TABLE 1
Quantitative cell cycle analysis of cultured KSL cells

Cell cycle	shRNA sequence	Means ± S.D. ($n = 5$)	p value
		%	
G_0/G_1 phase	Random	31.72 ± 3.53	
	Talin-A	34.12 ± 2.13	0.070
	Vin-B	35.02 ± 2.39	0.016[a]
	Vin-C	32.86 ± 4.21	0.46
S phase	Random	59.0 ± 3.29	
	Talin-A	51.5 ± 11.0	0.13
	Vin-B	45.9 ± 5.35	0.0013[a]
	Vin-C	55.3 ± 3.83	0.0384[a]
G_2/M phase	Random	2.82 ± 1.93	
	Talin-A	3.06 ± 1.71	0.66
	Vin-B	3.16 ± 1.59	0.64
	Vin-C	3.06 ± 1.67	0.76
Apoptotic cells	Random	5.44 ± 1.43	
	Talin-A	6.44 ± 3.73	0.49
	Vin-B	14.08 ± 3.71	0.0026[a]
	Vin-C	7.44 ± 1.41	0.0036[a]

[a] $p < 0.05$ compared with the control experiment (random) under the same conditions (Student's t test).

until 2 weeks after the transplantation (Fig. 6, *C* and *D*). The early luciferase activity derived from KSL cells expressing the Vin-B sequence was slightly attenuated (Fig. 6*C*). This was expected from the results of the cell proliferation experiments (Fig. 4*A*). It was notable that the luciferase activities derived from HSCs lacking vinculin seemed to gradually decrease, compared with the activities derived from control and talin-silenced cells (Fig. 6, *C* and *D*). These results support the idea that vinculin is an indispensable factor for HSC repopulation in the BM.

Loss of Talin-1, but Not Vinculin, Impairs Adhesion of KSL Cells to the ECM—The effect of vinculin silencing on HSC function, through integrin expression and adhesion to the ECM, was investigated. As shown in Fig. 7*A*, the expression levels of integrins $\beta1$ and $\beta3$ in KSL cells were unaffected after transduction of LentiLox vectors equipped with shRNA sequences for talin-1 and vinculin. Interestingly, adhesion to the ECM in the static condition was significantly inhibited in talin-deficient transduced KSL cells but not in vinculin-deficient transduced KSL cells (Fig. 7*B*). Cell spreading onto fibronectin was also investigated by confocal microscopy. The silencing of talin-1, but not of vinculin, resulted in a failure of cells to spread on fibronectin after adhesion (Fig. 7*C* and supplemental Fig. 5). These results indicate that vinculin is not involved in the attachment and spreading of KSL cells to the ECM in the static condition and that its role in HSC repopulation is independent of integrin function.

DISCUSSION

HSCs are the most thoroughly characterized type of adult stem cells, and the hematopoietic system has served as the principal model for stem cell biology. Transplantation of HSC populations has been shown to be sufficient for long term multilineage reconstitution, not only in experimental animal models but also in clinical patients with hematological malignancies. HSCs must undergo several steps to achieve engraftment after transplantation, including transendothelial migration into the BM (homing), settling in the BM niche (lodging and retention), and intra-BM proliferation and multilineage differentiation (repopulation) (30). The present study showed that HSCs lacking vinculin were unable to reconstitute the hematopoietic sys-

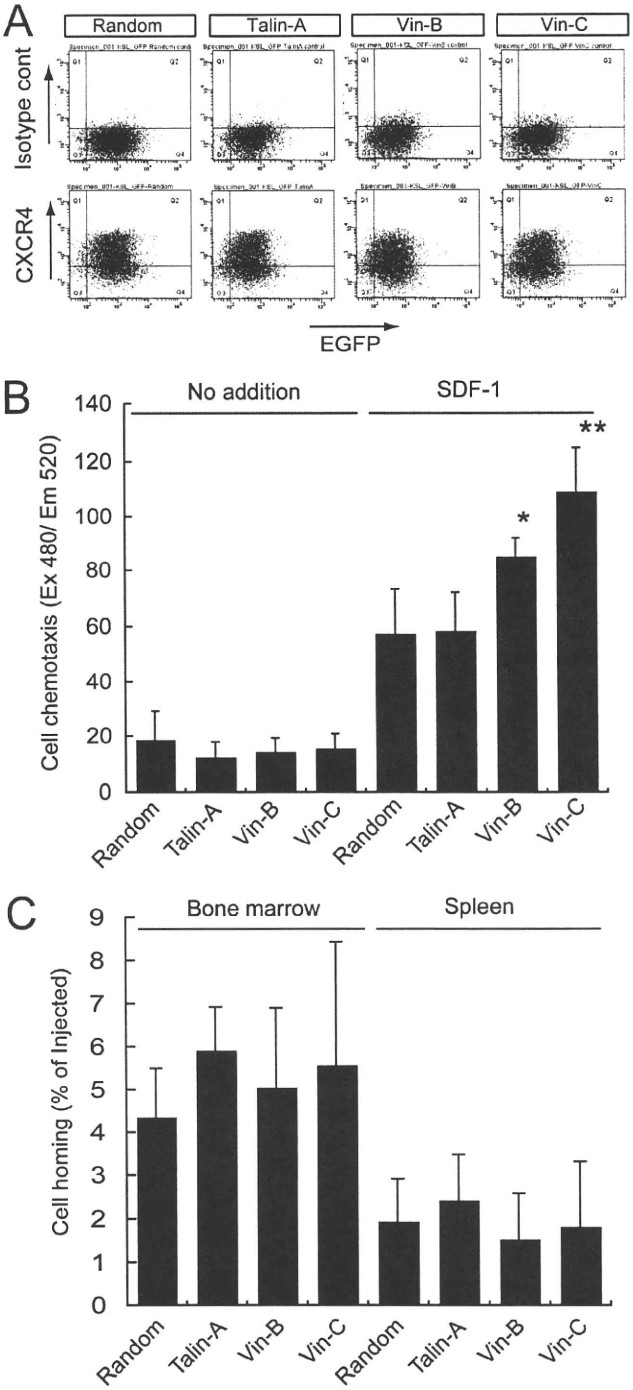
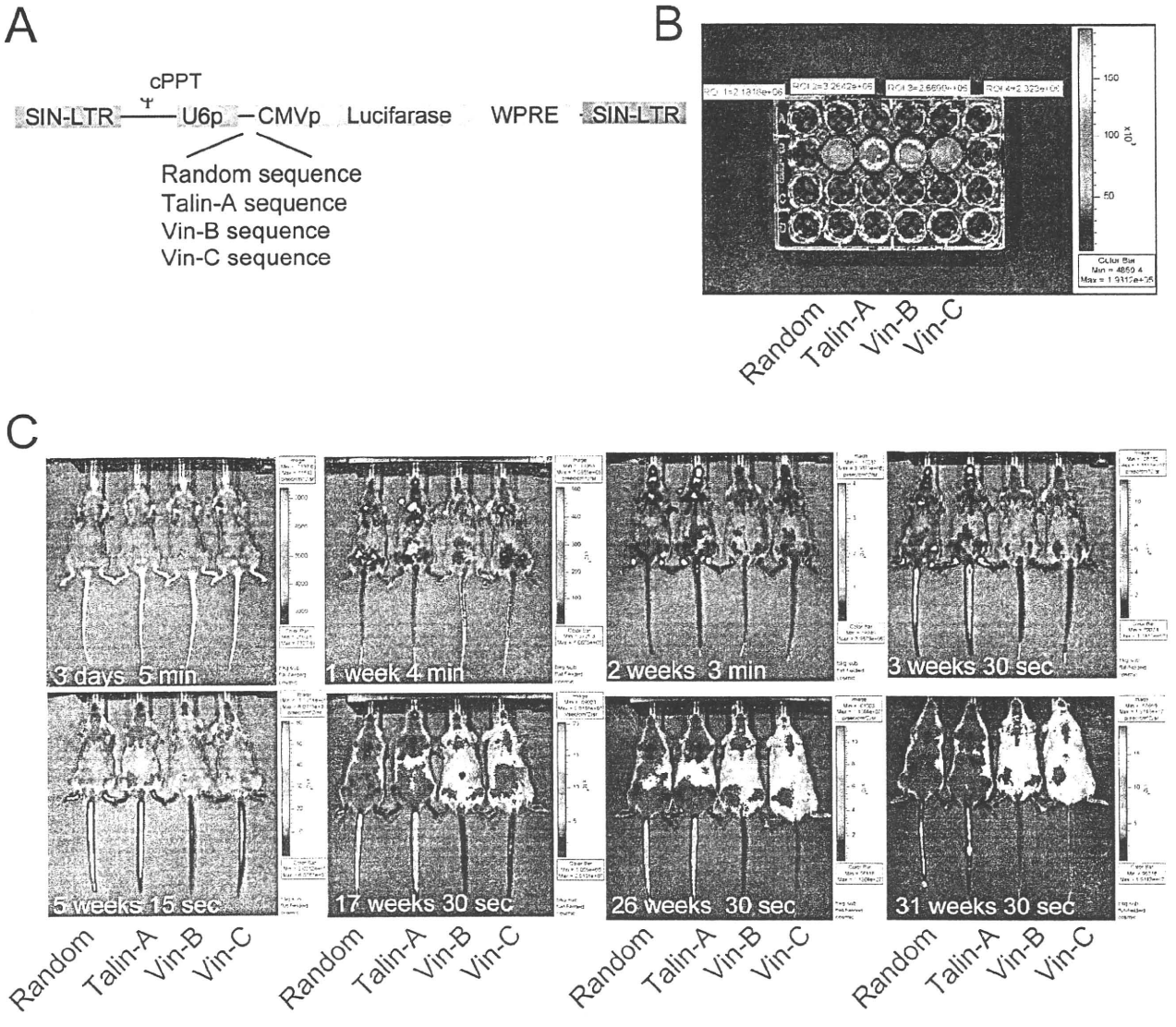


FIGURE 5. **Roles of talin and vinculin in the homing of KSL cells.** *A*, the expression of CXCR4 in KSL cells transduced with the indicated LentiLox vector was evaluated by FACS (*lower panels*). The results for an isotype-matched control Ab are also shown (*upper panels*). *B*, transduced KSL cell migration *in vitro* was assessed using a modified Boyden chamber assay. Stromal cell-derived factor 1 (100 ng/ml) was placed in the lower chamber (or not added), and the cells were allowed to migrate for 4 h. The *columns* and *error bars* represent the means ± S.D. ($n = 4$). *C*, transduced KSL cells were injected into lethally irradiated recipient mice. The cells from the BM and spleen were collected for CFU-GM assays at 16–20 h after injection. The total numbers of CFU-GM recovered from the BM and spleen were counted manually and expressed as percentages of the number of CFU-GM from the injected KSL cells. The *columns* and *error bars* represent the means ± S.D. ($n = 4$). *, $p < 0.05$; **, $p < 0.01$, compared with the control experiment (random) under the same conditions (two-tailed Student's t test).



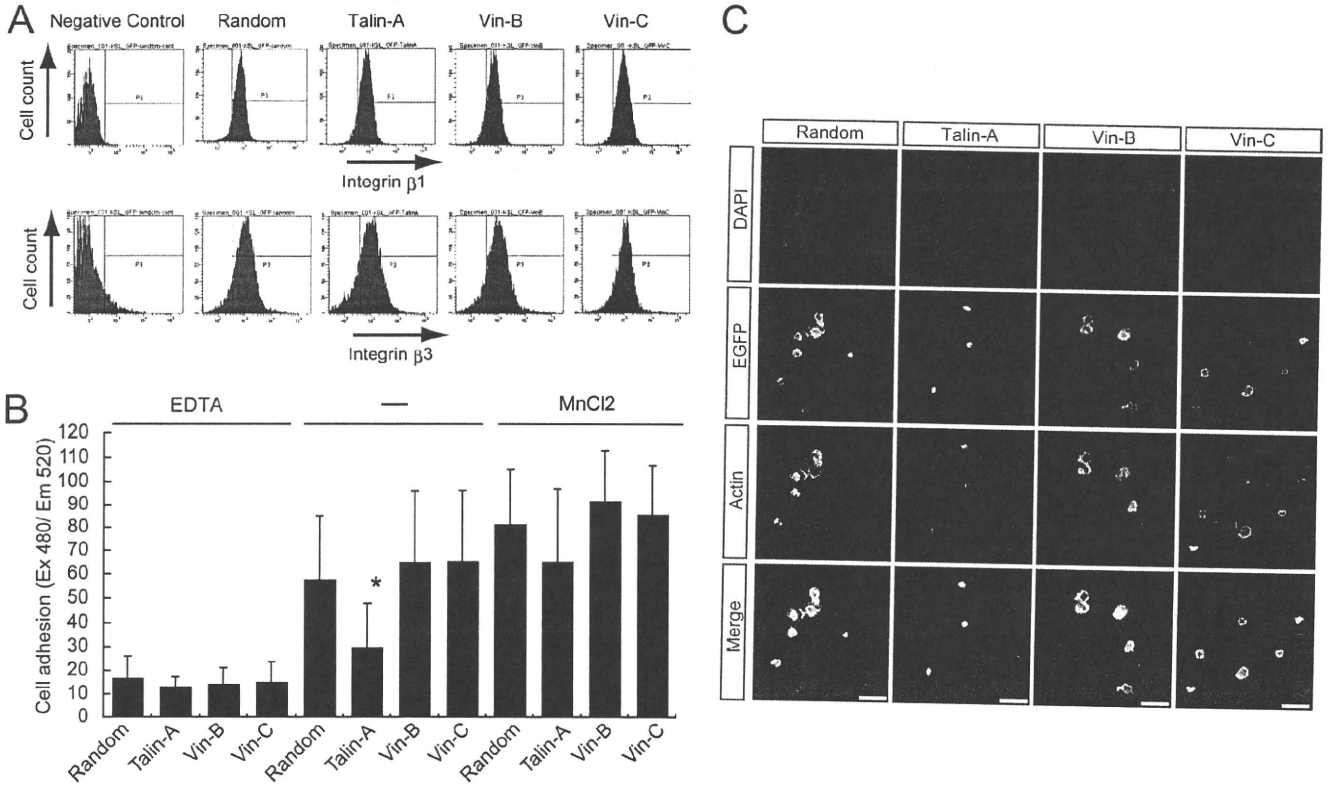


FIGURE 7. **Loss of vinculin does not affect integrin expression and adhesion of KSL cells to the ECM.** KSL cells were transduced with the indicated LentiLox vectors. *A*, the expression levels of integrin β1 (*upper panels*) and integrin β3 (*lower panels*) were examined by FACS. The results for an isotype-matched control Ab are also shown. *B*, cell adhesion to fibronectin (10 μg/ml) in the absence or presence of 2 mM EDTA or 2 mM $MnCl_2$ were assessed as described under "Experimental Procedures." The *columns* and *error bars* represent the means ± S.D. ($n = 4$). *, $p < 0.05$, compared with the control experiment (random) under the same conditions (two-tailed Student's *t* test). *C*, the transduced KSL cells were placed on immobilized fibronectin (10 μg/ml) for 3 h. The cells were fixed and then stained with DAPI (*blue*), anti-GFP antibody (*green*), and rhodamine-conjugated phalloidin (*red*). *Bar*, 40 μm. The data are representative of three independent experiments.

tem efficiently and failed to maintain their self-renewal capacity on BM stromal cell layers. However, the loss of vinculin did not inhibit the potential of HSCs to differentiate into granulocytic and monocytic lineages, *in vitro* migration toward stromal cell-derived factor 1α, and *in vivo* homing to the BM. In addition, HSC adhesion to the ECM was abolished by expression of an shRNA sequence against talin-1, but not vinculin. This suggests that vinculin controls repopulation by HSCs in the BM microenvironment, independent of integrin function.

