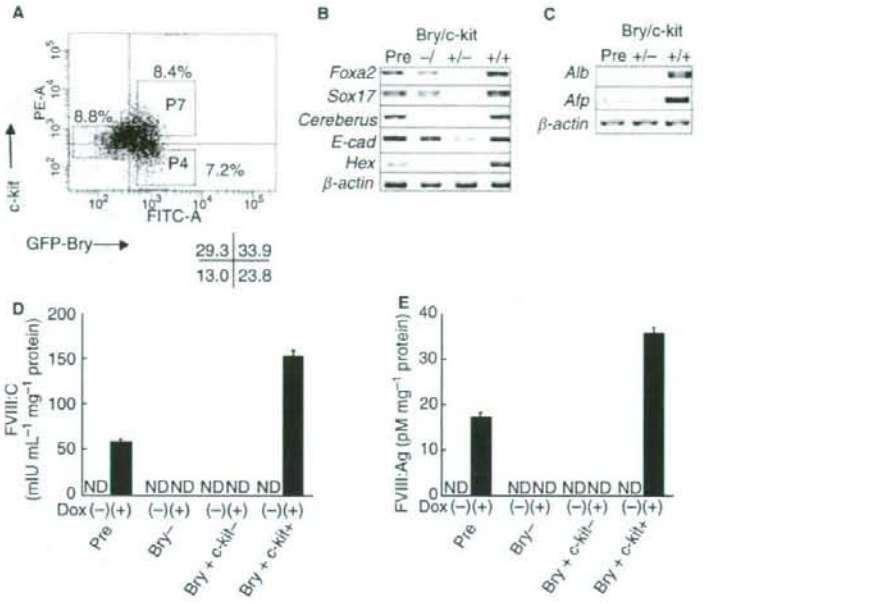

Fig. 4. GFP–Bry$^+$/c-kit$^+$ cells contain the definitive endoderm progenitors and efficiently secrete FVIII. (A) FACS profile for GFP–Bry and c-kit among day 3.5 embryoid bodies cultured in serum-containing medium. (B) Reverse transcription polymerase chain reaction analysis demonstrating the presence of endoderm-related genes in populations derived from presorted cells (pre) or cells sorted on the basis of GFP–Bry and c-kit. (C) Cells from presorted populations or those sorted on the basis of GFP–Bry and c-kit were reaggregated for 1 day, and then cultured in serum replacement medium and replated on day 10. *Alb* and *Afp* mRNA was expressed in GFP–Bry$^+$/c-kit$^+$ cells on day 15. (D, E) Levels of FVIII:C (D) and FVIII:Ag (E) in medium conditioned by presorted cells and those sorted on the basis of GFP–Bry and c-kit, with or without doxycycline (Dox) (1 μg mL^{-1}) induction. The data presented are means of three independent experiments; the error bars represent the SEM. ND, not detected; FITC, fluorescein isothiocyanate; PE, phycoerythrin; GFP, green fluorescent protein; Bry, brachyury.

and GFP–Bry$^+$/c-kit$^-$ cells did not secrete FVIII at all, even after induction with Dox.

Discussion

ES cells capable of secreting human FVIII may represent a unique source for a future cell-based treatment protocol for hemophilia. In the present study, we were able to establish mouse ES cells secreting functional human FVIII with coagulant activity. Tet-WT-*F8* ES cells were established by integrating full-length human *F8* cDNA under the control of the tet operator, which enabled *F8* transcription to be induced by Dox stimulation. We found that levels of FVIII secretion depended on the conditions under which the ES cells were differentiated, regardless of *F8* mRNA expression. Among the conditions that we evaluated, those leading to development of endoderm/liver EBs were the most suitable for efficient FVIII secretion. Furthermore, the efficacy of FVIII secretion was dramatically improved by using 226aa/N6 cDNA, a recently described B-domain variant of *F8* [13]. To our knowledge, this is the first report of an ES/EB system that secretes detectable levels of active human FVIII *in vitro*.

We found it noteworthy that FVIII was present in the supernatant of liver-like EBs, but not in that of undifferentiated ES cells or hematopoietic-like EBs, although the induction of *F8* mRNA was detected in all conditions. It has previously been shown that the transcriptional activity of *F8* is not a critical determinant of plasma FVIII levels, and that mRNA levels are not, themselves, sufficient to predict FVIII secretion [22]. The primary FVIII translation product must be translocated into the lumen of the endoplasmic reticulum (ER), where folding and N-linked glycosylation occurs. Improperly folded FVIII molecules are recognized by chaperones and are not released, but are instead transferred into degradative pathways [23]. Our results indicate that cells with this capacity only appear during differentiation of liver-like EBs, making them more suitable for FVIII secretion than undifferentiated ES cells or hematopoietic-like EBs. Although liver-like EBs expressed hepatocyte-specific marker gene such as *Alb*, *Ttr* and *Tat*, mouse *F8* mRNA was not induced. The reason for this is currently unclear, but liver-like EBs may be still have an immature phenotype for endogeneous *F8* expression. Previous reports have demonstrated that platelets are good targets for the lentivirus-mediated gene therapy of FVIII production [24]. Our hematopoietic EBs were previously showed to contain megakaryocytes, but not platelets [11]. Thus, hematopoietic EBs probably fail to produce FVIII because of the immature differentiation of platelets from megakaryocytes.

We also observed that the 226aa/N6 construct is an extremely useful tool for FVIII production from ES cells. It is known that expression of BDD-*F8* results in a seventeen-fold increase in mRNA levels over WT-*F8*, although it yields only a 1.3-fold increase in the amount of secreted protein [22]. As the reason for imbalance between mRNA and protein levels, BDD-*F8* may have a defect in efficient transfer of the primary translation product from the ER to the Golgi via interaction with the 53-kDa ER–Golgi intermediate compartment protein [25]. To overcome this problem, Miao *et al.* [13] created another bioengineered construct, 226aa/N6. They showed that transfecting COS-1 and CHO cells with 226aa/N6 resulted in a 4–11-fold increase in FVIII secretion, as compared to transfection with BDD-*F8*. Consistent with those studies, we found that BDD-*F8* improved FVIII secretion only about 1.5-fold, as compared to WT-*F8*, whereas tet-226aa/N6 ES cells showed a ten-fold increase in FVIII secretion, as compared to tet-WT-*F8* ES cells. Thus 226aa/N6 appears to provide a significant advantage over BDD-*F8* with respect to FVIII production from ES cells, making it the optimal construct for FVIII secretion.

In our data, the levels of FVIII:C seem to be higher than those of FVIII:Ag, especially in tet-226aa/N6 ES cells. To investigate this discrepancy, we also assessed FVIII:C by a COAtest chromogenic assay (Chromogenix, Mölndal, Sweden) with recombinant FVIII as a standard. In this experiment, FVIII:C was detected at lower levels (about 40–50%) than that evaluated by plasma standard (data not shown). These results were in good accordance with previous reports that FVIII:C level against a plasma standard was higher than that against a recombinant FVIII standard by the chromogenic assay [26]. Thus, the discrepancy between FVIII:C and FVIII:Ag may result from the overestimation of FVIII:C by the APTT clotting assay with plasma standard.

Recently, Gouon-Evans *et al.* [20] demonstrated that the GFP–Bry$^+$/c-kit$^+$ cell population contained definitive endoderm progenitors when ES cells were differentiated in serum-free medium with activin stimulation. Using serum differentiation, we also found that the GFP–Bry$^+$/c-kit$^+$ cell population contained endoderm progenitors and that cells with the liver marker genes *Alb* and *Afp* appeared in this fraction. We further showed that cells differentiated from endoderm progenitor (GFP–Bry$^+$/c-kit$^+$) cells secreted FVIII more efficiently on day 14 of differentiation than presorted cells. By contrast, the sorted GFP–Bry$^-$ (ectoderm progenitor) and GFP–Bry$^+$/c-kit$^-$ (mesoderm progenitor) fractions secreted no FVIII, even after induction with Dox. These findings suggest that cells with the capacity for FVIII production are probably present within endoderm-derived tissue such as liver.

When we consider applying these strategies for human therapy, safety issues will be a big concern. An earlier study showed that grafts containing the undifferentiated ES cells rapidly form teratomas, even when only 0.2% of the cells within the transplanted clusters are positive for the undifferentiated marker SSEA-1 [27]. A recent study succeeded in transplanting ES-derived cardiomyocytes without evidence of teratoma formation in *in vivo* mouse models when selectable markers were employed to eliminate undifferentiated ES cells [28]. Thus, it will probably be necessary to develop a system involving selection markers in our tet-226aa/N6 ES cells for further *in vivo* studies.

In conclusion, we established ES cells secreting human FVIII with tetracycline regulation. The combination of endoderm progenitors, liver condition and 226aa/N6 cDNA could improve production to a significant level of human FVIII from ES cells. Our *in vitro* findings will be the first step for ES cell-based therapy as a potentially useful approach to the treatment of hemophilia A. Further *in vivo* studies are anticipated.

Addendum

S. Kasuda performed laboratory studies, data analysis and interpretation and drafted the manuscript. A. Kubo and Y. Sakurai designed the study, interpreted the data and drafted the manuscript. S. Irion and S. W. Pipe contributed vital new reagents and edited the manuscript. K. Ohashi and K. Tatsumi performed laboratory studies and edited the manuscript. Y. Nakajima, Y. Saito and K. Hatake helped to design the study, interpret the analyses and edit the manuscript. A. Yoshioka and M. Shima contributed critical analytical tools and data interpretation, and edited the manuscript.

Acknowledgements

We thank J. Kato and Y. Bessho for their excellent technical assistance and H. Kinoshita for critically reading the manuscript. We also thank G. Keller in the McEwen Centre for Regenerative Medicine (Toronto, Canada) for critically reading the manuscript and GFP–Bry Ainv18 ES cells. This work was supported in part by Health and Labour Sciences Research Grants for Research on HIV/AIDS from the Ministry of Health, Labour and Welfare (A. Yoshioka), a Bayer Hemophilia Award 2006 (M. Shima), the Mitsubishi Pharma Research Foundation (Y. Sakurai), the Leading Projects from the Scientific Research from the Ministry of Education, Science, Sport and Culture of Japan (K. Ohashi) and the Uehara Memorial Foundation (A. Kubo).

Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

References

1 Antonarakis SE. Molecular genetics of coagulation factor VIII gene and haemophilia A. *Haemophilia* 1998; **4**(Suppl. 2): 1–11.
2 Manco-Johnson M. Hemophilia management: optimizing treatment based on patient needs. *Curr Opin Pediatr* 2005; **17**: 3–6.
3 Lillicrap D, Vandendriessche T, High K. Cellular and genetic therapies for haemophilia. *Haemophilia* 2006; **12**(Suppl. 3): 36–41.
4 Sarkar R, Tetreault R, Gao G, Wang L, Bell P, Chandler R, Wilson JM, Kazazian HH Jr. Total correction of hemophilia A mice with canine FVIII using an AAV 8 serotype. *Blood* 2004; **103**: 1253–60.

