうち，75歳以上か，脳卒中・TIAの既往，高血圧，心不全，糖尿病，冠動脈疾患を有する患者にはワルファリンの適応があり，これらの危険因子がない65～75歳の患者にはワルファリンでもアスピリンでもよく，60～65歳の患者にはアスピリンが適応となり，60歳未満の患者は無治療でよい．心房細動，左室血栓，急性心筋梗塞，人工弁置換例を除く脳梗塞にワルファリンの適応根拠はない．

Key words：脳梗塞，一過性脳虚血発作，心房細動，抗血小板療法，抗凝固療法

はじめに

脳梗塞や一過性脳虚血発作（TIA）の大多数は血栓による脳動脈の閉塞により生じるので抗血栓療法はこれらの虚血性脳血管障害のもっとも本質的な治療法であるといえる．抗血栓療法には抗凝固療法と抗血小板療法があり，広義の抗血栓療法には血栓溶解療法も含まれるが，紙面の余裕がないので省略する．本稿では，脳梗塞の急性期と慢性期における抗凝固療法と抗血小板療法に関する最新のエビデンスとコンセンサスを述べてみたい．

I.　急性期の抗血栓療法

1.　抗凝固療法

著者も共同研究者の一人であるCochrane Stroke Groupが発症後2週間以内の脳梗塞患者合計23,427例を対象とした21件の抗凝固療法のRCTをmeta-analysisにより解析した成績によると，抗凝固薬は標準的な未分画ヘパリン，低分子ヘパリン，ヘパリノイド，経口抗凝血薬，トロンビン阻害薬が含まれたが，追跡最終時点での死亡または要介助の転帰不良が減少するというエビデンスは得られなかった（図1）[1]．また，抗凝固療法により脳梗塞は1,000例当たり9例減少したが，症候性頭蓋内出血が同じく1,000例当たり9例増加してしまった．脳

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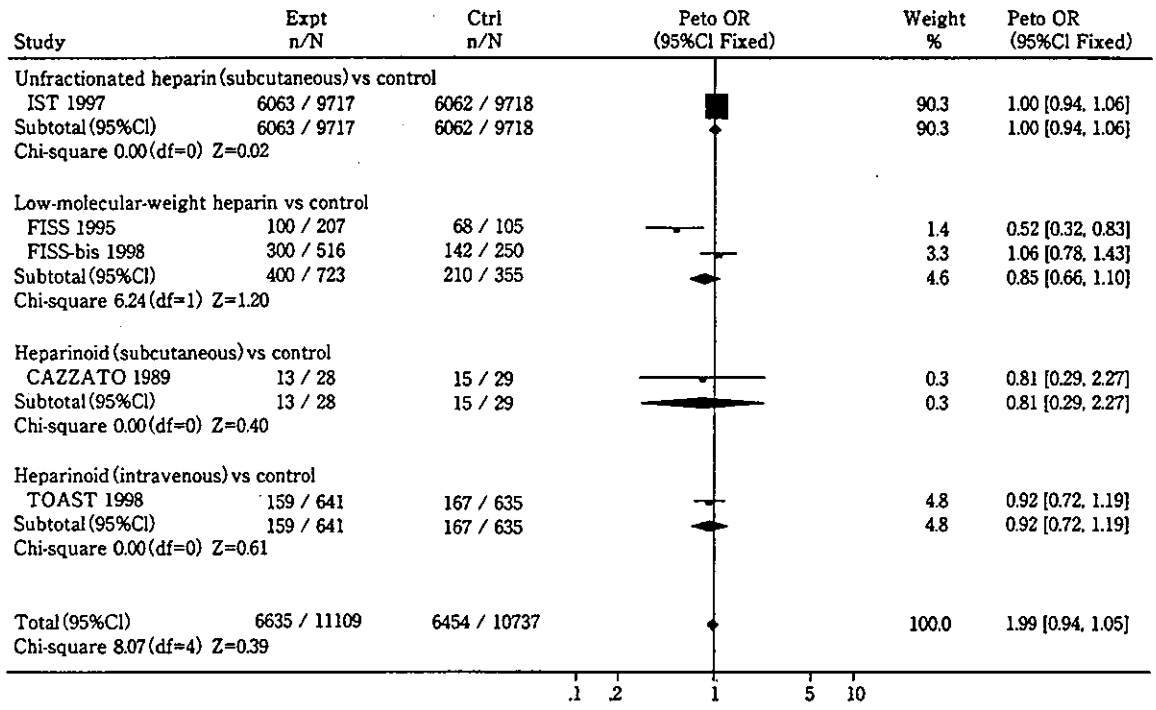