Cell-cell and cell-ECM interactions have been reported to be crucial for a number of HSC functions in the BM microenvironment (1, 2). Two different niches have been identified in the BM: the endosteal stem cell niche at the endosteum of the bone and the vascular niche in close proximity to the blood vessels. N-cadherin/β-catenin, Tie2/Ang-1, vascular cell adhesion molecule/integrin, and osteopontin/β1 integrin represent important adhesion molecules for the functions of these niches (1, 33). These molecules play roles either in the attachment of HSCs to the niche or in the migration of HSCs. Roles for integrins in HSC homing have been investigated in β1 integrin-knock-out mice and by blocking integrin function with anti-integrin antibodies (6–8). Osteopontin was recently found to contribute to HSC migration toward the endosteal region through its interaction with β1 integrin (34). Interactions between an integrin and the ECM are required for its transition from a low to a high affinity state via signaling referred to as "inside-out signaling" pathways (35). The interaction between an integrin and its ligand triggers signaling that promotes cytoskeletal changes, leading to cell spreading and stabilization through the process termed "outside-in signaling" (36, 37). Proteins that can directly bind to β-integrin cytoplasmic tails are important for these signaling pathways (see the Introduction) (12, 13). Inside-out and outside-in signaling of integrins are often considered separately, but some β integrin cytoplasmic proteins, including talin and cytoplasmic phospholipase A_2, may function in both (38, 39). The reconstitution of HSCs after

FIGURE 6. **Visualization of KSL cell fates *in vivo* in the absence of talin or vinculin.** *A*, schematic presentation of the lentiviral vector used in this experiment. The *EGFP* gene of the LentiLox lentiviral vector was replaced with a *luciferase* gene. *B–D*, KSL cells were transduced with a lentiviral vector expressing a control shRNA sequence (random) or shRNA sequences against talin (Talin-A) or vinculin (Vin-B and Vin-C). *B*, *ex vivo* bioluminescence images of transduced KSL cells before transplantation. *C*, transduced KSL cells together with competitor cells were transplanted into lethally irradiated recipient mice. The photons transmitted through the body were collected for the indicated lengths of time at the indicated days after transplantation using an IVIS Imaging System. *D*, quantitative data for *in vivo* bioluminescence imaging of the mice expressed as photon units (photons/s). The data are representative mean results obtained from two mice after transplantation in two independent experiments.

transplantation was partially impaired under conditions where the adhesion and spreading of HSCs onto ECM (outside-in signaling) were significantly inhibited by silencing of talin-1, whereas homing of HSCs to the BM was not affected. We cannot rule out the possibility that the discrepancy between the current and previous results (6) regarding the role of integrins in HSC homing could be the result of incomplete inhibition of integrin function by silencing of talin-1. However, our results suggest that signaling through integrins expressed in HSCs is involved in reconstitution in the BM microenvironment, although the contribution of talin-1 to integrin activation and HSC reconstitution was much weaker than that of vinculin.

The most important finding of the present study was the ability of vinculin to control HSC repopulation independently of integrin function. Activation of vinculin has been reported to be triggered by talin and actin polymerization, and this activation may strengthen the interactions between integrins and their ligands (40, 41). However, a reduction in the direct interaction between an integrin and its ligand alone cannot readily explain the phenotype of HSCs lacking vinculin, because although the loss of talin expression in HSCs caused severe impairment of integrin function, it only resulted in marginal failure of reconstitution. Vinculin also exists in cadherin-mediated cell-cell contacts and may therefore play an important role in cadherin-mediated cell-cell attachments in the niche, although conflicting results regarding the role of N-cadherin in HSC maintenance have been reported (42, 43). It is also possible that vinculin-deficient HSCs are unable to achieve a balance between self-renewal and differentiation; HSCs maintain the balance between stem cell and differentiated cell populations by choosing between several alternative fates in the BM, such as self-renewal and commitment to differentiation (44, 45). The former ensures preservation of the HSC fate upon cellular division, whereas the latter enables differentiation into multiple lineages (44). In the present study, the expression of shRNA sequences against vinculin significantly abolished the frequency of LTC-IC and the formation of cobblestone-like areas but not the ability of HSCs to differentiate into granulocytic lineage cells, indicating that vinculin may regulate the self-renewal potential of HSCs. A self-renewal division implies that an HSC is permissive in terms of cell cycle entry but restricted from engaging in differentiation, apoptosis, or senescence pathways (44). A number of genes involved in the cell cycle machinery and HOX proteins (INK4A, HoxB4, and HoxA9) have been shown to regulate intrinsic programs in HSCs during the self-renewal process (46). It is possible that vinculin is directly involved in these spatial and temporal control processes responsible for maintaining the intrinsic balance of HSC self-renewal. Indeed, silencing of vinculin induced cell cycle modulation and the induction of apoptosis in KSL HSCs. In a study of vinculin-deficient embryos, vinculin was found to be necessary for normal neural and cardiac development (47). Furthermore, a mouse model involving cardiac myocyte-specific deletion of the vinculin gene resulted in sudden death and dilated cardiomyopathy (48). Although these abnormalities are believed to result from perturbation of integrin-dependent cell functions, the involvement of a specific function of vinculin that is independent of integrins cannot be ruled out, as demonstrated for HSC functions. Further studies are required to address these issues.

Silencing of vinculin failed to inhibit the adhesive properties of HSCs in this study. This was consistent with the results of a recent study indicating that inhibition of vinculin by RNA interference did not affect cell attachment to ECM in epithelial cells (49). Furthermore, silencing of vinculin did not abolish cell spreading of Chinese hamster ovary cells transformed to express integrin αIIbβ3 (αIIbβ3-CHO cells) on fibrinogen (50). This suggests that vinculin may not be involved in the initial attachment to the ECM and outside-in signaling of integrin. However, vinculin might be important for integrin αIIbβ3 inside-out signaling, because the activated form of vinculin (the form inhibiting head-to-tail association) could induce binding of PAC-1 (a monoclonal Ab recognizing activated integrin αIIbβ3) to αIIbβ3-CHO cells (50). Further experiments to investigate the role of vinculin in inside-out signaling of integrins in platelet activation are now underway in our laboratory.

In summary, we have demonstrated that vinculin is required for HSC repopulation after HSC transplantation. These results provide the first evidence for vinculin as an important regulator of cellular functions, independent of integrin function. The manipulation of vinculin expression may represent a novel approach for not only regenerative and developmental medicine but also for the treatment of hematological malignancies. Further studies are needed to determine the precise cellular mechanisms whereby vinculin modulates HSC repopulation independently of integrin function and to apply vinculin-targeting therapies to the treatment of a variety of refractory disorders.

Acknowledgments—We acknowledge Dr. K. Eto (Tokyo University) for helpful discussions. We also thank N. Matsumoto and M. Ito for excellent technical assistance.

REFERENCES

1. Arai, F., and Suda, T. (2007) *Ann. N.Y. Acad. Sci.* **1106,** 41–53
2. Huang, X., Cho, S., and Spangrude, G. J. (2007) *Cell Death Differ.* **14,** 1851–1859
3. Arai, F., Hirao, A., Ohmura, M., Sato, H., Matsuoka, S., Takubo, K., Ito, K., Koh, G. Y., and Suda, T. (2004) *Cell* **118,** 149–161
4. Zhang, J., Niu, C., Ye, L., Huang, H., He, X., Tong, W. G., Ross, J., Haug, J., Johnson, T., Feng, J. Q., Harris, S., Wiedemann, L. M., Mishina, Y., and Li, L. (2003) *Nature* **425,** 836–841
5. Voura, E. B., Billia, F., Iscove, N. N., and Hawley, R. G. (1997) *Exp. Hematol.* **25,** 1172–1179
6. Potocnik, A. J., Brakebusch, C., and Fässler, R. (2000) *Immunity* **12,** 653–663
7. Priestley, G. V., Ulyanova, T., and Papayannopoulou, T. (2007) *Blood* **109,** 109–111
8. Papayannopoulou, T., Craddock, C., Nakamoto, B., Priestley, G. V., and Wolf, N. S. (1995) *Proc. Natl. Acad. Sci. U.S.A.* **92,** 9647–9651
9. van der Loo, J. C., Xiao, X., McMillin, D., Hashino, K., Kato, I., and Williams, D. A. (1998) *J. Clin. Invest.* **102,** 1051–1061
10. Yokota, T., Oritani, K., Mitsui, H., Aoyama, K., Ishikawa, J., Sugahara, H., Matsumura, I., Tsai, S., Tomiyama, Y., Kanakura, Y., and Matsuzawa, Y. (1998) *Blood* **91,** 3263–3272

11. Takada, Y., Ye, X., and Simon, S. (2007) *Genome Biol.* **8,** 215
12. Coller, B. S., and Shattil, S. J. (2008) *Blood* **112,** 3011–3025
13. Zamir, E., and Geiger, B. (2001) *J. Cell Sci.* **114,** 3583–3590
14. Tadokoro, S., Shattil, S. J., Eto, K., Tai, V., Liddington, R. C., de Pereda, J. M., Ginsberg, M. H., and Calderwood, D. A. (2003) *Science* **302,** 103–106
15. Honda, S., Shirotani-Ikejima, H., Tadokoro, S., Maeda, Y., Kinoshita, T., Tomiyama, Y., and Miyata, T. (2009) *Blood* **113,** 5304–5313
16. Ma, Y. Q., Qin, J., Wu, C., and Plow, E. F. (2008) *J. Cell Biol.* **181,** 439–446
17. Moser, M., Nieswandt, B., Ussar, S., Pozgajova, M., and Fässler, R. (2008) *Nat. Med.* **14,** 325–330
18. Tucker, K. L., Sage, T., Stevens, J. M., Jordan, P. A., Jones, S., Barrett, N. E., St-Arnaud, R., Frampton, J., Dedhar, S., and Gibbins, J. M. (2008) *Blood* **112,** 4523–4531
19. Ziegler, W. H., Liddington, R. C., and Critchley, D. R. (2006) *Trends Cell Biol.* **16,** 453–460
20. Izard, T., and Vonrhein, C. (2004) *J. Biol. Chem.* **279,** 27667–27678
21. Papagrigoriou, E., Gingras, A. R., Barsukov, I. L., Bate, N., Fillingham, I. J., Patel, B., Frank, R., Ziegler, W. H., Roberts, G. C., Critchley, D. R., and Emsley, J. (2004) *EMBO J.* **23,** 2942–2951
22. Izard, T., Evans, G., Borgon, R. A., Rush, C. L., Bricogne, G., and Bois, P. R. (2004) *Nature* **427,** 171–175
23. Chen, H., Choudhury, D. M., and Craig, S. W. (2006) *J. Biol. Chem.* **281,** 40389–40398
24. Rubinson, D. A., Dillon, C. P., Kwiatkowski, A. V., Sievers, C., Yang, L., Kopinja, J., Rooney, D. L., Zhang, M., Ihrig, M. M., McManus, M. T., Gertler, F. B., Scott, M. L., and Van Parijs, L. (2003) *Nat. Genet.* **33,** 401–406
25. Ohmori, T., Kashiwakura, Y., Ishiwata, A., Madoiwa, S., Mimuro, J., and Sakata, Y. (2007) *Arterioscler. Thromb. Vasc. Biol.* **27,** 2266–2272
26. Ohmori, T., Mimuro, J., Takano, K., Madoiwa, S., Kashiwakura, Y., Ishiwata, A., Niimura, M., Mitomo, K., Tabata, T., Hasegawa, M., Ozawa, K., and Sakata, Y. (2006) *FASEB J.* **20,** 1522–1524
27. Rawls, A. S., Gregory, A. D., Woloszynek, J. R., Liu, F., and Link, D. C. (2007) *Blood* **110,** 2414–2422
28. Yang, L., Wang, L., Geiger, H., Cancelas, J. A., Mo, J., and Zheng, Y. (2007) *Proc. Natl. Acad. Sci. U.S.A.* **104,** 5091–5096
29. Qian, H., Georges-Labouesse, E., Nyström, A., Domogatskaya, A., Tryggvason, K., Jacobsen, S. E., and Ekblom, M. (2007) *Blood* **110,** 2399–2407
30. Ogaeri, T., Eto, K., Otsu, M., Ema, H., and Nakauchi, H. (2009) *Stem Cells* **27,** 1120–1129
31. Okada, S., Nakauchi, H., Nagayoshi, K., Nishikawa, S., Miura, Y., and Suda, T. (1992) *Blood* **80,** 3044–3050
32. Nakauchi, H., Sudo, K., and Ema, H. (2001) *Ann. N.Y. Acad. Sci.* **938,** 18–25
33. Yin, T., and Li, L. (2006) *J. Clin. Invest.* **116,** 1195–1201
34. Nilsson, S. K., Johnston, H. M., Whitty, G. A., Williams, B., Webb, R. J., Denhardt, D. T., Bertoncello, I., Bendall, L. J., Simmons, P. J., and Haylock, D. N. (2005) *Blood* **106,** 1232–1239
35. Bennett, J. S. (2005) *J. Clin. Invest.* **115,** 3363–3369
36. Takizawa, H., Nishimura, S., Takayama, N., Oda, A., Nishikii, H., Morita, Y., Kakinuma, S., Yamazaki, S., Okamura, S., Tamura, N., Goto, S., Sawaguchi, A., Manabe, I., Takatsu, K., Nakauchi, H., Takaki, S., and Eto, K. *J. Clin. Invest.* **120,** 179–190
37. Phillips, D. R., Nannizzi-Alaimo, L., and Prasad, K. S. (2001) *Thromb. Haemost.* **86,** 246–258
38. Prévost, N., Mitsios, J. V., Kato, H., Burke, J. E., Dennis, E. A., Shimizu, T., and Shattil, S. J. (2009) *Blood* **113,** 447–457
39. Nieswandt, B., Moser, M., Pleines, I., Varga-Szabo, D., Monkley, S., Critchley, D., and Fässler, R. (2007) *J. Exp. Med.* **204,** 3113–3118
40. Schwartz, M. A. (2009) *Science* **323,** 588–589
41. del Rio, A., Perez-Jimenez, R., Liu, R., Roca-Cusachs, P., Fernandez, J. M., and Sheetz, M. P. (2009) *Science* **323,** 638–641
42. Kiel, M. J., Acar, M., Radice, G. L., and Morrison, S. J. (2009) *Cell Stem Cell* **4,** 170–179
43. Kiel, M. J., Radice, G. L., and Morrison, S. J. (2007) *Cell Stem Cell* **1,** 204–217
44. Blank, U., Karlsson, G., and Karlsson, S. (2008) *Blood* **111,** 492–503
45. Martinez-Agosto, J. A., Mikkola, H. K., Hartenstein, V., and Banerjee, U. (2007) *Genes Dev.* **21,** 3044–3060
46. Zon, L. I. (2008) *Nature* **453,** 306–313
47. Xu, W., Baribault, H., and Adamson, E. D. (1998) *Development* **125,** 327–337
48. Zemljic-Harpf, A. E., Miller, J. C., Henderson, S. A., Wright, A. T., Manso, A. M., Elsherif, L., Dalton, N. D., Thor, A. K., Perkins, G. A., McCulloch, A. D., and Ross, R. S. (2007) *Mol. Cell. Biol.* **27,** 7522–7537
49. Peng, X., Cuff, L. E., Lawton, C. D., and DeMali, K. A. (2010) *J. Cell Sci.* **123,** 567–577
50. Ohmori, T., Kashiwakura, Y., Ishiwata, A., Madoiwa, S., Mimuro, J., Honda, S., Miyata, T., and Sakata, Y. (2010) *Biochem. Biophys. Res. Commun.*, in press