5 Kaiser J. Gene therapy. Side effects sideline hemophilia trial. *Science* 2004; **304**: 1423–5.
6 Schuettrumpf J, Liu JH, Couto LB, Addya K, Leonard DG, Zhen Z, Sommer J, Arruda VR. Inadvertent germline transmission of AAV2 vector: findings in a rabbit model correlate with those in a human clinical trial. *Mol Ther* 2006; **13**: 1064–73.
7 Ko S, Tanaka I, Kanehiro H, Kanokogi H, Ori J, Shima M, Yoshioka A, Giles A, Nakajima Y. Preclinical experiment of auxiliary partial orthotopic liver transplantation as a curative treatment for hemophilia. *Liver Transpl* 2005; **11**: 579–84.
8 Ohashi K, Waugh JM, Dake MD, Yokoyama T, Kuge H, Nakajima Y, Yamanouchi M, Naka H, Yoshioka A, Kay MA. Liver tissue engineering at extrahepatic sites in mice as a potential new therapy for genetic liver diseases. *Hepatology* 2005; **41**: 132–40.
9 Kumaran V, Benten D, Follenzi A, Joseph B, Sarkar R, Gupta S. Transplantation of endothelial cells corrects the phenotype in hemophilia A mice. *J Thromb Haemost* 2005; **3**: 2022–31.
10 Kyba M, Perlingeiro RC, Daley GQ. HoxB4 confers definitive lymphoid-myeloid engraftment potential on embryonic stem cell and yolk sac hematopoietic progenitors. *Cell* 2002; **109**: 29–37.
11 Kubo A, Chen V, Kennedy M, Zahradka E, Daley GQ, Keller G. The homeobox gene HEX regulates proliferation and differentiation of hemangioblasts and endothelial cells during ES cell differentiation. *Blood* 2005; **105**: 4590–7.
12 Shibata M, Shima M, Morichika S, McVey J, Tuddenham EG, Tanaka I, Suzuki H, Nogami K, Minamoto Y, Hato T, Saenko EL, Scandella D, Yoshioka A. An alloantibody recognizing the FVIII A1 domain in a patient with CRM reduced haemophilia A due to deletion of a large portion of the A1 domain DNA sequence. *Thromb Haemost* 2000; **84**: 442–8.
13 Miao HZ, Sirachainan N, Palmer L, Kucab P, Cunningham MA, Kaufman RJ, Pipe SW. Bioengineering of coagulation factor VIII for improved secretion. *Blood* 2004; **103**: 3412–19.
14 Fehling HJ, Lacaud G, Kubo A, Kennedy M, Robertson S, Keller G, Kouskoff V. Tracking mesoderm induction and its specification to the hemangioblast during embryonic stem cell differentiation. *Development* 2003; **130**: 4217–27.
15 Kubo A, Shinozaki K, Shannon JM, Kouskoff V, Kennedy M, Woo S, Fehling HJ, Keller G. Development of definitive endoderm from embryonic stem cells in culture. *Development* 2004; **131**: 1651–62.
16 Kaufman RJ, Wasley LC, Dorner AJ. Synthesis, processing, and secretion of recombinant human factor VIII expressed in mammalian cells. *J Biol Chem* 1988; **263**: 6352–62.
17 Tada S, Era T, Furusawa C, Sakurai H, Nishikawa S, Kinoshita M, Nakao K, Chiba T, Nishikawa S. Characterization of mesendoderm: a diverging point of the definitive endoderm and mesoderm in embryonic stem cell differentiation culture. *Development* 2005; **132**: 4363–74.
18 Pittman DD, Alderman EM, Tomkinson KN, Wang JH, Giles AR, Kaufman RJ. Biochemical, immunological, and in vivo functional characterization of B-domain-deleted factor VIII. *Blood* 1993; **81**: 2925–35.
19 Toole JJ, Pittman DD, Orr EC, Murtha P, Wasley LC, Kaufman RJ. A large region (approximately equal to 95 kDa) of human factor VIII is dispensable for in vitro procoagulant activity. *Proc Natl Acad Sci USA* 1986; **83**: 5939–42.
20 Gouon-Evans V, Boussemart L, Gadue P, Nierhoff D, Koehler CI, Kubo A, Shafritz DA, Keller G. BMP-4 is required for hepatic specification of mouse embryonic stem cell-derived definitive endoderm. *Nat Biotechnol* 2006; **24**: 1402–11.
21 Barbera JPM, Clements M, Thomas P, Rodriguez T, Meloy D, Kioussis D, Beddington RSP. The homeobox gene Hex is required in definitive endoderm tissues for normal forebrain, liver and thyroid formation. *Development* 2000; **127**: 2433–45.
22 Pittman DD, Marquette KA, Kaufman RJ. Role of the B domain for factor VIII and factor V expression and function. *Blood* 1994; **84**: 4214–25.
23 Pipe SW, Morris JA, Shah J, Kaufman RJ. Differential interaction of coagulation factor VIII and factor V with protein chaperones calnexin and calreticulin. *J Biol Chem* 1998; **273**: 8537–44.
24 Shi Q, Wilcox DA, Fahs SA, Fang J, Johnson BD, Du LM, Desai D, Montgomery RR. Lentivirus-mediated platelet-derived factor VIII gene therapy in murine haemophilia A. *J Thromb Haemost* 2007; **5**: 352–61.
25 Pipe SW, Saint-Remy JM, Walsh CE. New high-technology products for the treatment of haemophilia. *Haemophilia* 2004; **10**(Suppl. 4): 55–63.
26 Hubbard AR, Bevan SA, Weller LJ. Potency estimation of recombinant factor VIII: effect of assay method and standard. *Br J Haematol* 2001; **113**: 533–6.
27 Fujikawa T, Oh SH, Pi L, Hatch HM, Shupe T, Petersen BE. Teratoma formation leads to failure of treatment for type I diabetes using embryonic stem cell-derived insulin-producing cells. *Am J Pathol* 2005; **166**: 1781–91.
28 Kolossov E, Bostani T, Roell W, Breitbach M, Pillekamp F, Nygren JM, Sasse P, Rubenchik O, Fries JW, Wenzel D, Geisen C, Xia Y, Lu Z, Duan Y, Kettenhofen R, Jovinge S, Bloch W, Bohlen H, Welz A, Hescheler J, *et al.* Engraftment of engineered ES cell-derived cardiomyocytes but not BM cells restores contractile function to the infarcted myocardium. *J Exp Med* 2006; **203**: 2315–27.

Original Article

Progression of Non-Culprit Coronary Artery Atherosclerosis After Acute Myocardial Infarction in Comparison with Stable Angina Pectoris

Satoshi Okayama, Shiro Uemura, Taku Nishida, Yoshinobu Morikawa, Kenji Onoue, Yasuhiro Takemoto, Tsunenari Soeda, Satoshi Somekawa, Ken-ichi Ishigami, Yukiji Takeda, Manabu Horii, Hiroyuki Kawata, Minoru Takaoka, Tamio Nakajima, and Yoshihiko Saito

First Department of Internal Medicine, Nara Medical University, Nara, Japan.

Aim: We previously found, using a mouse model, that activation of proinflammatory cytokines after acute myocardial infarction (AMI) augments neointimal hyperplasia of a remote artery. The present study assessed the progression of luminal narrowing of non-culprit coronary arteries (NCCA) in patients following AMI.

Methods: The study group comprised 21 AMI patients successfully treated with bare-metal stents and 16 stable angina (SA) patients treated with sirolimus-eluting stents. Clinical backgrounds were similar for both groups. Quantitative coronary angiography was performed before and after stent implantation and at 6-months of follow-up.

Results: We evaluated 126 non-culprit coronary segments (73 in AMI and 53 in SA). The minimum lumen diameter (MLD) (mm) of NCCA decreased significantly from 2.61±0.79 to 2.44±0.71 in the AMI group, but changed only slightly from 2.02±0.56 to 2.02±0.50 in the SA group. The absolute change in the MLD of NCCA was significantly greater (0.17±0.53) in the AMI, than in the SA (0.0070±0.261) group.

Conclusion: luminal narrowing of non-culprit coronary segments progressed in AMI patients within 6 months of stent implantation, but progressed only slightly in SA patients.

J Atheroscler Thromb, 2008; 15:228-234.

Key words; Quantitative coronary angiography, Bare-metal stent, Sirolimus-eluting stent, Inflammation

Introduction

Percutaneous coronary intervention (PCI), first performed in 1968 [1], has dramatically improved the outcome of treatment for coronary artery disease (CAD). Even conventional plain balloon angioplasty has significantly improved ischemia-related symptoms, but, periprocedural complications such as acute artery occlusion and chronic restenosis occur in approximately 5 and 40% of cases, respectively [2-4]. Bare-metal stent (BMS) implantation and ticlopidine administration have significantly decreased acute procedural complications, but have been unable to prevent chronic in-stent restenosis [5-8]. Sirolimus-eluting stent (SES) implantation significantly decreased the chronic in-stent restenosis rate to 5-7% through its anti-inflammatory effects, inhibiting several regulators of cell-cycle progression, and the migration of vascular smooth-muscle cells [9, 10]. Although SES implantation has the potential risk of late thrombosis after the stopping of antiplatelet therapy [11, 12], treatment of the culprit coronary artery is nearly perfect. However, for further improvements of the long-term prognosis in CAD patients, it became apparent that an appropriate treatment strategy for non-culprit coronary arteries is critical.

The genetic background [13], pathophysiology [14-18], and prognosis [19] of AMI considerably differ from those of SA. AMI is more associated with an enhanced

Address for correspondence: Shiro Uemura, First Department of Medicine, Nara Medical University 840 Shijo-cho, Kashihara, Nara 634-8522, Japan.
E-mail: suemur@naramed-u.ac.jp
Received: September 14, 2007
Accepted for publication: May 9, 2008

inflammatory response and vulnerable plaques than SA. Furthermore, subsequent coronary events including recurrent AMI and in-stent restenosis after PCI are more frequent in patients with AMI than with SA.

The plasma concentrations of various proinflammatory cytokines and growth factors, including tumor necrotic factor-α (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6), vascular endothelial growth factor (VEGF), and hepatocyte growth factor (HGF) are significantly elevated for approximately 2 weeks after onset[20, 21] in the setting of AMI, but only slightly elevated in that of SA. We previously found, using a mouse model, that the activation of proinflammatory cytokines after AMI augments the neointimal hyperplasia of a remote artery[22]. However, the augmentation of neointimal hyperplasia of non-culprit coronary arteries in patients after AMI has not been investigated in detail.

The present study evaluates the progression of luminal narrowing among non-culprit coronary arteries in patients after AMI.

Subjects and Methods

Subjects

We examined consecutive patients with AMI and SA who underwent initial coronary angiography, were successfully treated by stent implantation for significant coronary artery stenosis (>75% luminal narrowing), and were assessed by 6-month follow-up angiography between June 2004 and June 2005. Acute myocardial infarction was defined as typical chest pain at rest lasting >30 minutes accompanied by both ST-segment and/or T-wave elevation on electrocardiography (ECG), elevation of creatine kinase to twice the upper normal limit, and positive response of the cardiac troponin T rapid assay. Stable angina pectoris was defined as effort-related chest pain without any clinical changes in the preceding 2 months and negative cardiac troponin T. We performed PCI when indicated from the results of an exercise electrocardiography test and myocardial scintigraphy. We excluded the following patients to minimize the inflammatory response induced by stimulation other than AMI, SA, and the first coronary stenting procedure: (1) patients with in-hospital death after the primary procedure; (2) patients with AMI within 2 weeks of the primary procedure (to exclude potential subacute stent thrombosis of the treated arterial segment); (3) patients treated with a debulking device such as a rotablator or directional coronary atherectomy and (4) patients that underwent a second elective PCI, coronary artery bypass grafting, or other surgery within the 6-month follow-up period. We finally enrolled 21 AMI and 16 SA patients in this study.

All of the enrolled patients were systematically administered both aspirin (81-100 mg/day) and ticlopidine (200 mg/day). In the event of adverse reactions to ticlopidine, cilostazol (150-200 mg/day) was prescribed instead. None of the patients was receiving hormone replacement therapy. There was no cardiac event in any of the patients throughout the study. This protocol was approved by our institutional ethics committee (#2002-009), and was performed in accordance with the Helsinki Declaration of 1975.