図 1 急性虚血性脳卒中患者における抗凝固薬投与群と対照群の最終追跡時（>1ヶ月）の死亡または要介助（文献1より引用）．黒い正方形の面積は各臨床試験の統計学的情報量（症例数），水平の実線は95%信頼区間（CI），ダイヤモンドの中央は小計のオッズ比（OR）（英国ではメタアナリシスの創始者であるOxford大学医学部のRichard Peto教授に敬意を表してPeto ORと呼んでいる），ダイヤモンドの両端はその95%信頼区間，縦の実線はOR 1.0を示す．水平の実線またはダイヤモンドの右端が縦の実線より左なら実薬群で対照群より有意に少なく，それらの左端が縦の実線より右なら実薬群で対照群より有意に多いことを示している．Expは期待値，Ctrlは対照，Weightは全体に占める各臨床試験の規模の比率を示す．

卒中の治療は死亡率の減少と後遺症の軽減を目標とすべきであるが，早期抗凝固療法により確かに脳梗塞の再発は減少したが，この効果は同じだけの頭蓋内出血の増加により相殺されてしまい，死亡や後遺症にいかなる効果ももたらさなかったことを示している．このような成績に基づき，海外の脳卒中急性期診療のガイドラインから抗凝固療法は削除されつつある[2]．

ただし，TOAST[3]の病型別解析によれば，アテローム血栓性脳梗塞では低分子ヘパリノイド（ダナパロイド）投与群でプラセボ投与群より3カ月後の転帰良好例が有意に多かったことから，この病型における有効性が示唆される．トロンビン阻害薬の成績は本邦で行われたアルガトロバンのRCT[4]しかなく，meta-analysisの対象とはならないが，本邦では発症後48時間以内のラクナ梗塞を除く脳血栓症260例を対象としてアルガトロバンとトロンボキサンA2合成酵素阻害薬オザグレルのRCTが行われ，1カ月後の全般改善度，神経症候改善度，日常生活動作改善度には有意差がなかったという結果が報告された[5]．また，アメリカでは発症後12時間以内の虚血性脳卒中180例を対象としてAPTTにより用量を調節したアルガトロバンの5日間持続静注療法の第2相用量探索試験（Argatroban in Ischemic Stroke I：ARGIS-I）が行われている．

2. 抗血小板療法

International Stroke Trial（IST）[6]とChinese Acute Stroke Trial（CAST）[7]を含む脳梗塞急性期患者におけるアスピリン療法のRCTをmeta-analysisにより解析した成績によれば，アスピリンは発症後48時間以内の脳梗塞患者において在院期間の再発予防効果と軽度

ながら有意な長期の転帰改善効果があったことから，脳梗塞を発症したらただちにアスピリン投与を開始したほうがよいと考えられる（図2）[7]．日本ではトロンボキサン A_2 合成酵素阻害薬オザグレルが発症後5日以内の脳血栓症患者において28日後の運動障害を有意に改善する効果のあることが示され[8]，適用承認されているが，海外ではRCTが行われておらず，アスピリンとの優劣は不明である[9]．

アスピリンの長期予後に及ぼす効果は有意ではあるもののわずかなので，より有効な抗血小板薬が待望されていたが，最近，発症後24時間以内の脳梗塞においてGPIIb/IIIaのモノクローナル抗体abciximab（c7E3 Fab）の米欧共同研究による第2相試験[10]が行われた．重篤な頭蓋内出血はみられず，3カ月後の予後良好例が治療群でプラセボ群より多かったことから，現在発症後6時間以内の脳梗塞を対象として第3相試験（Abciximab in Emergent Stroke Treatment Trial；AbESTT）[11]が開始された．