Thrombosis Research 125 (2010) 533–537

Contents lists available at ScienceDirect

Thrombosis Research

journal homepage: www.elsevier.com/locate/thromres

Regular Article

Mutant Macaque Factor IX T262A: A Tool for Hemophilia B Gene Therapy Studies in Macaques

Akira Ishiwata [a,1], Jun Mimuro [a,b,*,1], Hiroaki Mizukami [c], Yuji Kashiwakura [a], Atsushi Yasumoto [a], Asuka Sakata [a], Tsukasa Ohmori [a,b], Seiji Madoiwa [a,b], Fumiko Ono [d,e], Midori Shima [f], Akira Yoshioka [f], Keiya Ozawa [b,c], Yoichi Sakata [a,b,*]

[a] Divisions of Cell and Molecular Medicine, Center for Molecular Medicine, Jichi Medical University, Tochigi-ken 329-0498, Japan
[b] Hematology Division of Department of Medicine, Jichi Medical University, Tochigi-ken 329-0498, Japan
[c] Division of Genetic Therapeutics, Center for Molecular Medicine, Jichi Medical University, Tochigi-ken 329-0498, Japan
[d] The Corporation for Production and Research of Laboratory Primates, Tsukuba, Ibaraki-ken 305-0843, Japan
[e] Tsukuba Primate Research Center, National Institute of Biomedical Innovation, Tsukuba, Ibaraki-ken 305-0843, Japan
[f] Department of Pediatrics, Nara Medical University, Kashihara, Nara-ken 634-8522, Japan

ARTICLE INFO

Article history:
Received 9 November 2009
Received in revised form 12 January 2010
Accepted 25 January 2010
Available online 18 February 2010

ABSTRACT

Introduction: Gene therapy is expected to be the next generation therapy for hemophilia, and a good animal model is required for hemophilia gene therapy preclinical studies.
Methods: Taking advantage of the human factor IX (FIX) specificity of monoclonal antibody 3A6, the epitope of which resides in the amino acid polypeptide segment including Ala 262 of human FIX, mutant macaque FIX with an amino acid substitution of Thr 262 to Ala (macaque FIX T262A) was generated and its reactivity to monoclonal antibody 3A6, biological activity and expression *in vivo* were studied.
Results: Enzyme-linked immunosorbent assays (ELISAs) and Western blot analyses showed that monoclonal antibody 3A6 bound to human FIX and macaque FIX T262A but not to wild-type macaque FIX. Recombinant macaque FIX T262A exhibited a comparable coagulation activity to wild-type macaque FIX and human FIX. High expression of macaque FIX T262A was achieved in mice by injection of AAV8 vectors carrying the macaque FIX T262A gene and reached levels of up to 31.5 μg/mL (1050% of the normal human FIX concentration). Macaque FIX T262A expressed in the liver of mice was as biologically active as that expressed *in vitro*. In addition, the macaque FIX T262A concentrations determined by a 3A6-based ELISA were not influenced by the presence of normal macaque plasma.
Conclusions: The results of the present study suggest that macaque FIX T262A may be processed appropriately *in vivo* and that the macaque FIX T262A concentration in the macaque circulation can be quantified precisely by a monoclonal antibody 3A6-based ELISA.

A wide variety of disorders are caused by genetic abnormalities, thereby giving rise to enthusiasm for gene therapy as the next generation therapeutics for many diseases [1–4]. Indeed, many gene therapy clinical trials have been conducted, and some have achieved great successes [5–7]. However, others have been unsuccessful. Furthermore, unpredicted adverse effects occurred in some trials [8,9]. To establish gene therapy technologies, good animal models are required. Advances in developmental biotechnology have allowed us to create a variety of mouse disease models, transgenic mice and gene-targeted mice. However, there are significant species differences between humans and mice, thus making it difficult under certain circumstances to extrapolate data obtained in mice to human patients [10]. Factor IX (FIX)-deficient mice (hemophilia B mice) and natural hemophilia B dogs have been used to study gene therapy approaches for the treatment of hemophilia B [3,11–13]. However, better animal models may be required because of the limited success in human trials [9,10]. Primates are used successfully as models in disease applications, but there are presently no hemophilic primates available for gene therapy studies. If one can distinguish human molecules from primate molecules *in vivo*, non-human primates may be used for hemophilia gene therapy preclinical studies [11,14,15], despite the fact that this genetic abnormality is not indigenous to these species.

Rhesus macaques have been proposed as a good primate model for hemophilia B gene therapy studies because of the amino acid sequence similarity between human FIX and macaque FIX and the low immunogenicity of human FIX in rhesus macaques [16]. However,

* Corresponding authors. Division of Cell and Molecular Medicine, Center for Molecular Medicine, Jichi Medical University, Tochigi-ken 329-0498, Japan. Tel.: +81 285 58 7398; fax: +81 285 44 7817.
E-mail addresses: mimuro-j@jichi.ac.jp (J. Mimuro), yoisaka@jichi.ac.jp (Y. Sakata).
[1] These authors contributed equally to this work.

0049-3848/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.thromres.2010.01.049

rhesus macaques still developed antibodies against human FIX upon receiving adenoviral vectors carrying the human FIX gene despite the high amino acid sequence homology [17], thus making it difficult to study the long-term expression of transgene-derived FIX. We previously reported that a human FIX-specific monoclonal antibody, 3A6, which can distinguish human FIX from cynomolgus macaque FIX in enzyme immunoassays and Western blot analyses, binds to the amino acid segment including the Ala residue at position 262 of the human FIX molecule [18]. Only one amino acid residue at position 262 in this segment differs between macaque FIX and human FIX [16,19]. Therefore, the Thr residue at position 262 of macaque FIX was mutated to Ala (macaque FIX T262A) to examine whether the human FIX-specific monoclonal antibody 3A6 could bind to the mutant macaque FIX in the present study. Here, we show that macaque FIX T262A binds to monoclonal antibody 3A6 and is as active as wild-type macaque FIX and human FIX, that macaque FIX T262A can be efficiently expressed *in vivo* in mice by injection of AAV8 vectors carrying the mutant macaque FIX gene, and that quantification of macaque FIX T262A by a 3A6-based ELISA was not influenced by the presence of macaque plasma. These findings raise the possibility of a potential advantage of mutant macaque FIX T262A over human FIX to study the long-term expression of a FIX transgene in macaques.

Materials and Methods

Cloning of macaque FIX cDNA

Total RNA was isolated from the cynomolgus macaque liver using an RNeasy Mini Kit (Qiagen Inc., Valencia, CA). The isolated RNA was subjected to RT-PCR to amplify the macaque FIX cDNA based on the nucleotide sequence of a macaque FIX cDNA [19] using *pfu* Turbo DNA polymerase (Stratagene, La Jolla, CA) and a primer pair (5′-AGG TTA TGC AGC GCG TGA AC-3′/5′-CCA TCT TTC ATT AAG TGA GCT TTG-3′). The DNA fragment of the macaque FIX cDNA was cloned into the plasmid vector pCR Blunt II using a Zero Blunt TOPO PCR Cloning Kit (Invitrogen Co., Carlsbad, CA) and sequenced. The macaque FIX cDNA was subjected to site-directed mutagenesis to create the cDNA expressing macaque FIX T262A using a QuikChange Site-directed Mutagenesis Kit (Stratagene) and a primer pair (5′-CCT CAT CAC AAC TAC AAT GCA GCT ATT AAT AAG TAC AAC CAT G-3′/5′-CAT GGT TGT ACT TAT TAA TAG CTG CAT TGT AG T TGT GAT GAG G-3′).

Construction of FIX minigenes

A human FIX cDNA was a generous gift from Dr. George G. Brownlee (Chemical Pathology Unit, University of Oxford, Oxford, UK). The DNA fragment spanning part of exon 1, intron 1 and part of exon 2 of the human FIX gene was amplified from the human gene by PCR and sequenced. After removing part of intron 1 of the FIX gene by Pvu II treatment, the modified DNA fragment spanning parts of exon 1, intron 1 and exon 2 of the human FIX gene was excised with Bcl I and cloned into the Bcl I recognition sequence of the human FIX cDNA in the appropriate orientation to create a FIX minigene that was shown to express human FIX more efficiently than the FIX cDNA [10]. Similarly, the modified DNA fragment spanning parts of exon 1, intron 1 and exon 2 of the human FIX gene was cloned into the Bcl I recognition sequences of the macaque FIX cDNA to create a chimeric macaque FIX minigene.

Expression of macaque FIX in vitro

The human FIX minigene, macaque FIX minigene and mutant macaque FIX T262A minigene were cloned into plasmid p1.1c (Avigen Inc., Alameda, CA) in the appropriate orientation to create the plasmids p1.1c-hFIX, p1.1c-macFIX and p1.1c-macFIXT262A expressing human FIX, wild-type macaque FIX, and mutant macaque FIX T262A, respectively, under the control of the CMV promoter. Human embryo kidney (HEK) 293 cells were transfected with p1.1c-hFIX, p1.1c-macFIX and p1.1c-macFIXT262A in the presence of vitamin K (10 μg/mL), and the human FIX and macaque FIX expression levels in the conditioned media of the HEK 293 cells were analyzed by enzyme-linked immunosorbent immunoassays (ELISAs) and Western blot analyses. The FIX clotting activities in the conditioned media were determined by the APTT method using FIX-deficient human plasma (Dade Behring, Siemens Healthcare Diagnostics, Deerfield, IL).

Cloning of the hepatic control region (HCR) of the ApoE/C-I gene locus and the human α1 antitrypsin promoter (HAAT)

The 325-bp DNA fragment spanning the HCR region of the ApoE/C-I locus (nucleotides 1-325) [20–23] was amplified by PCR with a primer pair (sense primer, 5′-CAC TAG TCT GCA GGC TCA GAG GCA CAC-3′; antisense primer, 5′-GAA CCC GGA CCC TCT CAC ACT AC-3′) and cloned into the plasmid vector pCR Blunt II. The 297-bp DNA fragment spanning the HAAT promoter (nucleotides -270 to +27) was amplified by PCR and cloned as described previously [24].

AAV vector production

Plasmid vector p1.1c was excised with Spe I and Eco RI to remove the DNA fragments spanning the CMV promoter and the growth factor gene intron 1, and the DNA fragment of HCR was inserted into the same position. Subsequently, the DNA fragment of the HAAT promoter was cloned into the Eco RI site in the appropriate orientation to create the plasmid p1.1HCRHAAT. The macaque FIX T262A minigene was cloned on the 3′ side of the HAAT promoter of p1.1HCRHAAT in the appropriate orientation to create p1.1HCRHAAT-macFIXT262A. The DNA fragments spanning the promoter, LacZ gene and polyadenylation signal sequence of pAAV2-LacZ (Stratagene) were replaced with DNA fragments spanning the CMV promoter, macaque FIX T262A minigene and SV40 polyadenylation signal sequences of p1.1c-macFIXT262A to create the gene transfer vector pAAV2-CMV-macFIXT262A, in which these DNA fragments were flanked by ITR sequences of AAV serotype 2 (AAV2) as described previously [24,25]. Similarly, pAAV2-HCRHAAT-macFIXT262A was constructed by replacing the DNA fragment between the two ITRs of pAAV2-LacZ with the DNA fragment spanning the HCRHAAT, macaque FIX T262A minigene and polyadenylation signal sequences of p1.1HCRHAAT-macFIXT262A. The vector production system and HEK 293 cells were kindly supplied by Avigen Inc. The AAV vectors were packaged with the AAV8 capsid by pseudotyping. The chimeric packaging plasmid for AAV8 capsid pseudotyping was a generous gift from Dr. James M. Wilson (Division of Medical Genetics, Department of Medicine, University of Pennsylvania, Philadelphia, PA). For virus vector purification, virus particle-containing samples were treated with DNase (Benzonase; Merck Japan, Tokyo, Japan) and subjected to two rounds of cesium chloride-density gradient ultracentrifugation in HEPES-buffered saline (pH 7.4) in the presence of 25 mM EDTA at 21 °C as described [24,25]. Titration of the recombinant AAV vectors was carried out by quantitative PCR as described previously [24] with the primers 5′-GGT TGT TGG TGG AGA AGA TGC-3′ and 5′-GAT AGA GCC TCC ACA GAA TGC A-3′, and the probe 5′-FAM- GAT AGA GCC TCC ACA GAA TGC A-3′.