Coronary Risk Factors

Peripheral venous blood samples were withdrawn from patients after at least a 12-h fast, in the morning, upon admission, and at 6-months follow-up to evaluate the following coronary risk factors: age, gender, body mass index (BMI), smoking habit, blood pressure (BP), glycated hemoglobin A1c (HbA1c), triglyceride (TG), total cholesterol (T-Cho), high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C). Levels of HbA1c were measured by high-performance liquid chromatography and those of TG, T-Cho, and HDL-C, by standard enzymatic methods. Levels of LDL-C were calculated using the Friedwald formula (T-cho − HDL-C − TG/5), because TG concentrations were <400 mg/dL in all patients. Hypertension was defined as repeated measurements ≥140 mmHg systolic BP and/or ≥90 mmHg diastolic BP and/or on permanent antihypertensive drug therapy. Dyslipidemia was defined as TG ≥150 mg/dL and/or HDL-C <40 mg/dL and/or LDL-C ≥140 mg/dL[23]. Diabetes mellitus (DM) was defined as a fasting blood glucose concentration of ≥126 mg/dL and/or HbA1c ≥6.5% and/or receiving antihyperglycemic drug therapy.

In all patients, coronary risk factors were treated according to Japanease studies and guidelines[24-26]. No restrictions were placed on the administration of medication.

Inflammatory Markers

Serial changes in inflammatory markers were determined in peripheral venous blood samples that were withdrawn from patients with AMI in the morning after at least a 12-h-fast. The white blood cell (WBC) count and the C-reactive protein (CRP) concentration were measured everyday until they reached peak values after stent implantation, using an automated hematology analyzer and an automated latex immunoturbidimetric assay, respectively. The WBC counts and CRP concentrations in the SA patients determined at the

first day after stent implantation were defined as the peak values respectively, when within normal ranges.

Cardiac Function

B-type natriuretic peptide (BNP) was measured and echocardiography was performed at 6-months of follow-up, to evaluate cardiac function. Venous plasma BNP concentrations were measured using a radioimmunoassay (Shionoria BNP kit, Shionogi Co Ltd, Osaka, Japan). Echocardiograms were obtained using an ACUSON Sequoia Ultrasound Imaging System (Siemens, Erlangen, Germany). Ejection fraction (EF) was calculated using the modified Sympson's rule[27].

Quantitative Coronary Angiography

We used the Judkins percutaneous femoral artery approach technique for coronary angiography. Multiple views of the right and left coronary arteries were routinely recorded on a full digital imaging system equipped with a flat-panel detector (AXIOM Artis FC/BC, Siemens, Erlangen, Germany; Allura Xper FD10, Philips, Eindhoven, the Netherlands). Angiographic images were obtained after the administration of 200 μg of intracoronary nitroglycerine.

Quantitative coronary angiographic (QCA) analysis was performed by a trained observer blind to the clinical background of subjects, using QCA-CMS software (MEDIS Medical Imaging Systems, Leiden, The Netherlands), in which coronary lumen diameters were measured using an automated edge-contour detection system. We evaluated both culprit and non-culprit coronary arteries. A culprit coronary artery was determined from ECG and echocardiographic findings, and the presence of stenosis and residual thrombosis in the artery. Non-culprit coronary arteries were defined as two coronary arteries (excluding the stented culprit coronary artery), and were limited to the proximal and mid coronary segments (AHA #1, 2, 3, 6, 7, 11, 12, 13). Angiographic images before (pre-procedure) and immediately after (post-procedure) stent implantation, as well as at the 6-month follow-up (chronic phase) were selected in the same view to clearly visualize narrowed lesions.

The lengths of the lesions were calibrated based on the image of the contrast-filled guiding catheter. The reference diameter (mm), minimal lumen diameter (MLD) (mm), and diametric stenosis (%) were measured in each segment of the culprit and non-culprit coronary arteries. The diametric stenosis was defined as [(reference diameter − MLD)/reference diameter] × 100. The diameter gained by treatment (acute gain = post-procedure MLD − pre-procedure MLD) (mm) and the luminal loss in the chronic phase (late loss = post-procedure MLD − chronic phase MLD) (mm) were determined in the culprit coronary artery. We also determined the absolute change in MLD (mm) and diametric stenosis (%) between pre-procedure and chronic phase in the non-culprit coronary arteries. A coronary diametric stenosis of >50% was considered clinically significant.

Table 1. Patient characteristics (baseline)

	AMI (n=21)	Stable angina (n=16)	p value
Age (years)	64.2 ± 11.1	66.8 ± 10.1	0.47
Men	17 (80.9%)	6 (37.5%)	<0.01
BMI (kg/m^2)	23.8 ± 2.6	23.8 ± 2.5	0.99
Past and current smoking	16 (76.1%)	9 (56.2%)	0.19
Hypertension	10 (47.6%)	14 (87.5%)	<0.05
Dyslipidemia	15 (71.4%)	13 (81.2%)	0.27
Diabetes mellitus	8 (38.0%)	6 (37.5%)	0.97

Values are presented as means ± standard deviations.
AMI = acute myocardial infarction, BMI = body mass index.

Statistics

Statistical analysis was performed using StatView 4.5 MDSU statistical software (Abacus Concept, Inc., Berkeley, CA, USA). Data are presented as the mean ± standard deviation (SD). Continuous quantitative data were compared using a matched Student's t test and discontinuous quantitative data were compared using the χ^2 test. Correlations between continuous variables were determined using a linear regression analysis. Statistical significance was established when $p<0.05$.

Results

Patient Characteristics

The baseline clinical background, excluding gender and hypertension, did not significantly differ between the two groups (**Table 1**). The clinical background at 6-months of follow-up, excluding systolic BP and EF on echocardiograms, was also similar between the two groups, and coronary risk factors were controlled well (**Table 2**). Systolic BP was significantly lower in patients with AMI than with SA, probably because the EF was significantly lower, and treatment with a angiotensin-converting enzyme inhibitor was somewhat more frequent in those with AMI.

Stenting Procedural Characteristics

Table 3 shows that the patients with AMI were implanted with a total of 22 BMS (BX velocity stent, 6; Duraflex stent, 6; Penta stent, 4; Driver stent, 3; Zeta

Table 2. Patient characteristics at 6-month follow-up

	AMI (n=21)	Stable angina (n=16)	p value
Current smoking	4 (19.0%)	2 (12.5%)	0.59
Systolic BP (mmHg)	120.5±14.2	131.3±16.6	<0.01
Diastolic BP (mmHg)	68.5±6.9	71.3±10.1	0.32
HbA1c (%)	5.5±0.63	5.6±0.70	0.76
TG (mg/dL)	130.5±59.2	102.9±29.8	0.097
T-Cho (mg/dL)	194.7±35.5	198.8±42.3	0.74
HDL-C (mg/dL)	52.8±16.0	59.6±16.5	0.22
LDL-C (mg/dL)	115.0±30.7	115.2±32.6	0.98
BNP (pg/mL)	95.9±191.7	59.2±68.2	0.48
Echocardiogram-EF (%)	55.9±10.4	68.6±6.5	<0.001
Medication			
ACE inhibitor	10 (47.6%)	3 (18.7%)	0.068
ARB	9 (42.8%)	7 (43.7%)	0.95
Statin	12 (57.1%)	11 (68.7%)	0.47
Fibrate	1 (4.7%)	0 (0%)	0.37
α-glucosidase inhibitor	3 (14.2%)	3 (18.7%)	0.66
Pioglitazone	0 (0%)	1 (6.2%)	0.24

Values are presented as means±standard deviations.
AMI=acute myocardial infarction, BP=blood pressure, HbA1c=glycated hemoglobin A1c, TG=triglyceride, T-Cho=total cholesterol, HDL-C=high-density lipoprotein cholesterol, LDL-C=low-density lipoprotein cholesterol, BNP=brain natriuretic peptide, ACE=angiotensin-converting enzyme, ARB=angiotensin II receptor blocker.

Table 3. Characteristics of stenting procedures

	AMI (n=21)	Stable angina (n=16)	p value
Total stent number	22	21	
Stent type			
Cypher	0	21	
BX velocity	6	0	
Duraflex	6	0	
Penta	4	0	
Driver	3	0	
Zeta	2	0	
S670	1	0	
Stent diameter (mm)	3.29±0.42	2.82±0.37	<0.001
Stent length (mm)	18.1±4.9	19.3±3.4	0.38
Pressure (atm)	16.0±4.6	16.4±3.58	0.75
Inflammation after PCI			
Peak WBC count ($\times 10^3/\mu L$)	12.1±2.8	6.1±2.0	<0.0001
Peak CRP concentration (mg/dL)	8.8±5.3	0.8±1.5	<0.0001

Values are presented as means±standard deviations.
AMI=acute myocardial infarction, PCI=percutaneous coronary intervention, WBC=white blood cell, CRP=C-reactive protein.

Table 4. Angiographic data for culprit lesions

	AMI (n=21)	Stable angina (n=16)	p value
Number of coronary artery stenoses			
1 vessel	19 (90.4%)	12 (75.0%)	0.40
2 vessels	2 (9.6%)	4 (25.0%)	
Location of stented coronary artery			
LAD	12	13	
LCX	1	3	0.33
RCA	9	5	
Reference diameter (mm)	3.02±0.59	2.20±0.31	<0.0001
MLD (mm)			
Pre-procedure	0.35±0.48	0.92±0.66	<0.01
Post-procedure	2.90±0.59	2.81±0.31	0.56
Chronic phase	1.96±0.78	2.57±0.35	<0.01
Acute gain	2.55±0.69	1.89±0.66	<0.01
Late loss	0.94±0.67	0.24±0.35	<0.001
Diametric stenosis (%)			
Pre-procedure	86.9±16.7	63.8±16.5	<0.0001
Post-procedure	10.9±8.0	10.4±5.3	0.8124
Chronic phase	31.7±21.5	9.6±5.4	<0.001

Values are presented as means±standard deviations.
AMI=acute myocardial infarction, LAD=left anterior descending artery, LCX=left circumflex artery, RCA=right coronary artery, MLD=minimal lumen diameter.

stent, 2; s670 stent, 1), and those with SA were implanted with a total of 21 SES. The procedural characteristics, excluding stent diameter, were similar between the two groups. Peak WBC counts and peak CRP concentrations after PCI were significantly higher in the AMI than SA group ($p<0.0001$, $p<0.0001$, respectively). Peak WBC counts and peak CRP concentrations in the SA group were within the normal ranges.

Coronary Artery Characteristics

Culprit Coronary Arteries

Table 4 shows the angiographic findings of culprit coronary arteries. The number of coronary artery stenoses and the location of stented coronary arteries were similar between the two groups. Most patients had single vessel stenosis because those with multivessel stenosis underwent elective PCI or coronary artery bypass grafting, and were excluded from the present study. The reference diameter was significantly larger in the AMI than SA group (3.02±0.59 vs. 2.20±0.31, $p<0.0001$), and pre-procedure MLD was significantly smaller in the AMI group (0.35±0.48 vs. 0.92±0.66, $p<0.01$). Post-procedure MLD was sufficiently large in both the groups, and did not significantly differ

Table 5. Angiographic data for non-culprit lesions

	AMI (n=21)	Stable angina (n=16)	p value
Total number of segments	73	53	
MLD (mm)			
Pre-procedure	2.61±0.78	2.02±0.56	<0.0001
Chronic phase	2.44±0.71	2.03±0.50	<0.001
Absolute change	0.17±0.53	0.0070±0.26	<0.05
Diametric stenosis (%)			
Pre-procedure	19.3±12.1	27.9±11.7	<0.0001
Chronic phase	23.4±14.9	23.1±10.9	0.92
Absolute change	4.85±18.9	1.58±15.0	<0.05

Values are presented as means ± standard deviations.
AMI = acute myocardial infarction, MLD = minimal lumen diameter.

between them (2.90±0.59 vs. 2.81±0.31, p=0.56). Chronic phase MLD was significantly smaller in the AMI than SA group (1.96±0.78 vs. 2.57±0.35, p< 0.01), and late loss was significantly larger in the AMI group (0.94±0.67 vs. 0.24±0.35, p<0.001).