II. 慢性期の抗血栓療法

1. 抗血小板療法

Antiplatelet Trialists' Collaboration（APT）のmeta-analysisにより脳梗塞・TIAを含むアテローム血栓性疾患における抗血小板療法の有効性は確立されている[12]．APTは抗血小板療法のRCTのみならず抗凝固療法のRCTをも解析対象とするAntithrombotic Trialists' Collaboration（ATT）となり，1997年に共同研究者会議が開催され，その解析結果が最近発表された[13]．ATTの解析対象となったのは266件のRCTで無作為化された約20万例であり，このうち日本で行われ，meta-analysisの解析対象となったRCTは6件，症例数は合計1,458例であったが，いずれも虚血性脳血管障害であった．

ATTのmeta-analysisでは94年に発表されたAPTの2倍以上の症例が解析対象となったが，脳梗塞・TIA患者における抗血小板療法の再発予防効果が再確認された[13]．抗血小板薬別の解析では，アスピリンが23%，チクロピジンが32%，アスピリンとジピリダモールの併用が30%の有意な血管イベント低減効果を認めた（図3）[13]．

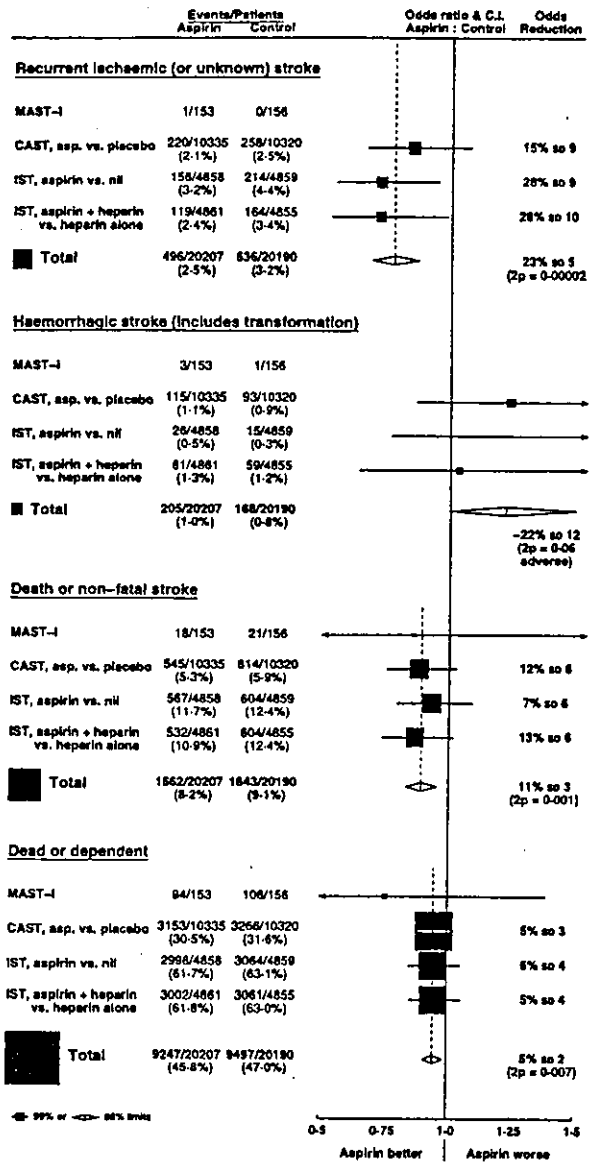

図2　急性虚血性脳卒中における早期アスピリン療法のメタアナリシス（文献7より引用）．CAST；Chinese Acute Stroke Trial, IST；International Stroke Trial, MAST-I；Multicenter Acute Stroke Trial-Italy．予定治療期間内（CASTは4週間，ISTは2週間，MAST-Iは10日間）の虚血性脳卒中の再発，出血性脳卒中，死亡または非致死的脳卒中．

アスピリンの至適用量に関してはいまだに議