Animal experiments

Male C57BL/6 wild-type mice were purchased from SLC Inc. (Hamamatsu, Japan) and maintained under standard lighting conditions in a clean room. All surgical procedures were carried out in accordance with guidelines approved by the Institutional Animal Care and Concern Committee at Jichi Medical University. Before and after AAV vector injection, blood was drawn from the cervical vein plexus

of the mice and mixed with a 1/10 volume of 3.8% sodium citrate, before platelet-poor plasma was prepared by centrifugation. The AAV vectors were injected into the cervical vein plexus of the mice under anesthesia with isoflurane. Cyclophosphamide (100 μg/body/day; Sigma-Aldrich Japan, Tokyo, Japan) and tacrolimus (12.5 μg/body/day; Fujisawa Pharmaceuticals Co., Tokyo, Japan) were subcutaneously administered to the mice 5 times a week for 12 weeks after the vector injection as an immunosuppressant. Mouse plasma samples were subjected to an ELISA for human FIX to determine the plasma concentrations of macaque FIX T262A. Macaque plasma and macaque liver tissues were obtained from macaques under anesthesia according to the Institutional Animal Care and Concern Committee at Tsukuba Primate Research Center.

ELISA for human FIX

The ELISA that detects human FIX but not wild-type macaque FIX was performed as described previously [18]. Monoclonal antibody 3A6-coated microtiter plates (Maxisorp; Nalge Nunc, Rochester, NY) were blocked with 5% casein and then incubated with FIX-containing samples (conditioned media or mouse plasma samples) in phosphate-buffered saline (pH 7.4) containing 1% casein and 0.1% Triton X-100. Monoclonal antibody 3A6-bound human FIX or macaque FIX was detected with a horseradish peroxidase (HRP)-conjugated goat anti-human FIX polyclonal antibody (Affinity Biologicals Inc., Hamilton, Ontario, Canada). Purified human FIX was used as a standard. The ELISA that detects human FIX, wild-type macaque FIX and macaque FIX T262A was carried out using a sheep anti-human FIX polyclonal antibody (Cedarlane Laboratories Ltd., Hornby, Ontario, Canada) and an HRP-conjugated goat anti-human FIX polyclonal antibody (Affinity Biologicals Inc., Ancaster, Canada). Briefly, microtiter plates coated with the sheep anti-human FIX polyclonal antibody (1 μg/mL) were incubated with samples containing FIX. After washing, the microtiter plate-bound FIX was detected with the HRP-conjugated goat anti-human FIX polyclonal antibody.

Results

Binding of monoclonal antibody 3A6 to macaque FIX T262A

Monoclonal antibody 3A6 has been shown to be able to distinguish human FIX from wild-type macaque FIX [18]. Furthermore, it binds to wild-type FIX but not to mutant human FIX with an amino acid substitution of Ala to Thr (the amino acid residue at position 262 of macaque FIX is Thr), suggesting that the epitope for 3A6 resides in the segment including the Ala residue at position 262 of human FIX. Based on these data, we isolated a macaque FIX cDNA and expressed wild-type macaque FIX and mutant macaque FIX with an amino acid substitution of Thr to Ala at position 262 (macaque FIX T262A). Wild-type macaque FIX, mutant macaque FIX T262A and wild-type human FIX were expressed in HEK 293 cells and evaluated for their biological activities and reactivity with monoclonal antibody 3A6. The antigen concentrations of recombinant FIX were quantified by ELISAs using a sheep polyclonal antibody against human FIX and an HRP-conjugated goat polyclonal antibody against human FIX. The polyclonal antibody-based ELISA detected wild-type macaque FIX and mutant macaque FIX T262A, and all the FIX molecules had coagulation activities with similar specific activities (Table 1). Wild-type macaque FIX was not detected by the 3A6-based ELISA, whereas macaque FIX T262A and wild-type human FIX were quantified in a similar manner by this ELISA. The antigen concentrations of macaque FIX T262A determined by the polyclonal antibody-based ELISA were slightly higher than those by 3A6-based ELISA. This may be due to FIX fragments found in the supernatant of vector-transfected 293 cells.

The above data were confirmed by Western blot analyses (Fig. 1), which also showed that 3A6 bound to human FIX and macaque FIX T262A, but not to wild-type macaque FIX, while the polyclonal anti-FIX antibody bound to all the FIX molecules.

Table 1
Recombinant factor IX expression *in vitro*.

	Activity (U/mL)	Antigen (ng/mL)	
		Polyclonal Ab ELISA	3A6 ELISA
Wild-type human FIX	0.031	260	100
Wild-type macaque FIX	0.017	120	0
Macaque FIX T262A	0.024	200	101

Polyclonal Ab ELISA: the solid-phase (catching) antibody was a polyclonal anti-human FIX antibody.
3A6 ELISA: the solid-phase (catching) antibody was monoclonal anti-human FIX antibody 3A6.
The normal human plasma FIX concentration is 3 μg/mL by the 3A6 ELISA.

Expression of mutant macaque FIX T262A in vivo

AAV8 vectors carrying the macaque FIX T262A gene under the control of the CMV promoter (AAV8-CMV-macFIXT262A) or HCRHAAT promoter (AAV8-HCRHAAT-macFIXT262A) (Fig. 2) were injected into wild-type mice, and the expression of macaque FIX T262A was analyzed by the 3A6-based ELISA. Macaque FIX T262A was efficiently expressed in the mice using the AAV8 vectors, and high macaque FIX T262A expression was observed for more than 50 weeks (Fig. 3). In particular, the concentration of macaque FIX T262A in mouse plasma increased to supernormal levels (maximum: 14.4-31.5 μg/mL, 480-1050% of the normal plasma human FIX concentration) with AAV8-HCRHAAT-macFIXT262A at a dose of 5×10^9 vector genome/g. Such high FIX transgene expression was also achieved with the AAV9 vector carrying the same promoter and the macaque FIX T262A gene (data not shown). The mouse plasma samples containing macaque FIX T262A at 5.4-14.4 μg/mL (180-480% of the normal plasma human FIX concentration) were diluted and subjected to the coagulation assay for FIX to study the biological activity of macaque FIX T262A expressed *in vivo*. After subtraction of the basal mouse FIX activity, the FIX activity of mouse plasma containing macaque FIXT 262A at the immunological concentration of 10.9 ± 3.9 μg/mL ($n=5$) was 4.7 ± 9.3 U/mL ($n=5$), indicating that macaque FIX T262A expressed in mice was biologically active. The plasma levels of macaque FIX T262A in AAV8-HCRHAAT-macFIXT262A-injected mice were approximately 100-fold higher than those in AAV8-CMV-macFIXT262A-injected mice, suggesting that the HCRHAAT promoter worked more efficiently than the CMV promoter *in vivo*.

Detection of macaque FIX T262A in the presence of macaque plasma

To confirm that the 3A6-based ELISA could distinguish macaque FIX T262A from wild-type macaque FIX, recombinant macaque FIX T262A-containing samples were subjected to the 3A6-based ELISA in

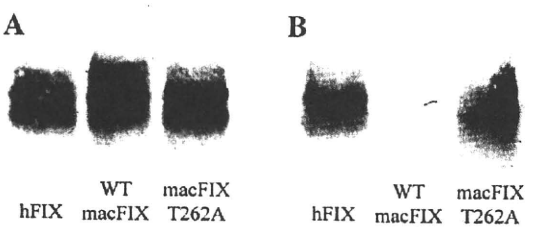


Fig. 1. Western blotting analyses of FIX molecules. Human FIX (hFIX), wild-type macaque FIX (WT macFIX) and macaque FIX T262A (macFIXT262A) expressed in the conditioned media of HEK 293 cells transfected with plasmid vectors carrying the corresponding FIX genes were analyzed by Western blotting with a polyclonal antibody against human FIX (A) and monoclonal antibody 3A6 (B).

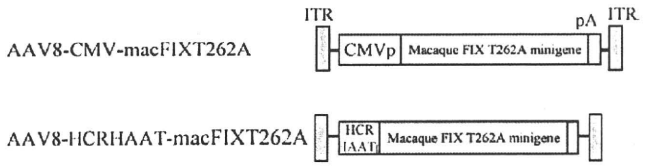


Fig. 2. Schematic representations of AAV8 vectors carrying the macaque FIX T262A gene. The AAV8 vectors carrying the macaque FIX T262A gene used in the present study are schematically illustrated. The promoter sequences, macaque FIX T262A gene and SV40 polyadenylation signal sequences are flanked by AAV2 ITRs.

the presence of increasing concentrations of macaque plasma to analyze the effect of wild-type macaque FIX in the plasma. As shown in Fig. 4, the presence of macaque plasma exhibited no inhibitory effects on the quantification of recombinant macaque FIX T262A expressed *in vivo* by the 3A6-based ELISA. These data confirm that the 3A6-based ELISA can be used to quantify macaque FIX T262A expressed in macaques.

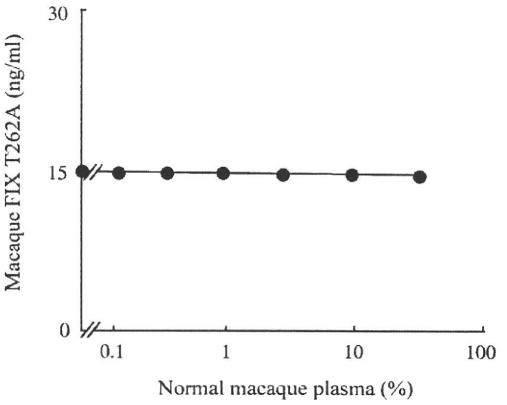


Fig. 4. Macaque plasma has no effect on the quantification of macaque FIX T262A by the 3A6-based ELISA. Mouse plasma containing macaque FIX T262A was diluted with buffer to adjust the macaque FIX T262A concentration to 30 ng/mL, mixed with equal amounts of buffer containing increasing concentrations of normal macaque plasma, and subjected to the 3A6-based ELISA for macaque FIX T262A quantification.

Discussion

To develop gene therapy technologies, good animal models are required. Hemophilia B mice (FIX-deficient mice) and natural hemophilia B dogs are available and have been used to study gene therapy approaches for hemophilia B. In a previous FIX gene transfer study with AAV2 vectors, a vector dose of 1.8×10^{12} vector genome/kg yielded plasma FIX levels of more than 1% in mice, whereas the same vector dose yielded circulating FIX levels of 0.2-0.4% in dogs. In humans, no significant increase in the FIX level was observed with the same vector dose [10]. One possible explanation for the differences in these results is that the transduction efficiency of the type 2 AAV vectors into the skeletal muscles of humans differs from those in the animal models. Unpredicted adverse effects occurred in patients who received an AAV2 vector for FIX gene expression in the liver [9]. In this regard, a primate model may be required to more closely mimic the situation in humans, though non-human primate experiments may not reflect perfectly human situations.

Cynomolgus macaques are native to southern Asia and have been used as simian models in medical research, such as gene therapy studies for Parkinson's disease [26]. As reported previously, human FIX may be immunogenic in macaques under certain conditions, such as expression of human FIX in rhesus macaques with adenoviral vectors and cynomolgus macaques receiving repeated subcutaneous injections of human FIX in the presence of Freund's adjuvant [17,19]. Therefore, as long as antibodies against human FIX develop in macaques during transduction with vectors carrying the human FIX gene, long-term studies of human FIX expression will be impossible. In this context, FIX molecules that are less immunogenic in macaques may be suitable for long-term expression of FIX transgenes in macaques. Possible candidate FIX molecules for this purpose would be tagged macaque FIX or mutant macaque FIX proteins that can be distinguished from the native wild-type macaque FIX. Anti-FIX monoclonal antibodies were screened for their inability to bind to simian FIX. One antibody was identified, which forms the basis for an ELISA that can quantify human FIX in macaque plasma down to a concentration of 1.7 ng/mL (0.06% of the normal plasma FIX concentration) [18]. In the present study, we developed a mutant macaque FIX that can be detected accurately with this specific anti-human FIX monoclonal antibody.

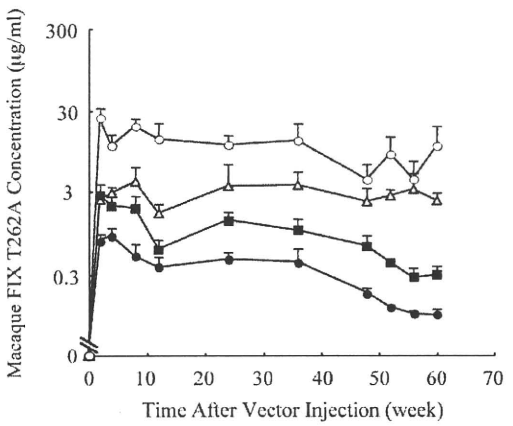


Fig. 3. Expression of macaque FIX T262A in mice using AAV8 vectors. The macaque FIX T262A levels in plasma of mice transduced with AAV8-HCRHAAT-macFIXT262A (open circles, 5×10^9 vector genome/g; open triangles, 5×10^8 vector genome/g) or AAV8-CMV-macFIXT262A (closed squares, 5×10^{10} vector genome/g; closed circles, 5×10^9 vector genome/g) were quantified by the 3A6-based ELISA.

Macaque FIX is highly homologous to human FIX, with the amino acid sequence identity of 97.1% [16]. Among the 415 amino acid residues of mature FIX, 12 amino acid residues at 11 distinct positions of human FIX are different from those in macaque FIX [16]. Human FIX has two potential N-glycosylation sites, while macaque FIX has three potential N-glycosylation sites, similar to the case for murine FIX, porcine FIX, and bovine FIX. Of the three potential N-glycosylation sites in macaque FIX, two are located at the same positions as the sites in human FIX. Human FIX lacks the potential N-glycosylation site located at position 260 of macaque FIX [18]. Since Thr at position 262 was substituted with Ala in macaque FIX T262A, the consensus sequence Asn-X-Thr for N-glycosylation at this position was mutated, indicating that macaque FIX T262A may loose this potential N-glycosylation site. Asn 260 of macaque FIX may be glycosylated because macaque FIX T262A migrated faster than wild-type macaque FIX on Western blotting. Regarding the carbohydrate composition, macaque FIX T262A may be humanized, but has only a single amino acid substitution. Therefore, the amino acid sequence of macaque FIX T262A may be closer to wild-type macaque FIX than to human FIX. In terms of the coagulation activity of macaque FIX T262A, it has almost the same coagulation activity as wild-type macaque FIX and human FIX. This observation suggests that the macaque FIX T262A conformation may not be significantly altered and that the N-glycosylation at position N260 may not contribute significantly to the coagulation activity of macaque FIX.