Non-Culprit Coronary Arteries

Table 5 shows the angiographic findings of 126 non-culprit coronary segments (AMI, 73 segments; SA, 53 segments). Pre-procedure MLD (mm) was significantly larger in the AMI than SA group (2.61±0.78 vs. 2.02±0.56, p<0.0001). At the 6-month follow-up, the decrease in MLD was more frequent in the AMI (42 segments, 57.5%) than SA (25 segments, 47.1%) group. The frequency of an MLD decrease per patient was greater in the AMI (54.2±31.7%) than SA (44.0 ±36.1%) group, although this difference was not significant. The MLD (mm) decreased significantly from 2.61±0.79 to 2.44±0.71 in patients with AMI (p< 0.01), but changed only slightly from 2.02±0.56 to 2.02±0.50 in SA patients (p=0.84). The absolute change (mm) in MLD between pre-procedure and the chronic phase was significantly greater in the AMI than the SA group (0.17±0.53 vs. 0.0070±0.26, p<0.05).

Neither peak WBC count, peak CRP concentration, TG, T-Cho, HDL-C, nor LDL-C correlated with the MLD decrease in each group.

Discussion

Culprit Lesions

The reference diameter of the culprit lesion was significantly larger in the AMI than SA group, which is consistent with the findings of Hong *et al.*[28]. Late loss (mm) was 0.94±0.67 after BMS implantation in the AMI group, and 0.24±0.35 after SES implantation in the SA group. These findings were compatible with those of others[5-10] and support the accuracy of the QCA used in the present study.

Non-Culprit Lesions

The present results support the notion that the activation of proinflammatory cytokines after AMI augments the neointimal hyperplasia of remote arteries. In patients with AMI whose WBC count and CRP concentration were elevated, the MLD (mm) of non-culprit coronary arteries significantly decreased from 2.61±0.79 to 2.44±0.71 within 6 months after stent implantation (p<0.01), whereas the change was minimal in SA patients without such elevations (from 2.02 ±0.56 to 2.02±0.50; p=0.84).

We used the WBC count and CRP concentration as inflammatory markers because of the absence of surplus blood samples, although we previously reported that activated TNF-α and IL-6 augment neointimal hyperplasia[22]. Hasegawa *et al.* reported that the WBC count is closely related to other conventional coronary risk factors and is an important indicator of chronic inflammation[29]. The CRP concentration becomes elevated after balloon angioplasty[30] and stent implantation[31], and predicts in-stent restenosis within 12-months follow-up[32]. Therefore, the WBC count and the CRP concentration are considered appropriate for evaluating inflammation after AMI. Hong *et al.* compared coronary plaque rupture between AMI and SA using three-vessel intravascular ultrasound[28], and reported that both non-culprit and culprit coronary plaque rupture are more frequent in patients with AMI than with SA. Plaque rupture in AMI patients was associated with a high CRP concentration, whereas that in SA patients was associated with diabetes. These findings are consistent with those of the present study, suggesting that inflammation after AMI increases the vulnerability of non-culprit arteries to atheromatous plaques. Despite the reduction in in-stent restenosis rates by SES, this does not demonstrate an improvement of long-term prognosis[10, 12]. One cause of this discrepancy might be the progression of atherosclerosis in non-culprit arteries. For the reasons stated above, careful management of non-culprit coronary arteries is critical to further improve the long-term prognosis of patients with AMI.

In contrast, systemic effects of the elution of sirolimus remain controversial. An implanted SES was reportedly associated with exercise-induced paradoxical coronary vasoconstriction of the adjacent vessel segments[33]. Furthermore, the implantation of SES suppressed, whereas the implantation of BMS induced, the mobilization and differentiation into both smooth

muscle-like and endothelium-like cells of bone marrow cells, possibly leading to restenosis[34]. However, the reported peak sirolimus concentration after SES implantation is approximately 0.86-2.00 ng/mL, far lower than the effective concentration when sirolimus is orally administered as an immunosuppressive agent[35].

In the present study, SA patients received SES, and AMI patients received BMS. Therefore, systemic effects after SES implantation might have inhibited atherosclerosis in non-culprit coronary arteries.

Study Limitations

The present study has four potential limitations. First, we retrospectively analyzed a relatively small sample of patients. Second, we routinely use 4F coronary catheters for coronary angiography to minimize the invasive effect on patients. The use of small catheters as a calibration standard may induce measurement bias for QCA. Third, QCA cannot clarify the cause of luminal narrowing in non-culprit coronary arteries. Ehara *et al.* reported that plaque morphology and pathology are closely related to progression characteristics[18]. Fourth, we used the peak WBC count and peak CRP concentration as inflammatory markers, although they are non-specific in this context. Moreover, we could not calculate the area under the curve (AUC) for these markers. Thus, we could indicate that the inflammation is related to the progression of non-culprit coronary artery atherosclerosis, but could not clarify whether the degree of inflammation is related to the progression and/or severity of the atherosclerosis.

Finally, our results should be confirmed by further prospective studies with large cohorts using specific inflammatory markers and other imaging methods, such as coronary computed tomography, cardiac magnetic resonance imaging, intravascular ultrasound (IVUS), and optical coherence tomography (OCT).

Conclusion

Within 6 months of stent implantation, luminal narrowing of non-culprit coronary segments had clearly progressed in patients with AMI, but progressed minimally in patients with SA.

Acknowledgments and Notice of Grant Support

None to declare.

References

1) Gruntzig AR, Senning A, and Siegenthaler WE: Nonoperative dilatation of coronary -artery stenosis: percutaneous transluminal coronary angioplasty. N Engl J Med, 1979; 301:61-68
2) Gruentzig AR, King SB 3rd, Schlumpf M, and Siegenthaler W: Long-term follow-up after percutaneous transluminal coronary angioplasty. The early zurich experience. N Engl J Med, 1987; 316:1127-1132
3) Simpfendorfer C, Belardi J, Bellamy G, Galan K, Franco I, and Hollman J: Frequency, management and follow-up of patients with acute coronary occlusion after percutaneous transluminal coronary angioplasty. Am J Cardiol, 1987; 59:267-269
4) Nobuyoshi M, Kimura T, Nosaka H, Mioka S, Ueno K, Yokoi H, Hamasaki N, Horiuchi H, and Ohishi H: Restenosis after successful percutaneous transluminal coronary angioplasty: serial angiographic follow-up of 229 patients. J Am Coll Cardiol, 1988; 12:616-623
5) Fischman DL, Leon MB, Baim DS, Schatz RA, Savage MP, Penn I, Detre K, Veltri L, Ricci D, Nobuyoshi M, Cleman M, Heuser R, Almond D, Teirstein PS, Fish RD, Colombo A, Brinker J, Moses J, Shaknovich A, Hirshfeld J, Bailey S, Ellis S, Ranke R, and Goldberg S: A randomized comparison of coronary-stent placement and balloon angioplasty in the treatment of coronary artery disease. Stent Restenosis Study Investigators. N Engl J Med, 1994; 331:496-501
6) Serruys PW, de Jaegere P, Kiemeneij F, Macaya C, Rutsch W, Heyndrickx G, Emanuelsson H, Marco J, Legrand V, Materne P, Belardi J, Sigwart U, Colombo A, Goy JJ, Heuvel PVD, Delcan J, and Morel MA: A comparison of balloon-expandable-stent implantation with balloon angioplasty in patients with coronary artery disease. Benestent Study Group. N Engl J Med, 1994; 331:489-495
7) Schömig A, Neumann FJ, Kastrati A, Schühlen H, Blasini R, Hadamitzky M, Walter H, Zitzmann-Roth EM, Richardt G, Alt E, Schmitt C, and Ulm K: A randomized comparison of antiplatelet and anticoagulant therapy after the placement of coronary-artery stents. N Engl J Med, 1996; 334:1084-1089
8) Leon MB, Baim DS, Popma JJ, Gordon PC, Cutlip DE, Ho KK, Giambartolomei A, Diver DJ, Lasorda DM, Williams DO, Pocock SJ, and Kuntz RE: A clinical trial comparing three antithrombotic-drug regimens after coronary-artery stenting. Stent Anticoagulation Restenosis Study Investigators. N Engl J Med, 1998; 339:1665-1671
9) Moses JW, Leon MB, Popma JJ, Fitzgerald PJ, Holmes DR, O'Shaughnessy C, Caputo RP, Kereiakes DJ, Williams DO, Teirstein PS, Jaeger JL, and Kuntz RE: SIRIUS Investigators. Sirolimus-eluting stents versus standard stents in patients with stenosis in a native coronary artery. N Engl J Med, 2003; 349:1315-1323
10) Morice MC, Serruys PW, Sousa JE, Fajadet J, Ban Hayashi E, Perin M, Colombo A, Schuler G, Barragan P, Guagliumi G, Molnàr F, and Falotico R: RAVEL Study Group. Randomized Study with the Sirolimus-Coated Bx Velocity Balloon-Expandable Stent in the Treatment of Patients with de Novo Native Coronary Artery Lesions. A randomized comparison of a sirolimus-eluting stent with a standard stent for coronary revascularization. N Engl J Med, 2002; 346:1773-1780