We created macaque FIX with a FLAG sequence at the C-terminal end (FLAG-tagged macaque FIX) and analyzed its properties.

Recombinant FLAG-tagged macaque FIX expressed in HEK 293 cells was efficiently secreted from the cells and detected by both a polyclonal antibody against human FIX and anti-FLAG antibody M2. However, its coagulation activity was significantly decreased compared with wild-type macaque FIX, macaque FIX T262A and human FIX expressed in HEK 293 cells (data not shown). These data suggest that the conformation of FLAG-tagged macaque FIX is altered. It is also possible that the FLAG sequence is immunogenic in macaques. Therefore, macaque FIX T262A may be closer to wild-type macaque FIX than tagged macaque FIX.

The mice that had high macaque FIX T262A expression survived more than 1 year without any events such as a sudden death. However, effect of over expression of FIX on the thrombogenecity may need to be studied carefully and precisely with the AAV vectors carrying the mouse FIX gene, since there may be a significant species difference in interaction of coagulation factors. Macaques with high FIX expression by the vector used in this study might also be good models for studying the effect of supra-physiological FIX level on the coagulation system. These may be the future studies.

In conclusion, macaque FIX T262A bound to the specific anti-human FIX monoclonal antibody 3A6, was efficiently expressed after gene transfer to the liver *in vivo*, and was quantified by the 3A6-based ELISA in the presence of wild-type macaque FIX. Macaque FIX T262A may have advantages over human FIX for studying the long-term expression of transgene-derived FIX in macaques. Therefore, macaque FIX T262A may be useful as a tool for FIX gene transfer studies in macaques.

Conflict of interest statement

The authors declare that they had no affiliation with or financial involvement in any organization or entity with a direct financial interest in the subject matter or materials discussed in the manuscript.

Acknowledgments

This study was supported by Grants-in-Aid for Scientific Research (20591155, 21591249 and 21790920) and the Support Program for Strategic Research Infrastructure from the Japanese Ministry of Education and Science, and Health Labor and Science Research Grants for Research on HIV/AIDS and Research on Intractable Diseases from the Japanese Ministry of Health, Labor and Welfare.

References

[1] Kay MA, Glorioso JC, Naldini L. Viral vectors for gene therapy: the art of turning infectious agents into vehicles of therapeutics. Nat Med 2001;7:33–40.
[2] Cavazzana-Calvo M, Fischer A. Gene therapy for severe combined immunodeficiency: are we there yet? J Clin Invest 2007;117:1456–65.
[3] Hasbrouck NC, High KA. AAV-mediated gene transfer for the treatment of hemophilia B: problems and prospects. Gene Ther 2008;15:870–5.
[4] Verma IM, Weitzman MD. Gene therapy: twenty-first century medicine. Annu Rev Biochem 2005;74:711–38.
[5] Cavazzana-Calvo M, Hacein-Bey S, de Saint Basile G, Gross F, Yvon E, Nusbaum P, et al. Gene therapy of human severe combined immunodeficiency (SCID)-X1 disease. Science 2000;288:669–72.
[6] Aiuti A, Slavin S, Aker M, Ficara F, Deola S, Mortellaro A, et al. Correction of ADA-SCID by stem cell gene therapy combined with nonmyeloablative conditioning. Science 2002;296:2410–3.
[7] Hacein-Bey-Abina S, Le Deist F, Carlier F, Bouneaud C, Hue C, De Villartay JP, et al. Sustained correction of X-linked severe combined immunodeficiency by ex vivo gene therapy. N Engl J Med 2002;346:1185–93.
[8] Hacein-Bey-Abina S, Von Kalle C, Schmidt M, McCormack MP, Wulffraat N, Leboulch P, et al. LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1. Science 2003;302:415–9.
[9] Manno CS, Pierce GF, Arruda VR, Glader B, Ragni M, Rasko JJ, et al. Successful transduction of liver in hemophilia by AAV-Factor IX and limitations imposed by the host immune response. Nat Med 2006;12:342–7.
[10] Manno CS, Chew AJ, Hutchison S, Larson PJ, Herzog RW, Arruda VR, et al. AAV-mediated factor IX gene transfer to skeletal muscle in patients with severe hemophilia B. Blood 2003;101:2963–72.
[11] Nathwani AC, Gray JT, Ng CY, Zhou J, Spence Y, Waddington SN, et al. Self-complementary adeno-associated virus vectors containing a novel liver-specific human factor IX expression cassette enable highly efficient transduction of murine and nonhuman primate liver. Blood 2006;107:2653–61.
[12] Hoffman BE, Dobrzynski E, Wang L, Hirao L, Mingozzi F, Cao O, et al. Muscle as a target for supplementary factor IX gene transfer. Hum Gene Ther 2007;18:603–13.
[13] Lu Y. Recombinant adeno-associated virus as delivery vector for gene therapy–a review. Stem Cells Dev 2004;13:133–45.
[14] Nathwani AC, Davidoff AM, Hanawa H, Hu Y, Hoffer FA, Nikanorov A, et al. Sustained high-level expression of human factor IX (hFIX) after liver-targeted delivery of recombinant adeno-associated virus encoding the hFIX gene in rhesus macaques. Blood 2002;100:1662–9.
[15] Nathwani AC, Gray JT, McIntosh J, Ng CY, Zhou J, Spence Y, et al. Safe and efficient transduction of the liver after peripheral vein infusion of self-complementary AAV vector results in stable therapeutic expression of human FIX in nonhuman primates. Blood 2007;109:1414–21.
[16] Lozier JN, Metzger ME, Donahue RE, Morgan RA. The rhesus macaque as an animal model for hemophilia B gene therapy. Blood 1999;93:1875–81.
[17] Lozier JN, Metzger ME, Donahue RE, Morgan RA. Adenovirus-mediated expression of human coagulation factor IX in the rhesus macaque is associated with dose-limiting toxicity. Blood 1999;94:3968–75.
[18] Mimuro J, Mizukami H, Ono F, Madoiwa S, Terao K, Yoshioka A, et al. Specific detection of human coagulation factor IX in cynomolgus macaques. J Thromb Haemost 2004;2:275–80.
[19] Tomokiyo K, Teshima K, Nakatomi Y, Watanabe T, Mizuguchi J, Nozaki C, et al. Induction of acquired factor IX inhibitors in cynomolgus monkey (Macaca fascicularis): a new primate model of hemophilia B. Thromb Res 2001;102:363–74.
[20] Allan CM, Taylor S, Taylor JM. Two hepatic enhancers, HCR.1 and HCR.2, coordinate the liver expression of the entire human apolipoprotein E/C-I/C-IV/C-II gene cluster. J Biol Chem 1997;272:29113–9.
[21] Simonet WS, Bucay N, Lauer SJ, Taylor JM. A far-downstream hepatocyte-specific control region directs expression of the linked human apolipoprotein E and C-I genes in transgenic mice. J Biol Chem 1993;268:8221–9.
[22] Dang Q, Walker D, Taylor S, Allan C, Chin P, Fan J, et al. Structure of the hepatic control region of the human apolipoprotein E/C-I gene locus. J Biol Chem 1995;270:22577–85.
[23] Miao CH, Ohashi K, Patijn GA, Meuse L, Ye X, Thompson AR, et al. Inclusion of the hepatic locus control region, an intron, and untranslated region increases and stabilizes hepatic factor IX gene expression in vivo but not in vitro. Mol Ther 2000;1:522–32.
[24] Ishiwata A, Mimuro J, Mizukami H, Kashiwakura Y, Takano K, Ohmori T, et al. Liver-restricted expression of the canine factor VIII gene facilitates prevention of inhibitor formation in factor VIII-deficient mice. J Gene Med 2009;11:1020–9.
[25] Ishiwata A, Mimuro J, Kashiwakura Y, Niimura M, Takano K, Ohmori T, et al. Phenotype correction of hemophilia A mice with adeno-associated virus vectors carrying the B domain-deleted canine factor VIII gene. Thromb Res 2006;118:627–35.
[26] Muramatsu S, Fujimoto K, Ikeguchi K, Shizuma N, Kawasaki K, Ono F, et al. Behavioral recovery in a primate model of Parkinson's disease by triple transduction of striatal cells with adeno-associated viral vectors expressing dopamine-synthesizing enzymes. Hum Gene Ther 2002;13:345–54.

Pediatr Transplantation 2010: 14: 369–376

Pediatric Transplantation
DOI: 10.1111/j.1399-3046.2009.01248.x

Impact of acute cellular rejection on coagulation and fibrinolysis biomarkers within the immediate post-operative period in pediatric liver transplantation

Mimuro J, Mizuta K, Kawano Y, Hishikawa S, Hamano A, Kashiwakura Y, Ishiwata A, Ohmori T, Madoiwa S, Kawarasaki H, Sakata Y. Impact of acute cellular rejection on coagulation and fibrinolysis biomarkers within the immediate post-operative period in pediatric liver transplantation.
Pediatr Transplantation 2010: 14: 369–376. © 2009 John Wiley & Sons A/S.

Abstract: We studied restoration of the coagulation and fibrinolysis system in pediatric patients following liver transplantation and biomarkers of blood coagulation and fibrinolysis for suspecting the occurrence of acute cellular rejection. Coagulation activity recovered rapidly within two days following transplantation, but it took approximately 21–28 days for full recovery of the coagulation and fibrinolysis factors synthesized in the liver. PAI-1 levels were significantly higher in patients at the time of acute cellular rejection compared with levels after control of AR, and levels on days 14 and 28 in patients without AR. Plasma protein C and plasminogen levels at the time of rejection were significantly lower than those on day 14 in patients without AR. Statistical analysis suggested that an increase in plasma PAI-1 at a single time point in the post-operative period is a reliable marker among the coagulation and fibrinolysis factors for suspecting the occurrence of acute cellular rejection. These data suggested that appropriate anticoagulation may be required for 14 days after liver transplantation in order to avoid vascular complications and measurement of plasma PAI-1 levels may be useful for suspecting the occurrence of acute cellular rejection in pediatric patients following liver transplantation.

Jun Mimuro[1], Koichi Mizuta[2], Yoichi Kawano[2], Shuji Hishikawa[2], Akiei Hamano[1], Yuji Kashiwakura[1], Akira Ishiwata[1], Tsukasa Ohmori[1], Seiji Madoiwa[1], Hideo Kawarasaki[2] and Yoichi Sakata[1]

[1]Division of Cell and Molecular Medicine, Center for Molecular Medicine, Jichi Medical University, School of Medicine, Shimotsuke, Tochigi-ken, Japan, [2]Division of Transplant Surgery, Jichi Medical University, School of Medicine, Shimotsuke, Tochigi-ken, Japan

Key words: liver transplant rejection – coagulation – fibrinolysis – plasminogen activator inhibitor 1

Jun Mimuro, MD, PhD, Division of Cell and Molecular Medicine, Center for Molecular Medicine, Jichi Medical University, School of Medicine, Shimotsuke, Tochigi-ken, 329-0498, Japan
Tel.: +81-285-58-7397
Fax: +81-44-7817
E-mail: mimuro-j@jichi.ac.jp

Accepted for publication 3 August 2009

Vascular thrombosis and immunological rejection of the transplanted liver in patients undergoing liver transplantation are frequent and serious post-operative complications (1). The majority of coagulation factors, factors regulating coagulation, and fibrinolysis factors are synthesized in the liver, and plasma coagulation factor levels may therefore fall for a short period after transplantation, but may return to normal levels upon regeneration of the grafted liver. Anastomosis of the vascular system of the grafted liver and the recipient vessels is carried out during liver transplantation, and anticoagulants are commonly administered for a period of time following surgery. However, restoration of the coagulation and fibrinolysis system following liver transplantation in pediatric patients has not been well studied (2, 3). In addition, the

Abbreviations: ADAMTS13, a disintegrin-like and metalloprotease with thrombospondin type 1 motif 13; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AR, acute rejection; AST, aspartate aminotransferase; ELISA, enzyme linked immunosorbent assay; LDH, lactate dehydrogenase; NAR, no acute rejection; PAI-1, plasminogen activator inhibitor 1; PELD, pediatric model for end-stage liver disease; PT-INR, prothrombin time-international normalized ratio; sES, soluble E-selectin; TM, thrombomodulin; vWF, von Willebrand factor; γ-GTP, γ-glutamyl transpeptidase.

thrombogenic state after liver transplantation is not well understood. We performed a single center study to investigate the coagulation and fibrinolysis system and the relationship between coagulation markers and acute cellular rejection following liver transplantation from living-related donors.

Materials and methods

Patients and study protocol

Sixty-three pediatric patients with liver failure due to biliary atresia (n = 59), ornithine transcarbamidase deficiency (n = 2), or Wilson's disease (n = 2) underwent living-related liver transplantation from April 2001 to March 2006 and were enrolled in this study. Most of the patients with biliary atresia had previously undergone hepatic porta-jejunostomies.

Description of patients

The patients were classified into two patient groups: one with acute cellular rejection (group AR, n = 24) and one with no acute cellular rejection (group NAR, n = 39). The diagnosis of acute cellular rejection was made by liver biopsy. There were no significant differences between group AR and group NAR in terms of age, gender, basal diseases, or the use of calcineurin inhibitors (data not shown). The PELD scores (AR, 13.0 ± 7.8; NAR, 15.1 ± 9.5), the amount of blood loss (AR, 85.0 ± 127.8 mL/kg; NAR, 125.8 ± 176.0 mL/kg), the amount of total blood transfusion (AR, 162.1 ± 109.1 mL/kg; NAR, 161.8 ± 170.8 mL/kg), the amount of plasma transfusion (AR, 64.1 ± 49.1 mL/kg; NAR, 94.2 ± 89.1 mL/kg), the cold ischemic time of graft liver (AR, 149.75 ± 126.4 min; NAR, 121.1 ± 69.1 min), and the warm ischemic time of graft liver (AR, 64.9 ± 18.1 min; NAR, 65.1 ± 13.3 min) upon operation were not significantly different between group AR and group NAR. Patients with severe infections or major bleeding episodes at the time of blood sampling for analysis were excluded from the analysis.