11) McFadden EP, Stabile E, Regar E, Cheneau E, Ong AT, Kinnaird T, Suddath WO, Weissman NJ, Torguson R, Kent KM, Pichard AD, Satler LF, Waksman R, and Serruys PW: Late thrombosis in drug-eluting coronary stents after discontinuation of antiplatelet therapy. Lancet, 2004; 364:1519-1521
12) Stone GW, Moses JW, Ellis SG, Schofer J, Dawkins KD, Morice MC, Colombo A, Schampaert E, Grube E, Kirtane AJ, Cutlip DE, Fahy M, Pocock SJ, Mehran R, and Leon MB: Safety and efficacy of sirolimus- and paclitaxel-eluting coronary stents. N Engl J Med, 2007; 356:1059-1060
13) Hon-Kan Yip, Pei-wen Wang, Li-Teh Chang, Ali A. Youssef, Jiunn-Jye Sheu, Fan-Yen Lee, and Chiung-Jen Wu: Cytotoxic T lymphocyte antigen 4 gene polymorphism associated with ST-segment elevation acute myocardial infarction. Circ J, 2007; 71:1213-1218
14) Wojakowski W, Maslankiewicz K, Ochala A, Wyderka R, Zuk-Popiolek I, Flak Z, Mroz I, and Tendera M: The pro- and anti-inflammatory markers in patients with acute myocardial infarction and chronic stable angina. Int J Mol Med, 2004; 14:317-322
15) Ashida K, Miyazaki K, Takayama E, Tsujimoto H, Ayaori M, Yakushiji T, Iwamoto N, Yonemura A, Isoda K, Mochizuki H, Hiraide H, Kusuhara M, and Ohsuzu F: Characterization of the expression of TLR2 (toll-like receptor 2) and TLR4 on circulating monocytes in coronary artery disease. J Atheroscler Thromb, 2005; 12:53-60
16) Zhou RH, Shi Q, Gao HQ, and Shen BJ: Changes in serum interleukin-8 and interleukin-12 levels in patients with ischemic heart disease in a Chinese population. J Atheroscler Thromb, 2001; 8:30-32
17) Kawamura A, Miura S, Murayama T, Iwata A, Nishikawa H, Matsuo Y, Tsuchiya Y, Matsuo K, Tsuji E, and Saku K: Changes in the expression of leukocyte adhesion molecules throughout the acute phase of myocardial infarction. J Atheroscler Thromb, 2004; 11:29-34
18) Ehara S, Kobayashi Y, Yoshiyama M, Ueda M, and Yoshikawa J: Coronary artery calcification revisited. J Atheroscler Thromb, 2006; 13:31-37
19) Chen L, Chester MR, Crook R, and Kaski JC: Differential progression of complex culprit stenoses in patients with stable and unstable angina pectoris. J Am Coll Cardiol, 1996; 28:597-603
20) Frangogiannis NG, Smith CW, and Entman ML: The inflammatory response in myocardial infarction. Cardiovasc Res, 2002; 53:31-47
21) Aoki M, Morishita R, Taniyama Y, Kaneda Y, and Ogihara T: Therapeutic angiogenesis induced by hepatocyte growth factor: potential gene therapy for ischemic diseases. J Atheroscler Thromb, 2000; 7:71-76
22) Takaoka M, Uemura S, Kawata H, Imagawa K, Takeda Y, Nkatani K, Naya N, Horii M, Yamano S, Miyamoto Y, Yoshimasa Y, and Saito Y: Inflammatory response to acute myocardial infarction augments neointimal hyperplasia after vascular injury in a remote artery. Arterioscler Thromb Vasc Biol, 2006; 26:2083-2089
23) Teramoto T, Sasaki J, Ueshima H, Egusa G, Kinoshita M, Shimamoto K, Daida H, Biro S, Hirobe K, Funahashi T, Yokote K, and Yokode M; Japan Atherosclerosis Society (JAS). Committee for Epidemiology and clinical Management of Atherosclerosis: Diagnostic criteria for dyslipidemia. Executive summary of Japan Atherosclerosis Society (JAS) guideline for diagnosis and prevention of atherosclerotic cardiovascular diseases for Japanese. J Atheroscler Thromb, 2007; 14:155-158
24) Kinoshita M, Ishikawa K, Inoue H, Sakakibara H, Kanmatsuse K, Saito Y, Nonogi H, Hiramori K, Suzuki T, Nakamura Y, Katagiri T, Shirato K, Fujita M, Maruyama Y, and Miura M: Guidelines for secondary prevention of myocardial infarction. Jpn Circ J, 2000; 65 Supple. IV:863-867
25) Saito Y, Shirai K, Sasaki N, Shinomiya M, and Yoshida S: Prognosis of hypercholesterolemic patients taking pravastatin for five years: The Chiba lipid intervention program (CLIP) study. J Atheroscler Thromb, 2002; 9:99-108
26) Koizumi J, Shimizu M, Miyamoto S, Origasa H, and Mabuchi H: Effect of pravastatin-induced LDL-cholesterol reduction on coronary heart disease and cerebrovascular disease in Japanese: Hokuriku lipid coronary heart disease study-pravastatin atherosclerosis trial (Holicos-PAT). J Atheroscler Thromb, 2002; 9:251-259
27) Parisi AF, Moynihan PF, Feldman CL, and Folland ED: Approaches to determination of left ventricular volume and ejection fraction by real-time two-dimensional echocardiography. Clin Cardiol, 1979; 2:257-263
28) Hong MK, Mintz GS, Lee CW, Kim YH, Lee SW, Song JM, Han KH, Kang DH, Song JK, Kim JJ, Park SW, and Park SJ: Comparison of coronary plaque rupture between stable angina and acute myocardial infarction: A three-vessel intravascular ultrasound study in 235 patients. Circulation, 2004; 110:928-933
29) Hasegawa T, Negishi T, and Deguchi M: WBC count, atherosclerosis and coronary risk factors. J Atheroscler Thromb, 2002; 9:219-223
30) Azar RR, McKay RG, Kiernan FJ, Seecharran B, Feng YJ, Fram DB, Wu AH, and Waters DD: Coronary angioplasty induces a systemic inflammatory response. Am J Cardiol, 1997; 80:1476-1478
31) Gaspardone A, Crea F, Versaci F, Tomai F, Pellegrino A, Chiariello L, and Gioffré PA: Predictive value of C-reactive protein after successful coronary-artery stenting in patients with stable angina. Am J Cardiol, 1998; 82:515-518
32) Angioi M, Abdelmouttaleb I, Rodriguez RM, Aimone-Gastin I, Adjalla C, Guéant JL, and Danchin N: Increased C-reactive protein levels in patients with in-stent restenosis and its implications. Am J Cardiol, 2001; 87:1189-1193
33) Togni M, Windecker S, Cocchia R, Wenaweser P, Cook S, Billinger M, Meier B, and Hess OM: Sirolimus-eluting stents associated with paradoxic coronary vasoconstriction. J Am Coll Cardiol, 2005; 46:231-236
34) Inoue T, Sata M, Hikichi Y, Sohma R, Fukuda D, Uchida T, Shimizu M, Komoda H, and Node K: Mobilization of CD34-positive bone marrow-derived cells after coronary stent implantation. Impact on restenosis. Circulation, 2007; 115:553-561
35) Otsuka Y, Nakamura M, Yasuda S, Kozuma K, Hara H, Morii I, Kawamura A, Sase K, and Miyazaki S: Comparison of pharmacokinetics of the sirolimus-eluting stent in Japanese patients with those in American patients. J Cardiovasc Pharmacol, 2005; 46:468-473

ELSEVIER

Clinical Neurology and Neurosurgery 110 (2008) 500–501

Clinical Neurology and Neurosurgery

www.elsevier.com/locate/clineuro

Case Report

Odor abnormalities caused by bilateral thalamic infarction

Hirohide Asai [a,*], Fukashi Udaka [b], Makito Hirano [a], Satoshi Ueno [a]

[a] *Department of Neurology, Nara Medical University, 840 Shijo-cho, Kashihara, Nara 634-8522, Japan*
[b] *Department of Neurology, Sumitomo Hospital, Japan*

Received 12 July 2007; received in revised form 23 October 2007; accepted 20 January 2008

Abstract

Odor is the only sensation thought to be unrelated to the thalamus. However, accumulating evidence suggests that the dorsomedial nucleus (DM) of the thalamus is associated with odor. Although the thalamus is prone to ischemia, only a single patient with bilateral DM infarctions was reported to have odor abnormalities. We describe a second such patient with infarctions involving the left DM and the right ventral posterior nucleus and ventral lateral nucleus, nuclei adjacent to the DM, associated with transient edema. In contrast to the previous case, our patient had transient odor abnormality. These observations suggested that direct and/or indirect bilateral involvement of the DM might be associated with odor abnormalities in patients with thalamic infarction.

Keywords: Thalamic infarction; Odor abnormalities; Dorsomedial nucleus; Ventral posterior nucleus; Ventral lateral nucleus; Brain MRI

1. Introduction

Odor is the only sensation thought to be unrelated to the thalamus [1], since odor information is relayed via and likely modulated by the olfactory bulb instead of the thalamus [2–4]. However, electric regional stimulation has shown that the dorsomedial nucleus (DM) of the thalamus receives inputs from the olfactory bulbs and sends outputs to the odor perception cortex [5]. Unilateral involvement of the DM by tumor has been associated with ipsilaterally impaired odor discrimination [3]. These facts suggest that odor perception is associated with the thalamus. Although the thalamus is prone to ischemia, only a single patient with thalamic infarction was reported to have odor abnormalities [6]. We describe a second such patient, suggesting a topographic association of the thalamus with odor abnormalities.

2. Case report

A 71-year-old man was admitted because of sudden left hemiparesthesia. He had a history of diabetes mellitus, hyperlipidemia, and hypertension. Consciousness disturbances, dementia, oculomotor impairment, muscle weakness, ataxia, and otorhinolaryngological symptoms were absent. Although no objective odor testing was performed, an intravenous thiamine injection test showed normal findings, indicating that simple odor perceptions were retained. However, he had nausea and aversion on smelling any food. The taste was altered in all flavors. Odor and taste abnormalities were not lateralized. He hardly ate anything, and received intravenous fluid replacement for 2 weeks. Brain MRI on admission revealed acute bilateral infarctions only in the thalamus, including the right ventral posterior nucleus (VP), ventral lateral nucleus (VL), and left DM (Fig. 1) [7]. Oral aspirin was started. Odor and taste abnormalities gradually disappeared in 2 weeks. Two months later, diffusion-weighted MRI abnormalities were no longer detected.

3. Discussion

Our patient had odor abnormalities associated with bilateral thalamic infarctions involving the left DM and the right VP and VL. The DM sends outputs to the cingulate gyrus, a region closely related to emotion and memory [8]. Spinella suggested the involvement of the DM in emotional odor cognition [9]. The previous patient with odor abnormalities had bilateral DM infarctions. These findings suggested that

* Corresponding author. Tel.: +81 744 22 3051; fax: +81 744 24 6065.
E-mail address: asaihozon@yahoo.co.jp (H. Asai).

0303-8467/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.clineuro.2008.01.008

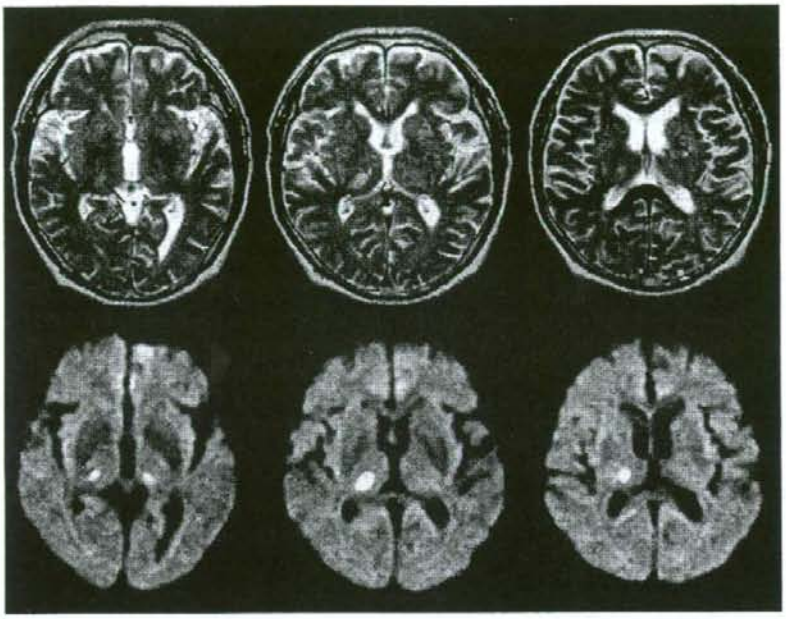

Fig. 1. MRI T2-weighted images (upper images, TR/TE = 4000/120 ms) and diffusion-weighted images (lower images, TR/TE = 3800/96, $b = 1000$ s/mm^2) obtained with a 1.5T Signa EXCIT HD system (GE Medical Systems, Milwaukee, Wis) showed bilateral hyperintensity in the thalami. Three-dimensional reconstruction using the Schaltenbrand and Wahren atlas (see Ref. [7]) showed that the lesions were located in the right ventral posterior and ventral lateral nuclei and the left dorsomedial nucleus.

DM involvement was associated with the odor abnormalities in our patient.

Clinically, the duration and symptoms of odor abnormalities and the locations of thalamic lesions differed between our patient and the previous patient. The latter (with bilateral DM infarctions) initially had odor deficit and later prolonged (>2 years) abnormalities in odor character, while our patient had sudden-onset, transient (<2 weeks) abnormalities in odor character. Infarction in the VP and VL of our patient was accompanied by MRI-proven transient cytotoxic edema, which probably transiently affected adjacent nuclei, including the DM. Thus, transient bilateral DM involvement may have occurred because of unilateral DM infarction and edema-induced contralateral DM dysfunction. The observed clinical differences may be partly attributed to the duration of bilateral DM involvement.

An important question is why odor abnormalities are rare although bilateral thalamic infarction is often associated with occlusion of the paramedian artery [10]. One reason is that odor evaluation is often precluded by consciousness or mental disturbances in such patients. Alternatively, mild odor deficit might be overlooked [11].

In summary, bilateral involvement of the DM without consciousness or mental disturbances may be associated with odor abnormalities in patients with thalamic infarction.