Immunosuppression and anticoagulation protocols

The standard protocol for immunosuppression was as follows. Both methylprednisolone and a calcineurin inhibitor (tacrolimus or cyclosporine) were used for immunosuppression. Intravenous administration of methylprednisolone (20 mg/kg) was started during the operation and the dosage was tapered to 3 mg/kg on day 1 and to 0.5 mg/kg on day 7 after liver transplantation. A calcineurin inhibitor was infused intravenously after transplantation and the blood concentration of tacrolimus or cyclosporine was adjusted to 18–20 ng/mL or 200–300 ng/mL till day 7 after liver transplantation, respectively. Intravenous injection of calcineurin inhibitor and methylprednisolone were converted to oral administration of these regimens after patient's oral intake had been fully confirmed and the blood concentration of tacrolimus or cyclosporine was adjusted 10–15 ng/mL or 100–150 ng/mL, respectively. The methylprednisolone dose was tapered to 0.06 mg/kg on day 30. Post-operative anticoagulation was performed with intravenous administration of dalteparin (low molecular weight heparin) at the dose of 2 U/kg/h, nafamostat mesilate (serine protease inhibitor with anticoagulant activity) at the dose of 0.1 mg/kg/h, and prostaglandin E1 at the dose of 0.01 μg/kg/min till day 7 after transplantation. Anticoagulation was continued with intravenous administration of heparin (unfractionated heparin) at the dose of 8 U/kg/h from day 8 to day 21 after liver transplantation.

Blood sample collection and analysis

All samples were obtained from patients with informed consent, according to the Declaration of Helsinki. Routine laboratory tests including complete blood counts, coagulation tests, blood chemistry analysis, and urinalysis were performed, and biomarkers of blood coagulation and fibrinolysis, i.e., PAI-1, TM, ADAMTS13, and sES were measured before and after liver transplantation on days 1, 3, 7, 10, 14, 21, and 28. Blood sampling was performed on days 35 and 49 in some patients. These were quantified using commercially available ELISAs (Mitsubishi Chemical Medience Co., Tokyo, Japan; Diaclone, Tepnel Research Products & Services, Cedex, France) (4, 5). The plasma activity levels of plasminogen and protein C were quantified using laboratory test kits (Siemens Healthcare Diagnostics Inc., Deerfield, IL, USA). Rationale for measurements of these biomarkers are as follows. PT-INR is currently used worldwide as a coagulation test to monitor the effects of anticoagulants such as coumarin in patients at risk of thrombosis. Protein C is a vitamin K-dependent protein synthesized in the liver that functions as an important regulatory factor for coagulation (6). Plasminogen is the zymogen of plasmin, a key enzyme in fibrinolysis, and is also synthesized in the liver (7). Therefore, plasma protein C and plasminogen levels were thought to be good markers for the restoration of the coagulation and fibrinolysis system following liver transplantation. Levels of these markers might correlate with protein synthesis in the liver, thereby reflecting regeneration of the graft liver. Additionally, measurement of these factors may also be important for patient management, because deficiency of protein C and type II plasminogen deficiency are thought to increase the risk of thrombosis (7, 8). The fibrin degradation product level, determined by the monoclonal antibody specific for degradation products of cross-linked fibrin, is a biomarker for the presence of a thrombus and is used to diagnose venous thrombosis and disseminated intravascular coagulation, however, the fibrin degradation product level may be affected by the presence of blood clots in the extravascular spaces (e.g., the peritoneal cavity), and may therefore not accurately reflect the thrombogenic state in the post-operative period. Thus, the soluble fibrin level was used to assess the thrombogenic state during the post-operative period following liver transplantation. PAI-1 is a primary regulator of fibrinolysis that is synthesized mainly in endothelial cells. Plasma PAI-1 levels change significantly in various pathological conditions (4). ADAMTS13 is the vWF cleaving protease that plays an important role in vWF multimer processing (9). It is synthesized in liver stellate cells and the liver is thought to be the primary source of ADAMTS13 in the circulation (9–11). In addition to the liver stellate cells, vascular endothelial cells in other organs may also be able to synthesize ADAMTS13 (12), and ADAMTS13 mRNA has been detected in the liver, kidneys and lungs in mice (13). ADAMTS13 deficiency results in platelet thrombus formation in the circulation, resulting in the development of a typical thrombotic microangiopathy (9). It is possible that ADAMTS13 deficiency might occur after liver transplantation, and plasma ADAMTS13 levels in patients were

therefore quantified following transplantation. TM, an important regulator of blood coagulation, is synthesized in vascular endothelial cells and is used as a marker of vascular injury (6). The sES level has been used as a marker for endothelial cell dysfunction (14). For example, the sES level is increased in systemic infections such as sepsis.

Diagnosis of acute cellular rejection

The diagnosis of acute cellular rejection was made by liver biopsy and was evaluated using the rejection activity index (3) scores (1, 15, 16). Patients suspected of suffering from acute cellular rejection because of deterioration of liver function (increased serum levels of bilirubin, AST, ALT, ALP, LDH, and γ-GTP compared with previous levels) were subjected to ultrasonography-guided liver biopsy. The liver biopsy specimens were examined for the presence of acute cellular rejection. Patients diagnosed with acute cellular rejection were subjected to intensive immunosuppressive therapy with intravenous methylprednisolone. Mycophenolate mofetil and/or OKT3 were also administered in some patients. Plasma samples obtained before starting administration of the intensive immunosuppressive regimens were evaluated in the following studies.

Statistical analysis

Statistical analyses were performed using SPSS software (SPSS Inc., Tokyo, Japan). Student *t*-tests were used to compare the mean values between groups. Multiple logistic regression analysis was used to investigate the association between biomarkers of blood coagulation and fibrinolysis and the occurrence of acute cellular rejection. p-values < 0.05 were considered statistically significant.

Results

Analysis of the coagulation and fibrinolysis system following liver transplantation

Changes in mean values of coagulation tests in patients without acute cellular rejection, vascular complications, or severe infections are shown in Fig. 1. The coagulation activity after liver transplantation was assessed by measuring prothrombin time (PT-INR). The mean PT-INR value rose to approximately 1.8 on day 1, but quickly fell again to < 1.5 on day 2, and then normalized gradually. These data suggest that the coagulation activity rapidly recovered after transplantation, once the graft liver started to function.

The mean protein C level of patients before liver transplantation decreased to 57.5% of the normal level. This may have been due to the decreased synthesis of protein C in the liver because most patients had liver failure. The mean protein C level fell to approximately 50% of the normal level on day 1 post-transplantation, and then increased gradually, reaching ≥80% of the normal level by day 14. The mean plasminogen level changed in a similar manner to protein C. By day 28, both protein C and plasminogen levels had returned to almost 90–100% of the normal levels. The nadir values of protein C and plasminogen on day 1 post-transplantation might

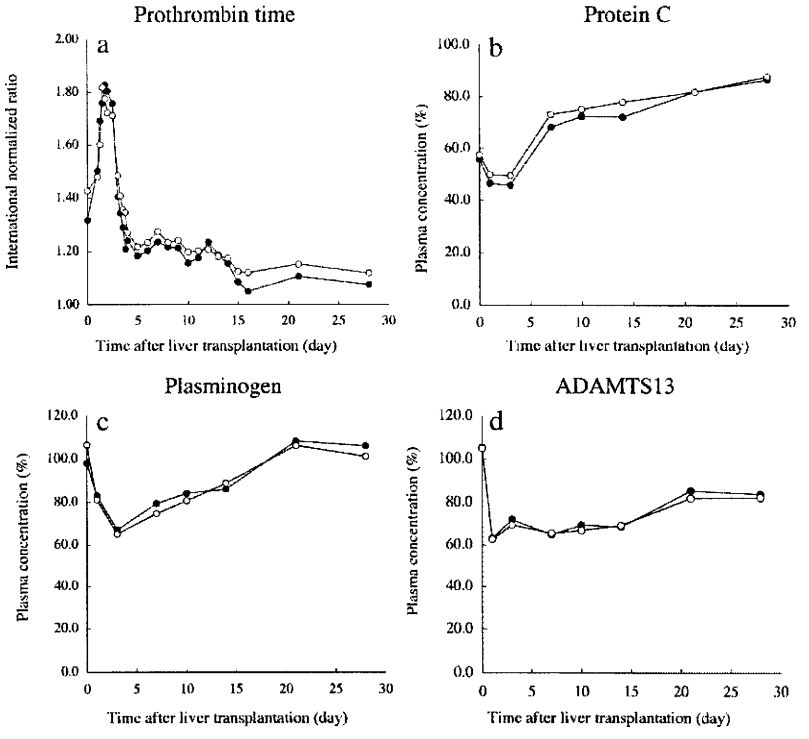


Fig. 1. Restoration of the coagulation and fibrinolysis system following liver transplantation. (a) The mean values of coagulation activity assessed by PT-INR and (b) plasma levels of protein C (normal range 67.1–129.0%), (c) plasminogen (normal range 85.0–120.0%), and (d) ADAMTS13 (normal range 100 ± 15%) of patients without complications (open circle) and of patients with acute cellular rejection (closed circle) are shown.

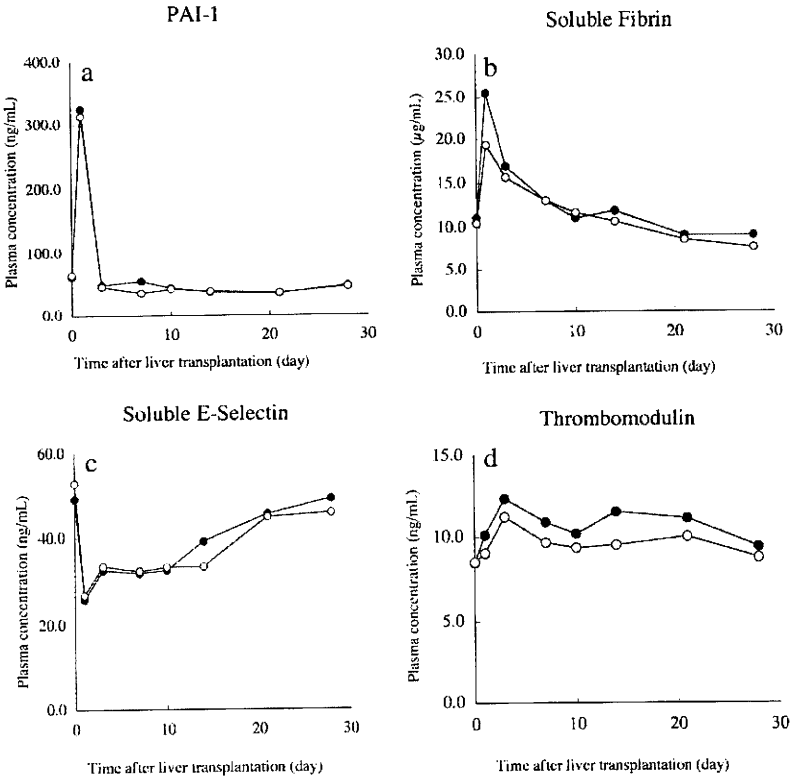


Fig. 2. Analysis of biomarkers of coagulation and fibrinolysis following liver transplantation. The mean plasma levels of PAI-1 (normal range 20–30 ng/mL), soluble fibrin (normal range: < 7.5 μg/mL), sES (normal range: < 37.5 ng/mL), and TM (normal range 4.46 ± 1.36 ng/mL) of patients without complications (open circle) and of patients with acute cellular rejection (closed circle) are shown.

be affected by plasma transfusion during and after surgery. These data suggest that the synthesis of coagulation factors in the graft liver may start on day 1, resulting in rapid recovery of coagulation activity, but it may take up to 14 days for recovery of the coagulation and fibrinolysis system to near normal levels, and 21–28 days for full restoration of the system after liver transplantation. These data also suggest that graft livers may regenerate to the appropriate size within four wk, though graft livers may vary in size depending on their recipients and donors. The average levels of the coagulation and fibrinolysis factors in patients with acute cellular rejection was not significantly different from those in patients without acute cellular rejection in the post-operative periods, but rate of restoration of the protein C and plasminogen levels on day 14 in group AR was slow.

Soluble fibrin levels in patients with no complications increased significantly on day 1 and then gradually decreased, normalizing by day 14 as shown in Fig. 2. These data suggest that the thrombotic state may continue for 14 days after liver transplantation, and that appropriate antithrombotic therapy may therefore be required during this period.

The mean plasma PAI-1 level was increased approximately 10-fold on post-operative day 1, compared with the normal level, but returned quickly to the normal level on day 3 after transplantation (Fig. 2). These data, together with the changes in the plasminogen level during the post-operative period (Fig. 1), suggest that fibrinolysis activity was suppressed on day 1 after liver transplantation.

The average plasma ADAMTS13 level decreased significantly on day 1 post-transplantation (Fig. 2), but the decrease was not as severe as that of protein C or plasminogen (Fig. 1). However, low levels of ADAMTS13 were maintained for 14 days after liver transplantation. These changes in plasma ADAMTS13 levels after liver transplantation did not parallel those of protein C or plasminogen (Fig. 1), reflecting the extrahepatic synthesis of ADAMTS13 and the possibility that ADAMS13 is synthesized not in hepatocytes, but in stellate cells in the liver. The plasma ADAMTS13 level fell to 28.4% of the normal level in one patient, but she showed no typical signs of thrombotic microangiopathy.

The TM level was increased on day 3 post-transplantation and remained at the upper limit of the normal range after day 7 (Fig. 2). The sES level was significantly increased in patients before liver transplantation (Fig. 2), which may be explained by the fact that many patients enrolled

in the study had undergone hepatic portajejunostomies and therefore had biliary tract infections before transplantation. The sES level was reduced post-transplantation, and remained almost within the normal range until day 14, but then was significantly increased on days 21 and 28 (Fig. 2). This increase in the sES level was not associated with the presence of infection or other disease states. The average changes of biomarkers of the coagulation and fibrinolysis system in patients with acute cellular rejection was not significantly different from those in patients without acute cellular rejection in the postoperative periods.

Three patients in this study suffered from hepatic artery thrombosis after liver transplantation, and an increased PT-INR (prolongation of prothrombin time) was detected in all three patients. Increase of plasma PAI-1 in the following samples of two patients was observed. Decrease of plasma protein C and plasma ADAMTS 13 in the following samples were observed in two patients. Other biomarkers did not change significantly. However, due to the small sample size, the predictive value of this test for the development of vascular complication was inconclusive.

Relationship between coagulation and fibrinolysis markers and acute cellular rejection

Patients were divided into two groups, group AR and group NAR, based upon the presence of acute cellular rejection as described above. The mean onset time of acute cellular rejection in group AR was on day 15 ± 8.7 after liver transplantation, while the mean time for data collection was on day 14 ± 7.9. Laboratory data and coagulation markers for each group at two time points were subjected to statistical analysis. Measurements taken immediately before the diagnosis of acute cellular rejection in group AR were compared with those taken after the cessation of rejection by intensive treatment with methylprednisolone in group AR, and those taken on days 14 and 28 in group NAR.

Statistical analysis of the mean levels of coagulation and fibrinolysis markers (Table 1) revealed that the PAI-1 level at the time of acute cellular rejection in group AR was significantly higher than that after cessation of rejection in group AR, and those on days 14 and 28 in group NAR (data for day 28 of group NR are not shown in Table 1).

The plasma protein C and plasminogen levels at the time of AR diagnosis in group AR were significantly lower than those on day 14 in group NAR.

Table 1. Coagulation and fibrinolysis biomarkers following liver transplantation

	Group AR (n = 24)		Group NAR (n = 39)
	Before*	After†	Day 14
PAI-1 (ng/mL)	79.3 ± 103.9‡	23.0 ± 10.7	38.5 ± 30.4
Plasminogen (%)	85.2 ± 22.8‡	99.4 ± 29.0	97.68 ± 13.8
Protein C (%)	65.7 ± 23.0‡	89.3 ± 37.9	87.2 ± 25.5
ADAMTS13 (%)	67.5 ± 24.1	77.8 ± 23.6	72.5 ± 17.4
ATIII (%)	96.3 ± 17.3	111.5 ± 57.4	99.3 ± 14.9
PT-INR	1.17 ± 0.21	1.08 ± 0.13	1.13 ± 0.13
Fibrinogen (mg/mL)	295.3 ± 116.4	296.3 ± 106.7	280.6 ± 74.0
Thrombomodulin (U/mL)	10.2 ± 3.8	10.8 ± 4.8	8.7 ± 5.2
Soluble E-selectin (μg/mL)	43.8 ± 16.7	46.4 ± 19.0	33.5 ± 17.2
Soluble fibrin (μg/mL)	13.57 ± 17.3	8.64 ± 14.9	10.2 ± 13.9

*Values at the time immediately before acute cellular rejection.
†Values after cessation of acute cellular rejection.
‡Values taken from the time point proximate to acute cellular rejection (before) are significantly different from those of group AR after cessation of acute cellular rejection (after) and those on day 14 in group NAR ($p < 0.01$).
Values are mean ± s.d.

The ADAMTS13 level at the diagnosis of AR in group AR appeared to be lower than that after cessation of rejection in group AR, and those on day 14 in group NR, though the differences were not statistically significant.

There were no significant differences between the levels of other coagulation and fibrinolysis markers in patients at the time of rejection diagnosis and after cessation of acute cellular rejection in group AR, or the levels on days 14 and 28 in group NAR.

The changes of the coagulation and fibrinolysis factors and biomarkers before the diagnosis of acute cellular rejection by liver biopsy were studied. These biomarkers levels of samples obtained from the patients proximate to the diagnosis of acute cellular rejection (AR-proximate sample in Fig. 3) were compared with those obtained before the AR-proximate sample (earlier sample in Fig. 3). The PAI-1 level in the AR-proximate samples were significantly higher than that in the earlier samples. The mean values of protein C, plasminogen, and ADAMTS13 in the AR-proximate samples was expected to be higher than those in the earlier samples, but they were lower than the earlier samples though the differences were not statistically significant. The mean values of other biomarkers in the two time points were not significantly different.

Multiple logistic regression analysis was performed to identify the coagulation and fibrinolysis markers for suspecting the occurrence of acute cellular rejection. Absolute values of coagulation and fibrinolysis factors (protein C, plasminogen, ADAMTS13) synthesized in the liver

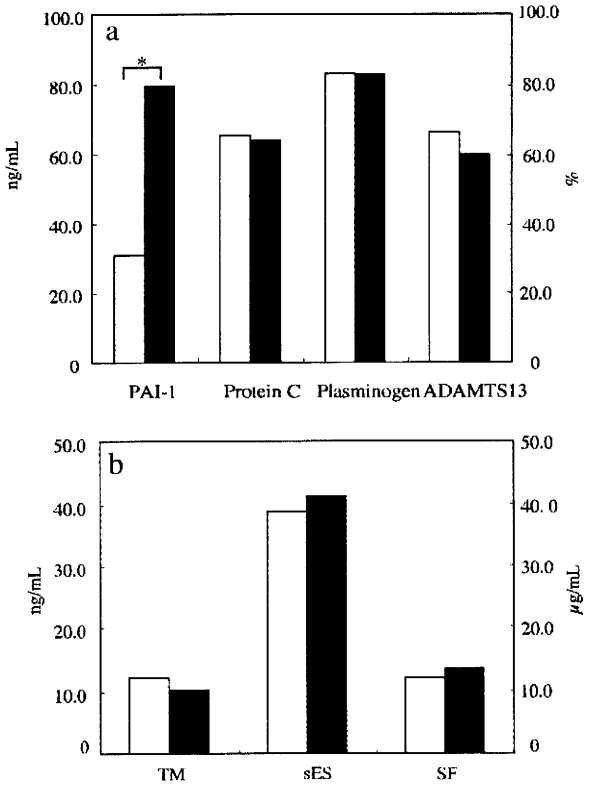


Fig. 3. Changes of coagulation and fibrinolysis factors and biomarkers in patients with acute cellular rejection. The mean plasma levels of coagulation and fibrinolysis factors and biomarkers obtained from patients with acute cellular rejection at two time points were shown. The AR-proximate samples (closed square) were obtained from the patients proximate to the diagnosis of acute cellular rejection. The earlier samples (open square) were obtained before the AR-proximate samples.

were difficult to ascertain using this method, and the changes in levels between time points were therefore analyzed. PAI-1 levels are independent of regeneration of the liver and an increase in plasma PAI-1 levels of >40 ng/mL at a single time point was therefore taken into account. Increases in soluble fibrin, TM, or sES since the previous time point, and above the normal range, were also taken into account. A summary of the multiple logistic regression analysis of coagulation and fibrinolysis markers is shown in Table 2. These data suggest that an increase in PAI-1 levels, and a decrease in protein C, plasminogen, or ADAMTS13 levels, were independently related to the occurrence of acute cellular rejection. Other markers were not related to the occurrence of acute cellular rejection (Table 2). Among these markers, an increase in plasma PAI-1 levels was observed in almost 80% of the patients in group AR.

Table 2. Multiple logistic regression analysis of biomarkers

	Odds ratio	Confidence interval	p-value
PAI-1	17.91	4.89–64.36	<0.001
ADAMTS13*	6.40	1.85–22.03	0.003
Protein C*	4.58	1.14–18.28	0.027
Plasminogen*	7.86	1.23–49.83	0.02
Soluble fibrin†	0.60	Not applicable	0.60
Soluble E-selectin†	0.60	Not applicable	0.65
Thrombomodulin†	0.421	Not applicable	0.42

*Decrease of marker values from the previous time point was adopted.
†Increase of marker values from the previous time point was adopted.

Discussion

The prevention and treatment of vascular thrombosis and immunological rejection of the transplanted liver during the post-operative period is a keystone of patient management. The present study analyzed the coagulation and fibrinolysis system following liver transplantation in pediatric patients to identify biomarkers for suspecting the occurrence of acute cellular rejection.

The present study suggests that the coagulation activity recovered rapidly once the graft liver started functioning, and that the graft liver might regenerate to the appropriate size in 21–28 days, with coincident full recovery of the coagulation and fibrinolysis system in pediatric patients undergoing liver transplantation. The present study also suggests that the hypercoagulable state persisted for 14 days after surgery, and that appropriate anticoagulation may therefore be required at least for 14 days post-transplant, even in the absence of any apparent vascular complications.

Recent advances in the management of patients with liver transplants have improved the clinical outcome of these patients. Adjustments in the doses of immunosuppressive drugs such as calcineurin inhibitors, based on their blood concentrations, are widely conducted after liver transplantation. However, immunological rejection of the transplanted liver still develops in a certain ratio of these patients, even when the blood calcineurin inhibitor concentration is within the appropriate therapeutic range (17, 18). A variety of methods for evaluating immune cell activation have been proposed as a basis for adjusting immunosuppressive therapy, and these have been shown to be useful for assessing the level of immunosuppression (19–22). Intensive treatment of acute cellular rejection with high dose methylprednisolone, with or without other medicines such as OKT3, is usually effective, though the prediction and rapid diagnosis of AR may be important for its effective treatment. In

this regard, the timely suspicion of acute cellular rejection using laboratory markers is a key indicator of the need for liver biopsy. Fluorescent-activated cell sorting analysis of CD25, CD28, and CD38 expression in peripheral lymphocytes is considered to be useful, not only for evaluation of the degree of immunosuppression, but also for the prediction of acute allograft cellular rejection (22). The present study showed that four coagulation and fibrinolysis markers, i.e., increase in PAI-1, decrease in protein C, decrease in plasminogen, and decrease in ADAMTS13, might be used as markers for suspecting the occurrence of acute cellular rejection. Statistical analysis suggested that an increase in the plasma PAI-1 level was the most reliable and sensitive marker for acute cellular rejection. Protein C, plasminogen, and ADAMTS13 are all synthesized in the liver, and their levels may therefore depend on the size and regeneration of the graft liver, and their plasma levels at any given time point might thus be less reliable as predictors of acute cellular rejection. PAI-1 is synthesized mainly in the vascular endothelial cells and its plasma level was elevated on day 1 after liver transplantation, and had returned to pretransplant levels after day 3. An increased plasma PAI-1 level at a single time point after day 1, together with a deterioration in liver function, may therefore be adopted as a predictive marker for acute cellular rejection.

Acute cellular rejection is characterized by portal inflammation, bile duct inflammation, and subendothelial cell inflammation (15, 16). Recent studies have suggested that not only T-cells, but also B-cells, are involved in acute cellular rejection, and cytokines and chemokines may also play roles in this process (23). As shown in a previous report, Toll-like receptor signaling through MyD88 may be involved in acute allograft rejection, indicating that toll-like receptors may be activated in the transplant setting causing inflammatory cytokine release (24). Therefore, the increase in PAI-1 levels seen during acute cellular rejection may be accounted for by immune cell-derived cytokine/chemokine activation of, and inflammation of, sinusoid-endothelial and portal vein endothelial cells. An increased PAI-1 level has previously been shown to be predictive for veno-occlusive disease developing after bone marrow transplantation (25), and this mechanism is thought to be responsible for busulfan-related toxic injury of sinusoidal endothelial cells (26, 27). The increase in plasma PAI-1 levels in patients with allograft cellular rejection is not as high as that seen in veno-occlusive disease, suggesting that the mechanisms and the outcomes of these PAI-1 increases may differ. Although the mechanisms of activation of endothelial cells may differ in veno-occlusive disease and in acute cellular rejection after allograft liver transplantation, both might result in increased plasma levels of PAI-1. Further studies are required to determine the precise mechanism responsible for the increase in PAI-1 levels occurring during acute cellular rejection.

Cytokines released from infiltrated immune cells in the liver, and inflammation in portal and sinusoid endothelial cells, might also inhibit the synthesis of ADAMTS13 in stellate cells, resulting in decreased plasma ADAMTS13 levels because the plasma ADAMTS13 level was significantly decreased in patients with sepsis-induced disseminated intravascular coagulation (5) and ADAMTS13 mRNA expression in the liver is decreased in endotoxin-injected mice (13). The decrease in protein C and plasminogen levels associated with acute cellular rejection might be due to their reduced synthesis by the graft hepatocytes, and a reduction in levels of these markers might therefore take time to become apparent. The decrease in plasminogen levels in patients with acute cellular rejection was less severe than that in protein C levels. These differences may be due to differences in the plasma half-lives of these molecules.

In conclusion, we have performed a comprehensive analysis of the coagulation and fibrinolysis system in pediatric patients undergoing orthotopic liver transplantation. Coagulation activity was quickly normalized by two days after liver transplantation. However, it took for 21–28 days for full restoration of the coagulation and fibrinolysis system. The post-operative thrombogenic state continued for approximately 14 days. PAI-1 may be used as predictive markers for acute cellular rejection in pediatric patients. These findings might also be applicable to adult liver transplant patients, though this needs to be confirmed by future prospective studies.

Acknowledgments

This study was supported by Grants-in-Aid for Scientific Research (20591155, 21591249, and 21790920) and Support Program for Strategic Research Infrastructure from the Japanese Ministry of Education and Science, and Health Labor and Science Research Grants for Research on HIV/AIDS and Research on Intractable Diseases from the Japanese Ministry of Health, Labor and Welfare.

References

1. Wiesner RH, Demetris AJ, Belle SH, et al. Acute hepatic allograft rejection: Incidence, risk factors, and impact on outcome. Hepatology 1998: 28: 638–645.