References

[1] Tanabe T, Yarita H, Iino M, Ooshima Y, Takagi SF. An olfactory projection area in orbitofrontal cortex of the monkey. J Neurophysiol 1975;38:1269–83.

[2] Potter H, Butters N. An assessment of olfactory deficits in patients with damage to prefrontal cortex. Neuropsychologia 1980;18:621–8.

[3] Shepherd GM. Perception without a thalamus how does olfaction do it? Neuron 2005;46:166–8.

[4] Kay LM, Sherman SM. An argument for an olfactory thalamus. Trends Neurosci 2007;30:47–53.

[5] Jackson JC, Benjamin RM. Unit discharges in the mediodorsal nucleus of the rabbit evoked by electrical stimulation of the olfactory bulb. Brain Res 1974;26:193–201.

[6] Rousseaux M, Muller P, Gahide I, Mottin Y, Romon M. Disorders of smell, taste, and food intake in a patient with a dorsomedial thalamic infarct. Stroke 1996;27:2328–30.

[7] Schaltenbrand G, Wahren W. Atlas for stereotaxy of the human brain. Stuttgart, Germany: Georg Thieme Publishers; 1977.

[8] Clark DL, Boutros NN. The brain and behavior: an introduction to behavioral neuroanatomy. Maldan: Blackwell Science; 1999. pp. 119–29.

[9] Spinella M. Correlations among behavioral measures of orbitofrontal function. Int J Neurosci 2002;112:1359–69.

[10] Bogousslavsky J, Regli F, Uske A. Thalamic infarcts: clinical syndromes, etiology, and prognosis. Neurology 1988;38:837–48.

[11] Heckmann JG, Heckmann SM, Lang CJ, Hummel T. Neurological aspects of taste disorders. Arch Neurol 2003;60:667–71.

INTERNAL MEDICINE

□ CASE REPORT □

Cerebral Infarction Associated with Heparin-Induced Thrombocytopenia in a Patient with Encephalitis

Kozue Saito[1], Makito Hirano[1], Miyuki Kajitani[1], Toshiaki Taoka[2], Kimihiko Kichikawa[2] and Satoshi Ueno[1]

Abstract

We report a patient who had cerebral infarction associated with heparin-induced thrombocytopenia (HIT) during treatment of aseptic encephalitis. In patients with intracranial inflammation, such as ours, the possibility of HIT has to be considered when heparin is used, since inflammatory cerebral lesions often cause vascular damage, which is an aggravating factor for HIT-associated thrombosis.

Key words: cerebral infarction, encephalitis, heparin-induced thrombocytopenia

(Inter Med 48: 71-74, 2009)
(DOI: 10.2169/internalmedicine.48.1486)

Heparin-induced thrombocytopenia (HIT) is a life-threatening thrombotic disorder caused by antibodies (HIT-Ab) against a complex of heparin and platelet factor 4 (1). Thrombotic complications include cerebral infarction and peripheral deep vein thrombosis (DVT), often occurring 5-15 days after the start of heparin (2). Encephalitis occasionally involves cerebral arteries, leading to cerebral infarction (2). However, whether heparin use triggers cerebral infarction is unknown. We report an encephalitis patient with HIT-associated cerebral infarction.

Case Report

A 73-year-old man was admitted to a local hospital because of headache, drowsiness, and mild left hemiparesis including the facial muscles, without apparent sensory disturbance. Abnormal MRI findings (Fig. 1A, B) initially suggested cerebral infarction, and he was given heparin (10,000 IU/day) for 2 days. He had no risk factors for cerebral infarction, including hypertension, hyperlipidemia, diabetes mellitus, hereditary coagulation disorders (Protein C, Protein S, AT III deficiencies), and cardiac diseases; there was no evidence of cerebral infarction on cerebral angiography (Fig. 1C). The next day, pleocytosis (187/mm^3, 58% lymphocytes), elevated protein (159 mg/dL), and normal glucose in the CSF suggested the diagnosis of encephalitis. Heparin was stopped. The patient was transferred to our hospital on day 4. He had almost normal blood chemistry and cell counts, including a normal platelet count (192×10^3/μL). Anticoagulation testing revealed slightly elevated levels of D-dimer (6.8 μg/mL [normal <1.0 μg/mL]) and FDP (11.6 μg/mL [normal <10 μg/mL]), but normal PT and APTT levels. On CSF testing, Herpes simplex and Varicella zoster DNA were negative. Serum antibody titers excluded infection with various bacteria, fungi, and viruses. No organisms (including *Mycobacterium tuberculosis*) were cultured from the CSF. Serum auto-antibodies against the nucleus, ds-DNA, cardiolipin, galactose, RNP, Sm, SSA, SSB, and neutrophil cytoplasm were negative. These findings suggested that the patient had aseptic encephalitis. On day 8, total parenteral nutrition was started, and heparin was flushed (100 IU) once daily to maintain central catheter patency. The patient gradually recovered consciousness so that he was able to eat meals by himself, and the CSF abnormalities improved (37 cells/mm^3 and 124 mg protein/dL) on day 21. On day 23, he suddenly became semicomatose and developed right hemiplegia with total aphasia. A brain diffusion-weighted image (DWI) showed high intensity in the left basal ganglia and corona radiata (Fig. 1D, E). On MRA, the main trunk of the left middle cerebral artery was occluded (Fig. 1F). Electrocardiography (ECG) including bedside ECG monitoring showed normal sinus rhythm during the entire disease

[1]Department of Neurology, Nara Medical University, School of Medicine, Kashihara and [2]Department of Radiology, Nara Medical University, School of Medicine, Kashihara
Received for publication July 8, 2008; Accepted for publication September 16, 2008
Correspondence to Dr. Kozue Saito, ksaito@naramed-u.ac.jp

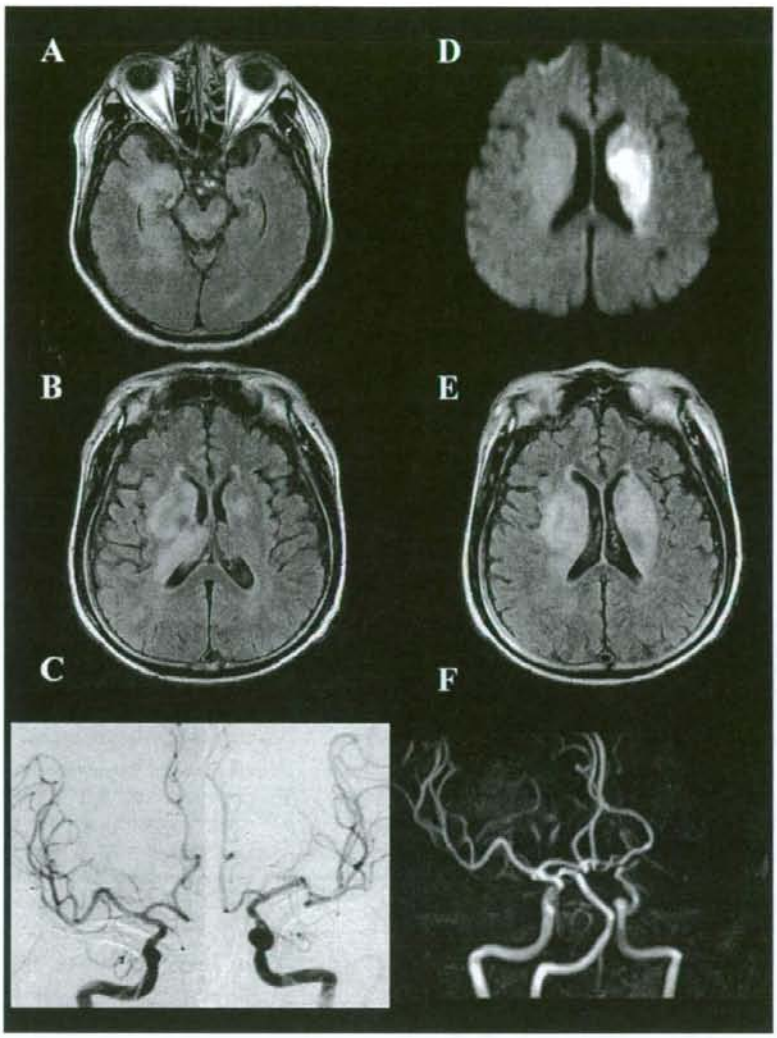

Figure 1. **Radiological findings before (A-C) and after (D-F) cerebral infarction associated with HIT. (A, B) FLAIR images (TE /TR=124/9000, 1.5-T whole-body magnetic resonance system, Magnetom Sonata A.G., Siemens, Erlangen, Germany) obtained 9 days after disease onset (day 9) show hyperintense lesions in the right hippocampus, temporal lobe, and corona radiata. (C) Cerebral angiography done 3 days after disease onset shows no stenosis or occlusion in bilateral internal carotid arteries. (D) DWI (TR/TE=180/96, b=1,000 s/mm^2, matrix 128×128, field of view 230 mm) on day 23 shows a hyperintense lesion in the left corona radiata. (E) A FLAIR image on day 23 shows a hyperintense lesion in the corona radiata bilaterally. (F) Brain MRA on day 23 shows occlusion of the main trunk of the left middle cerebral artery.**

course; transthoracic and transesophageal echocardiography revealed no cardiac thrombus, patent foramen ovale, or complicated aortic arch lesions. The platelet count remained normal (211×10^3/μL). On day 24, intravenous continuous heparin injection (8,000 IU/day) was resumed. On day 30, the platelet count decreased to 9.9×10^3/μL, with an extremely high D-dimer level (42.4 μg/mL) (Fig. 2). Venous ultrasonography showed DVT in the right popliteal vein on day 32. Since these findings suggested the possibility of HIT, we stopped heparin and started argatroban on day 34. HIT-Ab was subsequently found to be positive in the blood sample obtained at the onset of cerebral infarction (OD_{405}=0.652, normal <0.4, enzyme-linked immunosorbent assay GTI-PF4, Genetic Testing Institute, Waukesha WI, USA). According to a previous report, the clinical probability score at the onset of stroke was 4 points, indicating a intermediate probability

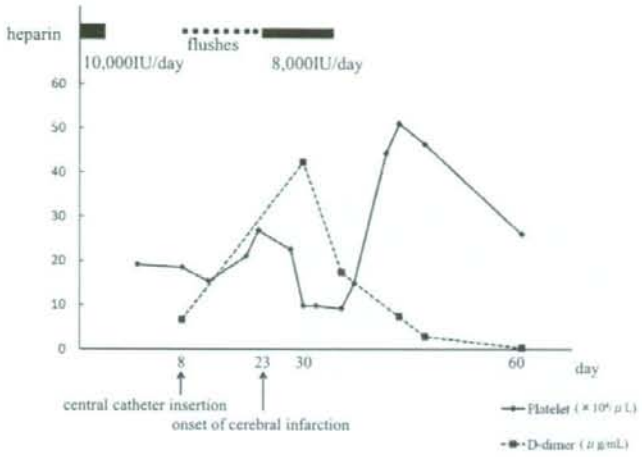

Figure 2. Time course of the platelet count and the D-dimer level with heparin use.

of HIT (low, 0-3; intermediate, 4-5; and high, 6-8) (3), and the score at the onset of thrombocytopenia increased to 6 points (high probability). On the other hand, the disseminated intravascular coagulation (DIC) score was 4 points, indicating a low probability of DIC. Argatroban therapy normalized the platelet count and the D-dimer level with negative HIT-Ab (OD_{405}=0.177), and the patient's condition improved within two weeks. Resolution of DVT seen on follow-up venous ultrasonography on day 56 also supported the effectiveness of argatroban.

Discussion

We report a patient with encephalitis and HIT, the combination of which has not been previously described. Even though encephalitis can cause cerebral infarction or inflammatory lesions with similar neurological deficits, the symptoms our patient which developed on day 23 were likely due to HIT-associated cerebral infarction for several reasons. First, HIT-Ab was positive with severe thrombosis and decreased platelets, and these findings were resolved by discontinuation of heparin and administration of argatroban. Second, the improved CSF findings and clinical recovery at the onset of cerebral infarction seemed to rule out aggravation of encephalitis. Furthermore, while inflammatory-associated cerebral infarction often occurs in the territories of small arteries, our patient had a new lesion in the main trunk of the left middle cerebral artery, with a high intensity DWI lesion matching the vascular territory. Development of cerebral infarction 7 days before thrombocytopenia in our patient also does not rule out HIT, since recent studies demonstrated that thrombocytopenia is often absent at the onset of thromboembolic complications and, sometimes, may be absent during the entire disease course (4). We cannot exclude the possibility that the HIT-associated cerebral infarction occurred independently of the encephalitis; however, the lack of other causes of vascular damage suggests that encephalitis may trigger or predispose to HIT-associated infarction.

The pathophysiological mechanism by which encephalitis causes HIT-associated cerebral infarction remains unknown. However, in encephalitis, vascular damage in cerebral arteries involves leukocyte infiltration into arterial walls (5). These activated leukocytes release chemokines, such as CCL 17 and CCL22, which may subsequently activate platelets, thereby releasing platelet factor 4 to form a complex with HIT-Ab and heparin (6). Thus, inflammation-mediated vascular damage in encephalitis may trigger thrombosis and subsequent HIT-associated cerebral infarction (6).

Some issues and limitations related to the present case report need to be considered. First, the dose of heparin that triggered HIT was low. A therapeutic dose (10,000 IU/day) was administered for two days, and heparin flushes (100 IU/day) were given for 15 days. It is true that HIT due to heparin flushes is rare (7), but several reports suggested a risk of HIT after exposures to small quantities of heparin from catheter flushes (8). The initial therapeutic dose may have triggered immune sensitization, and the daily small amount for heparin flush may have resulted in cerebral infarction. Second, HIT-Ab was measured by enzyme-immunoassays (EIAs) in the present case. EIAs have limited sensitivity and specificity compared to the serotonin release assay (SRA) that is considered the "gold standard" for the diagnosis of HIT (9). However, SRA is available in only a few laboratories in Japan. Although the laboratory findings (positive HIT-Ab by EIAs) did not demonstrate definite HIT in our patient, these findings in combination with a clinical score suggest the diagnosis of HIT (3). Third, the possibility of DIC and other drug-induced thrombocytopenia cannot be completely excluded. However, the low DIC score and successful treatment with argatroban, in addition to the continuation of the drugs for encephalitis, suggest that the possibil-

ity of DIC and other drug-induced thrombocytopenia was low.

Heparin is widely used as anticoagulation therapy to prevent worsening or recurrence of ischemic stroke, and it is often used as continuous infusion therapy for cardioembolic stroke. HIT sometimes causes recurrence of cerebral infarction and worsens neurological complications (10, 11). Recent reports have advocated the use of heparin to prevent DVTs in patients with a consciousness disturbance, since they have decreased spontaneous limb movements (12). However, HIT may develop with heparin therapy, especially in patients with intracranial inflammation.

Acknowledgement

The authors thank Drs. M. Matsumoto and Y. Fujimura (Departments of Blood Transfusion Medicine, Nara Medical University) for their helpful comments. We also thank Drs. T. Matsuo (Hyogo Prefectural Awaji Hospital), S. Suzuki, and K. Wanaka (Japan Clearinghouse for Heparin-induced Thrombocytopenia) for the measurement of HIT antibodies.

References

1. Visentin GP, Ford SE, Scott JP, Aster RH. Antibodies from patients with heparin-induced thrombocytopenia/thrombosis are specific for platelet factor 4 complexed with heparin or bound to endothelial cells. J Clin Invest **93**: 81-88, 1994.
2. Pohl C, Harbrecht U, Greinacher A, et al. Neurologic complications in immune-mediated heparin-induced thrombocytopenia. Neurology **54**: 1240-1245, 2000.
3. Lo GK, Juhl D, Warkentin TE, Sigouin CS, Eichler P, Greinacher A. Evaluation of pretest clinical score (4 T's) for the diagnosis of heparin-induced thrombocytopenia in two clinical settings. J Thromb Haemost **4**: 759-765, 2006.
4. Greinacher A, Farner B, Kroll H, Kohlmann T, Warkentin TE, Eichler P. Clinical features of heparin-induced thrombocytopenia including risk factors for thrombosis. A retrospective analysis of 408 patients. Thromb Haemost **94**: 132-135, 2005.
5. Sarazin L, Duong H, Bourgouin PM, et al. Herpes zoster vasculitis: demonstration by MR angiography. J Comput Assist Tomogr **19**: 624-627, 1995.
6. Walenga JM, Jeske WP, Messmore HL. Mechanisms of venous and arterial thrombosis in heparin-induced thrombocytopenia. Journal of Thrombosis and Thrombolysis **10** (Suppl 1): 13-20, 2000.
7. Warkentin TE, Greinacher A, Koster A, Lincoff AM. Treatment and prevention of heparin-induced thrombocytopenia: American College of Chest Physicians Evidence-Based Clinical Practice Guidelines (8th Edition). Chest **133**: 340S-380S, 2008.
8. Muslimani AA, Ricaurte B, Daw HA. Immune heparin-induced thrombocytopenia resulting from preceding exposure to heparin catheter flushes. Am J Hematol **82**: 652-655, 2007.
9. Lo GK, Sigouin CS, Warkentin TE. What is the potential for overdiagnosis of heparin-induced thrombocytopenia? Am J Hematol **82**: 1037-1043, 2007.
10. LaMonte MP, Brown PM, Hursting MJ. Stroke in patients with heparin-induced thrombocytopenia and the effect of argatroban therapy. Crit Care Med **32**: 976-980, 2004.
11. Ramirez-Lassepas M, Cipolle RJ, Rodvold KA, et al. Heparin-induced thrombocytopenia in patients with cerebrovascular ischemic disease. Neurology **34**: 736-740, 1984.
12. Kyrle PA, Eichinger S. Deep vein thrombosis. Lancet **365**: 1163-1174, 2005.

Original Paper

nephron
Experimental
Nephrology

Nephron Exp Nephrol 2008;109:e20–e28
DOI: 10.1159/000135386

Received: December 5, 2007
Accepted: February 25, 2008
Published online: May 28, 2008

Differential Effects of Transient Treatment of Spontaneously Hypertensive Rats with Various Antihypertensive Agents on the Subsequent Development of Diabetic Nephropathy

Kimiko Ishiguro Hiroyuki Sasamura Yusuke Sakamaki Kaori Hayashi
Takao Saruta Hiroshi Itoh

Department of Internal Medicine, School of Medicine, Keio University, Tokyo, Japan

Key Words
Transient antihypertensive treatment · Spontaneously hypertensive rats · Streptozotocin · Diabetic nephropathy

Abstract
Background/Aims: We have previously shown that treatment of spontaneously hypertensive rats (SHR) with an angiotensin receptor blocker (ARB) during the 'critical period' from age 3 to 10 weeks confers protection against L-NAME-induced renal injury later in life. The aim of this study was to examine the effects of transient prepubertal exposure to ARB on the development of nephropathy in streptozotocin-induced diabetic SHR and to compare the results with other antihypertensive agents including a mineralocorticoid receptor antagonist (MR-ant). ***Methods:*** Male SHR (n = 43) were transiently treated with candesartan (ARB), potassium canreonate (the active metabolite of the MR-ant spironolactone) or hydralazine (vasodilator) between 3 and 10 weeks of age with untreated rats serving as controls. An additional group was treated continuously with candesartan throughout the study. Rats were injected with streptozotocin to induce diabetes at age 16 weeks and followed until age 8 months. ***Results:*** Diabetic control rats showed signs of diabetic nephropathy including albuminuria and mesangial expansion. These changes were significantly suppressed in rats exposed to ARB or MR-ant. Systolic blood pressure was significantly reduced compared to controls in the ARB (transient) and ARB (sustained) groups, but not in the MR-ant or vasodilator groups. ***Conclusion:*** Transient prepubertal exposure to ARB or MR-ant, but not vasodilator, confers protection against the later development of diabetic nephropathy and involves blood pressure-independent protective mechanisms.

Introduction

Diabetic nephropathy is the leading cause of chronic renal failure leading to end-stage renal disease in many countries worldwide [1]. The economic cost of caring for these patients is substantial and is expected to increase because of the increased incidence of diabetes in the general population. The formulation of strategies to prevent the development of diabetic nephropathy is therefore an important goal of renal research.

KARGER

Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

1660–2129/08/1091–0020$24.50/0

Accessible online at:
www.karger.com/nee

Hiroyuki Sasamura, MD, PhD
Department of Internal Medicine, Keio University School of Medicine
35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582 (Japan)
Tel. +81 3 5363 3796, Fax +81 3 3359 2745
E-Mail sasamura@sc.itc.keio.ac.jp

The onset of diabetic nephropathy is heralded by the development of albuminuria, with histological changes characterized by expansion of the glomerular mesangial matrix. Interestingly, not all diabetic patients develop nephropathy. It has been estimated that only a subgroup (20–30%) of patients with insulin-dependent diabetes (IDDM) develop nephropathy [2] and this susceptibility may be genetically determined [3].

Multiple animal and clinical studies have shown that the use of inhibitors of the renin-angiotensin-aldosterone system (RAAS) is associated with a decrease in both the onset and progression of diabetic nephropathy indicating that the RAAS plays a central role in the development of diabetic nephropathy. An unresolved issue, however, is whether this protection is afforded by blood pressure (BP)-dependent mechanisms alone as suggested by Casas et al. [4] or whether BP-independent mechanisms are involved [5]. Previous studies from our and other laboratories have shown that prepubertal treatment of spontaneously hypertensive rats (SHR), stroke-prone SHR or Dahl salt-sensitive rats with either an angiotensin-converting enzyme inhibitor or angiotensin receptor blocker (ARB) during the 'critical period' from age 3 to 10 weeks results in a sustained suppression of the development of hypertension and renal injury [6–10]. We have also shown that administration of the agonist angiotensin II during this same 'critical period' results in an increased susceptibility to hypertension and L-NAME-induced renal injury later in life [10]. At present, the relative contribution of BP-dependent and -independent mechanisms is unclear.

In this study, our first aim was to determine whether treatment of SHR with an ARB during the 'critical period' would suppress the development of diabetic nephropathy in the streptozotocin (STZ) model of diabetes. A second aim was to examine the effects of transient treatment with other agents (vasodilator and MR-ant) during the 'critical period' in order to test the hypothesis that protection is conferred independent of changes in BP in this model.

Methods

Animal Treatment Protocols

The studies were conducted using 3-week-old male SHR (SHR/Izm) obtained from Sankyo Laboratory Services, Tokyo, Japan. All experiments were performed in accordance with the Animal Experimentation Guidelines of the Keio University School of Medicine.

The rats were randomly divided into 5 treatment groups as follows: Rats in group 1 (control group, n = 10) were control SHR. Rats in group 2 (ARB (transient) group, n = 9) were treated from age 3 to 10 weeks with the ARB candesartan cilexetil dissolved in the drinking water to deliver a dose of 1 mg/kg/day as described previously [10]. Rats in group 3 (MR-ant group, n = 7) were treated from age 3 to 10 weeks with the MR-ant potassium canrenoate in the drinking water (20 mg/kg/day). Rats in group 4 (VD group, n = 9) were treated from age 3 to 10 weeks with the vasodilator hydralazine in the drinking water (25 mg/kg/day). Rats in group 5 (ARB (sustained) group, n = 8) were treated continuously from age 3 to 33 weeks with candesartan cilexetil (1 mg/kg/day). Diabetes mellitus was induced by the intraperitoneal injection of STZ (65 mg/kg) at age 16 weeks and rats that did not become diabetic were excluded from the study.