2. Muller C, Fleischer J, Renger F, Wolff H. The blood coagulation system in liver diseases with special reference to liver transplantation. Z Gesamte Inn Med 1981: 36: 660–665.
3. Sato Y, Nakatsuka H, Yamamoto S, et al. Coagulation and fibrinolytic systems during liver regeneration in the early period after adult living related partial liver transplantation. Transplant Proc 2008: 40: 2501–2502.
4. Madoiwa S, Nunomiya S, Ono T, et al. Plasminogen activator inhibitor 1 promotes a poor prognosis in sepsis-induced disseminated intravascular coagulation. Int J Hematol 2006: 84: 398–405.
5. Ono T, Mimuro J, Madoiwa S, et al. Severe secondary deficiency of von Willebrand factor-cleaving protease (ADAMTS13) in patients with sepsis-induced disseminated intravascular coagulation: Its correlation with development of renal failure. Blood 2006: 107: 528–534.
6. Esmon CT. Inflammation and the activated protein C anticoagulant pathway. Semin Thromb Hemost 2006: 32(Suppl. 1): 49–60.
7. Sakata Y, Aoki N. Molecular abnormality of plasminogen. J Biol Chem 1980: 255: 5442–5447.
8. Miyata T, Iwanaga S, Sakata Y, Aoki N. Plasminogen Tochigi: inactive plasmin resulting from replacement of alanine-600 by threonine in the active site. Proc Natl Acad Sci USA 1982: 79: 6132–6136.
9. Moake JL. Thrombotic microangiopathies. N Engl J Med 2002: 347: 589–600.
10. Uemura M, Tatsumi K, Matsumoto M, et al. Localization of ADAMTS13 to the stellate cells of human liver. Blood 2005: 106: 922–924.
11. Zheng X, Chung D, Takayama TK, Majerus EM, Sadler JE, Fujikawa K. Structure of von Willebrand factor-cleaving protease (ADAMTS13), a metalloprotease involved in thrombotic thrombocytopenic purpura. J Biol Chem 2001: 276: 41059–41063.
12. Turner N, Nolasco L, Tao Z, Dong JF, Moake J. Human endothelial cells synthesize and release ADAMTS-13. J Thromb Haemost 2006: 4: 1396–1404.
13. Mimuro J, Niimura M, Kashiwakura Y, et al. Unbalanced expression of ADAMTS13 and von Willebrand factor in mouse endotoxinemia. Thromb Res 2008: 122: 91–97.
14. Yano Y, Ohmori T, Hoshide S, et al. Determinants of thrombin generation, fibrinolytic activity, and endothelial dysfunction in patients on dual antiplatelet therapy: Involvement of factors other than platelet aggregability in Virchow's triad. Eur Heart J 2008: 29: 1729–1738.
15. Ormonde DG, de Boer WB, Kierath A, et al. Banff schema for grading liver allograft rejection: Utility in clinical practice. Liver Transpl Surg 1999: 5: 261–268.
16. Anthony JD, Amar PD, Linda F, et al. Banff schema for grading liver allograft rejection: an international consensus document. Hepatology 1997: 25: 658–663.
17. Cakaloglu Y, Tredger JM, Devlin J, Williams R. Importance of cytochrome P-450IIIA activity in determining dosage and blood levels of FK 506 and cyclosporine in liver transplant recipients. Hepatology 1994: 20: 309–316.
18. Klintmalm GB, Nery JR, Husberg BS, Gonwa TA, Tillery GW. Rejection in liver transplantation. Hepatology 1989: 10: 978–985.
19. van den Berg AP, Twilhaar WN, Mesander G, et al. Quantitation of immunosuppression by flow cytometric measurement of the capacity of T cells for interleukin-2 production. Transplantation 1998: 65: 1066–1071.
20. Boleslawski E, Conti F, Sanquer S, et al. Defective inhibition of peripheral $CD8^+$ T-cell IL-2 production by anti-calcineurin drugs during acute liver allograft rejection. Transplantation 2004: 77: 1815–1820.
21. Kowalski RJ, Post DR, Mannon RB, et al. Assessing relative risks of infection and rejection: A meta-analysis using an immune function assay. Transplantation 2006: 82: 663–668.
22. Boleslawski E, Benothman S, Grabar S, et al. CD25, CD28 and CD38 expression in peripheral blood lymphocytes as a tool to predict acute rejection after liver transplantation. Clin Transplant 2008: 22: 494–501.
23. Tarlinton DM, Batista F, Smith KG. The B-cell response to protein antigens in immunity and transplantation. Transplantation 2008: 85: 1698–1704.
24. Goldstein DR, Tesar BM, Akira S, Lakkis FG. Critical role of the Toll-like receptor signal adaptor protein MyD88 in acute allograft rejection. J Clin Invest 2003: 111: 1571–1578.
25. Pihusch M, Wegner H, Goehring P, et al. Diagnosis of hepatic veno-occlusive disease by plasminogen activator inhibitor-1 plasma antigen levels: A prospective analysis in 350 allogeneic hematopoietic stem cell recipients. Transplantation 2005: 80: 1376–1382.
26. Carreras E, Rosinol L, Terol MJ, et al. Veno-occlusive disease of the liver after high-dose cytoreductive therapy with busulfan and melphalan for autologous blood stem cell transplantation in multiple myeloma patients. Biol Blood Marrow Transplant 2007: 13: 1448–1454.
27. Dix SP, Wingard JR, Mullins RE, et al. Association of busulfan area under the curve with veno-occlusive disease following BMT. Bone Marrow Transplant 1996: 17: 225–230.

Thrombosis Research 125 (2010) 128–133

Contents lists available at ScienceDirect

Thrombosis Research

journal homepage: www.elsevier.com/locate/thromres

Regular Article

Low level of factor V is associated with development of deep-vein thrombosis in Japanese patients

Etsuji Suehisa [a,*], Tomio Kawasaki [b], Masayuki Toku [a], Yoh Hidaka [a]

[a] Laboratory for Clinical Investigation, Osaka University Hospital, Japan
[b] Cardiovascular Surgery, Osaka University Graduate School of Medicine, Japan

ARTICLE INFO

Article history:
Received 10 March 2009
Received in revised form 7 May 2009
Accepted 14 May 2009
Available online 4 June 2009

Keywords:
deep-vein thrombosis
factor V level
factor V antigen
phospholipid

ABSTRACT

Background: Factor V, having two functions (procoagulant and anticoagulant), is a key factor in blood coagulation, and low plasma levels of factor V may be a risk factor for thrombosis.
Objective: The levels of plasma factor V antigen (FV:Ag), and the phospholipid binding capability of Factor V (FV:PL-bound) were evaluated in patients with deep-vein thrombosis (DVT).
Methods: Levels of FV:Ag, and FV:PL-bound were expressed as a percentage of the normal level found in pooled plasma from control subjects. One hundred and twenty-three Japanese patients with deep-vein thrombosis (DVT) were included, with 100 age and sex-matched healthy control subjects.
Results: The FV:Ag, and FV:PL-bound values were significantly lower in DVT patients than in healthy subjects ($p<0.05$ and $p<0.005$, respectively). Among the 123 patients, 30 for FV:Ag (24.4%), and 32 for FV:PL (26%) had less than the arbitrary cutoff point (set at the 5th percentile of the value for FV:Ag and FV:PL-bound from healthy subjects), and the odds ratios (ORs) were 6.1 (95% confidence interval [CI], 2.3-16.5) and 6.7 (95%CI, 2.5-17.9), respectively. When patients with a deficiency of natural anticoagulants (antithrombin, protein C, and protein S) were excluded from the analysis, the ORs increased for all patients (6.6 for FV:Ag (95%CI, 2.4-18.3) and 7.4 for FV:PL-bound (95%CI, 2.7-20.3). Moreover, twenty-one (17%) of the 123 DVT patients, and 1 (1%) of 100 control subjects had values below the cutoff points for both FV:Ag and FV:PL-bound, and the OR was 21.6 (95%CI, 2.85-163.1).
Conclusions: These results suggest that low levels of factor V are associated with development of DVT, and may be a predictor for DVT.

Introduction

Venous thromboembolism (VTE), such as deep-vein thrombosis (DVT) and pulmonary embolism, is a common disorder [1,2]. Despite much investigation into the causes of thrombosis, the causative factors for many cases remain unknown. A point mutation in the factor V (FV) gene (R506Q: FV Leiden) [3] results in a reduced sensitivity of plasma factor Va (FVa) to be inactivated by activated protein C (APC) [4] and is present in more than 20% of Caucasian patients with DVT [5]. However, this FV Leiden mutation has not been reported in the Japanese population up to now.

Blood coagulation FV has a central role in procoagulant and anticoagulant pathways: FV is activated by factor Xa (FXa) or thrombin and accelerates the activation of prothrombin by FXa before FVa is degraded by the APC [6]. APC limits thrombin generation by cleaving FVa, and also factor VIIIa (FVIIIa) inhibition by APC requires FV, but not FVa, as a cofactor [7–9]. Whether the level of FV in plasma affects the risk of thrombosis is unclear, although it is hypothetically possible that high or low levels of FV may increase the thrombotic risk.

High plasma levels of FV may lead to an increased prothrombinase activity and an increased risk of thrombosis, and high FV coagulant levels have been found to be an independent risk factor for myocardial infarction [10]. No significant association was found between the FV antigen level and venous thrombotic risk [11]. Low FV levels are associated with a reduced APC cofactor activity in the inactivation of FVIII/VIIIa [7–9]. This results in an APC-resistant phenotype and therefore might be associated with an increased risk of thrombosis. On the other hand, FV circulates in two forms, FV1 and FV2, having slightly different molecular masses and phospholipid-binding properties (the affinity to phospholipid of FVa1 is lower than FVa2 at low phospholipid concentration). FVa1 is inactivated by APC at 15-fold lower rates than FVa2, both in the absence and in the presence of

Abbreviations: VTE, venous thromboembolism; DVT, deep vein thrombosis; APC, activated protein C; FV:Ag, factor V antigen; FV:PL-bound, phospholipid bound factor V; TTBS, Tween20 in TBS; TBS, tris-buffered saline.

* Corresponding author. Laboratory for Clinical Investigation, Osaka University Hospital, 2-15 Yamada-oka, Suita-City, Osaka, 565-0871, Japan. Tel./fax: +81 06 6879 6655.
E-mail address: esuehisa@hp-lab.med.osaka-u.ac.jp (E. Suehisa).

0049-3848/$ – see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.thromres.2009.05.010

protein S, and in the process of generation, and down-regulation of FVa cofactor activity on physiological membranes, the overall procoagulant activity of FV1 can considerably exceed that of FV2 [12]. This result indicates that the low level of the binding capacity to phospholipid of FV may lead to thrombosis.

The aim of this study was to determine whether a plasma concentration of FV is associated with thrombosis. To investigate whether impaired binding of FV to phospholipid is associated with deep vein thrombosis, we developed an assay method for the phospholipid binding capacities of FV.

Material and methods

Patients and control subjects

The study group consisted of 123 patients (53 men and 70 women between 19 and 88 years of age (mean, 54 years) with DVT. Patients were referred to the Department of Cardiovascular Surgery at Osaka University Medical Hospital during an 88-month period. Blood samples were obtained from these patients at least 3 months after the thrombotic episode and at least 4 weeks after discontinuation of oral anticoagulants. Patients taking oral anticoagulants were excluded from the study. None of the patients received heparin during the investigation period. DVT was diagnosed on the basis of clinical manifestations and findings of duplex scanning, radioisotope venography, and contrast venography. We determined natural anticoagulant deficiencies according to the method of our previous study [13].

Healthy Japanese subjects (100 control subjects) without any history of venous thrombosis were randomly selected from volunteers. Patients and volunteers were informed that blood samples were being obtained for research purposes and that their privacy would be protected.

Blood samples

Venous blood samples were obtained by venipuncture with the use of a tourniquet. Blood was drawn into Vacutainer tubes (5.0 ml total volume, Sekisui, Yamaguchi, Japan) containing 3.13% (w/v) trisodium citrate (9:1, v/v). Platelet-poor plasma was prepared by centrifugation at 2800 g for 10 minutes. Aliquoted plasma was kept at −80 °C until use. Pooled normal plasma used in the study was obtained from 20 of the healthy volunteers who were not receiving medication. These individuals were not screened for abnormalities associated with an increased risk of venous thrombosis.

Measurement of FV antigen (FV:Ag)

FV antigen (FV:Ag) levels were quantified using an enzyme-linked immunosorbent assay (ELISA) specific for FV. Microtiter plates (Nunc-Immunoplate, Maxi-Sorp, Roskilde, Denmark) were coated overnight at 4 °C with 100 μl per well of anti-human Factor V sheep purified IgG (Cedarlane, Ontario, Canada) suspended at a concentration of 10 μg/ml in 50 mM carbonate at pH 9.6. The plates were incubated overnight at 4 °C with 100 μl of Tris-bufferd saline (TBS: 50 mmol/L Tris, 150 mmol/L NaCl, pH 7.4) containing 1% bovine serum albumin (BSA: Sigma, MO, USA), and then washed three times with 0.1% Tween 20 in TBS (TTBS). Then, 100 μl samples of diluted plasma (diluted 50 times with 1.0% BSA-TTBS) were added to each well. After a 2 hour incubation at room temperature, the plates were washed three times with TTBS. Bound FV antigen was detected using peroxidase conjugated anti-human Factor V sheep IgG (Cedarlane, Ontario, Canada). The plates were developed for 10 minutes with o-phenylenediamine (OPD: Sigma, MO, USA) and H_2O_2 diluted in 0.1 mol/L citrate-phosphate buffer at pH 5.0. The reaction was stopped by addition of 2.5 mol/L H_2SO_4 and the absorbance measured at 490 nm. Dilutions of pooled normal plasma (1 : 25 – 1 : 800 dilution) were used to calibrate the assay. Results were expressed as a percentage of that found in normal pooled plasma.

Assay for phospholipid bound FV (FV:PL-bound)

Phosphatidylserine (L-α-Phosphatidyl-L-serine: Sigma, MO, USA) in ethanol (25 μg/50 μl per well) was coated on the surface of wells of microtiter plates (Immuron 1B: Dynatech Laboratories Alexandria, VA) by evaporation under air. The plates were incubated overnight at 4 °C with 100 μl of TBS containing 1% BSA and then washed three times with 0.05% Tween 20 in TBS. Then, 50 μl of diluted plasma sample (diluted 50 times with 1.0% BSA-TTBS) was added to each well. After a 2 hour incubation at room temperature, plates were washed three times with 0.05% Tween 20 in TBS.

FV bound to phosphatydilserine (FV:PL-bound) was detected using peroxidase conjugated anti-human Factor V sheep IgG. The plates were developed for 10 minutes with o-phenylenediamine and H_2O_2 diluted in 0.1 mol/L citrate-phosphate buffer at pH 5.0. The reaction was stopped by addition of 2.5 mol/L H_2SO_4 and the absorbance measured at 490 nm. Dilutions of pooled normal plasma (1:25 – 1:800 dilution) were used to calibrate the assay. Results were expressed as a percentage of that found in normal pooled plasma.

Statistical analysis

The Mann-Whitney U test was used to compare the results for FV:Ag or FV:PL-bound between the DVT patients and controls. Between-group differences in age were examined by Student's *t*-test, and between-group differences in sex ratio were examined by the chi-square test. Odds ratios (ORs) with 95% confidence intervals (CIs) were calculated as an estimate of the relative risk for development of DVT. All analyses were performed with StatFlex ver. 5.0 software (Artech Inc., Osaka, Japan). A p value of <0.05 was considered statistically significant.

Results

Standard curve for the FV:PL-bound assay

For the standard curve, FV concentrations from 200% to 6.25% of normal were prepared with dilutions of pooled normal plasma (x25,

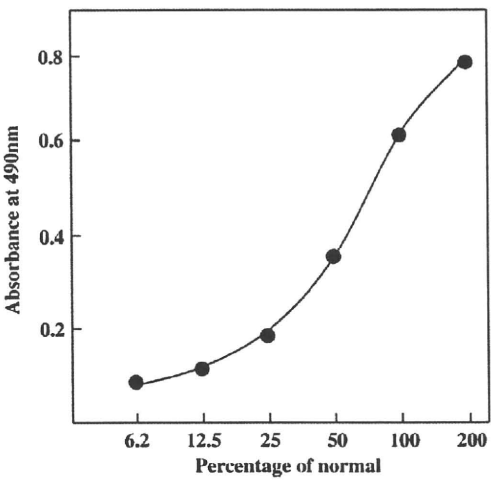


Fig. 1. Standard curve for phospholipid bound factor V. Standard samples were prepared by dilution of normal pooled plasma (200%, ×25. 100%, ×50. 50%, ×100. 25%, ×200. 12.5%, ×400. 6.25%, ×800).