Assays

The systolic blood pressure (SBP) and heart rate (HR) of conscious animals were measured by tail-cuff plethysmography using a Natsume KN-210 manometer (Natsume Inc., Tokyo, Japan). SBP measurements were made every 2 weeks after a period of training and acclimatization to the tail-cuff. 24-Hour urine collection was performed every 2 weeks using metabolic cages. At the end of the study the animals were handled gently until swift euthanasia by decapitation. Blood samples were immediately collected in chilled containers containing 10 mM EDTA and 0.1 mM PMSF (final concentration) and the centrifuged samples were stored at –20°C until analyzed. Plasma glucose and fructosamine levels were measured using commercial kits (Kanto Kagaku, Tokyo, Japan). Urea nitrogen (UN), creatinine and total cholesterol were measured using an autoanalyzer. Plasma renin activity (PRA) was determined by radioimmunoassay of angiotensin I formed by incubation of plasma for 1 h at 37°C, while plasma aldosterone concentrations (PAC) were determined by radioimmunoassay using commercially available kits (SRL Inc., Tokyo, Japan). The intra- and interassay coefficients of variation reported by the manufacturers are 4.3/5.9% and 6.7/6.7% for PRA and PAC, respectively. Plasma lipid peroxides were measured using the hemoglobin methylene blue method [11]. Rat urine albumin was measured using a direct competitive ELISA (Nephrat, Exocell, Pa., USA) as specified by the manufacturer.

Histological Studies

The kidneys and thoracic aortae were removed, fixed in 4% paraformaldehyde and embedded in paraffin blocks. Histologic sections from the rat kidneys were stained with PAS (the mesangial area stains purple), while the sections from the aortae underwent Azan staining (the medial muscle layer stains orange). Slides were examined by light microscopy and the mesangial expansion scores were assessed by a modified computerized image analysis as described previously [12]. In brief, images of 20–30 glomeruli per section (magnification ×200) were acquired using an optical microscope (Axioskop 2 plus, Zeiss, Göttingen, Germany) using Axiovision 4.4 software (Zeiss, Göttingen, Germany). The glomerular area stained purple by PAS stain was quantified by color channel analysis and pixel counting using Adobe Photoshop 7.0 software (Adobe Systems Inc., Calif., USA) and Scion Image software (Scion Corp., Md., USA). This value was then divided by the area of the glomerular tuft to obtain a semiquantitative estimate of glomerular mesangial expansion in the different groups.

Real-Time Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Assays

Total RNA was purified from the kidneys by the acid guanidine-phenol-chloroform method and quantified by measurement of absorbance at 260 nm in a spectrophotometer. Renin, AT1a receptor and AT2 receptor mRNA expression were analyzed by real-time RT-PCR using previously reported primers and probes and normalized to the corresponding values of GAPDH mRNA [13].

Statistics

Results were expressed as the mean ± SEM. Statistical comparisons were made by ANOVA. Comparison of time course data for BP and albuminuria was performed by repeated-measures ANOVA. p values <0.05 were considered to be statistically significant.

Results

Effects of Transient Exposure to Various Antihypertensive Agents on BP, Cardiovascular and Metabolic Parameters in STZ-Treated SHR

In the control group, the SBP was approximately 110 mm Hg at age 3 weeks but rose to approximately 220 mm Hg by age 12–16 weeks (fig. 1). In the rats treated with the ARB or vasodilator from age 3 to 10 weeks, the SBP was significantly ($p < 0.01$) reduced by 40–50 mm Hg during the treatment period compared to the values in the control group. In the case of the vasodilator (hydralazine), discontinuation of the drug at age 10 weeks resulted in the SBP rising to values similar to the control group by age 14 weeks. In contrast, the SBP in the ARB (transient) group remained significantly reduced at approximately 180 mm Hg throughout the experimental period. Interestingly, the rats in the MR-ant group exhibited a small non-significant tendency to decreased SBP during the treatment period with the BP at 34 weeks (211 ± 11 mm Hg) being comparable to both the control group (230 ± 7 mm Hg) and the vasodilator group (225 ± 6 mm Hg) at the end of the study. Statistical analysis by repeated-measures ANOVA confirmed that the BP time course was significantly reduced in the ARB (transient) and ARB (sustained) groups ($p < 0.01$) but no significant reduction was evident in the MR-ant or vasodilator groups.

The heart weight/body weight ratios were significantly reduced in the ARB (sustained) group compared to the control group, whereas the aorta media thickness and media/lumen ratios were not significantly changed. Plasma lipid peroxides were significantly reduced in the ARB (sustained) group. There were no significant differences in plasma glucose or fructosamine levels amongst the different groups at the end of the study (table 1).

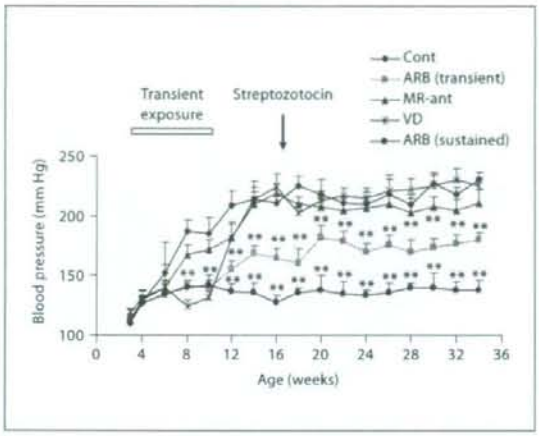

Fig. 1. Effects of transient exposure to various antihypertensive agents on BP in STZ-treated SHR. SHR were treated with various antihypertensive agents between 3 and 10 weeks of age and STZ was administered at 16 weeks. BP was monitored until 36 weeks of age. Treatments consisted of no treatment (control group = Cont), transient treatment with the angiotensin receptor blocker candesartan cilexetil [ARB (transient)], transient treatment with the mineralocorticoid receptor antagonist potassium canrenoate (MR-ant), transient treatment with the vasodilator hydralazine (VD) or continuous treatment with the angiotensin receptor blocker candesartan cilexetil [ARB (sustained)]. ** $p < 0.01$ vs. Cont.

Effects of Transient Exposure to Various Antihypertensive Agents on the Subsequent Renal Injury in STZ-Treated Diabetic SHR

Rats in the control group exhibited marked albuminuria which was clearly evident at age 28 weeks and reached a value of 489 ± 117 mg/day at the end of the study (fig. 2a). Analysis by repeated-measures ANOVA indicated that the development of albuminuria was markedly suppressed in the rats in both the ARB (sustained) group (24 ± 15 mg/day, $p < 0.01$ vs. control) and the ARB (transient) group (57 ± 33 mg/day, $p < 0.01$ vs. control). The rats in the MR-ant group exhibited a partial suppression of the albuminuria (165 ± 5 1 mg/day, $p < 0.05$ vs. control). The level of albuminuria in non-diabetic, untreated SHR was 19 ± 8 mg/day.

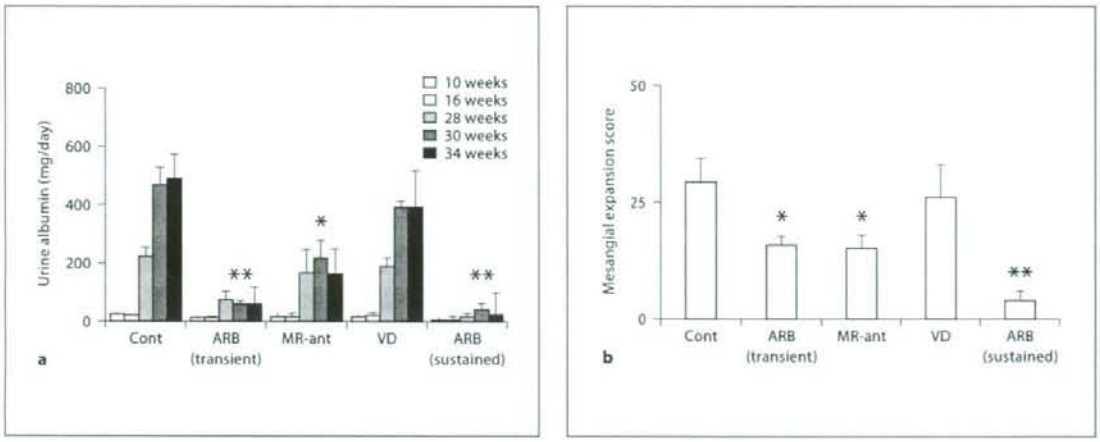

Fig. 2. Effects of transient exposure to various antihypertensive agents on albuminuria and mesangial expansion score in STZ-treated SHR. SHR were treated with various antihypertensive agents between 3 and 10 weeks of age and STZ was administered at 16 weeks. Treatments consisted of no treatment (control group = Cont), transient treatment with the angiotensin receptor blocker candesartan cilexetil [ARB (transient)], transient treatment with the mineralocorticoid receptor antagonist potassium canrenoate (MR-ant), transient treatment with the vasodilator hydralazine (VD) or continuous treatment with the angiotensin receptor blocker candesartan cilexetil [ARB (sustained)]. **a** Urine albumin excretion was determined at 10, 16, 28, 32 and 34 weeks of age. A beneficial effect upon albuminuria was evident in the ARB (transient), MR-ant and ARB (sustained) groups. **b** The mesangial expansion score was determined on PAS-stained tissue sections by computer image analysis at 36 weeks. * $p < 0.05$; ** $p < 0.01$ vs. Cont.

Table 1. Cardiovascular and biochemical parameters in the different groups

	Group 1	Group 2	Group 3	Group 4	Group 5
Treatment	Cont	ARB (transient)	MR-ant	VD	ARB (sustained)
Body weight (BW), g	256 ± 20	276 ± 23	263 ± 22	287 ± 28	294 ± 29
Heart weight (HW), g	1.62 ± 0.06	1.66 ± 0.08	1.61 ± 0.07	1.78 ± 0.09	1.57 ± 0.07
HW/BW × 100	0.66 ± 0.05	0.63 ± 0.06	0.57 ± 0.07	0.65 ± 0.05	0.55 ± 0.03*
Aorta media thickness, mm	0.18 ± 0.07	0.14 ± 0.01	0.13 ± 0.01	0.15 ± 0.02	0.13 ± 0.01
Aorta media/lumen × 100	7.85 ± 1.2	7.66 ± 0.29	7.29 ± 0.43	7.41 ± 0.81	7.18 ± 0.22
Plasma glucose, mg/dl	581 ± 40	451 ± 64	524 ± 72	496 ± 48	501 ± 73
Plasma fructosamine, μmol/l	336 ± 77	360 ± 132	357 ± 63	379 ± 59	411 ± 89
Plasma total cholesterol, mg/dl	83 ± 15	66 ± 5	87 ± 5	65 ± 7	71 ± 8
Plasma lipid peroxides, nmol/ml	1.11 ± 0.40	0.73 ± 0.09	0.79 ± 0.19	0.89 ± 0.10	0.59 ± 0.06*
Plasma urea nitrogen, mg/dl	28 ± 3	27 ± 3	32 ± 7	27 ± 6	33 ± 4
Plasma creatinine, mg/dl	0.29 ± 0.02	0.29 ± 0.03	0.28 ± 0.03	0.27 ± 0.02	0.30 ± 0.02

Results shown are means ± SEM. Cont = Control treated; ARB (transient) = treated transiently with candesartan cilexetil; MR-ant = treated transiently with potassium canrenoate; VD = treated transiently with hydralazine; ARB (sustained) = treated continuously with candesartan cilexetil.

* $p < 0.05$ vs. Cont.