DNA pull-down assay

We performed DNA pull-down assays (known as DNA affinity precipitation or DNAP assay) as described earlier (*17*). The nuclear extracts were incubated with biotin-labelled DNA probes and 15 μg of poly d(I-C) (Roche Applied Science, Penzberg, Germany) in DNAP buffer (20 mM HEPES-KOH pH 7.9, 80 mM KCl, 1 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 10% glycerol and 0.1% TritonX-100) on ice for 45 min. Subsequently, 500 μg of Dynabeads M-280 streptavidin (Invitrogen) were added and incubated at 4°C for further 1 h. The beads were washed, and bound proteins were eluted in SDS sample buffer, separated using a 10% SDS–PAGE gel and characterized by western blot analysis.

Chromatin immunoprecipitation assay

The chromatin immunoprecipitation (ChIP) assay was performed as described earlier (*18*). EAhy926 cells were fixed with 1.5% formaldehyde at 37°C for 10 min. For immunoprecipitation, the nuclear proteins were incubated at 4°C for 2 h with antibodies specific to the NF-κB subunits p65, p50, c-Rel or phospho-p65 (Ser536) or with non-specific IgG. The protein–antibody complexes were incubated with Dynabeads protein G (Invitrogen) at 4°C for 1 h. DNA was purified with a High Pure PCR cleanup micro kit (Roche) and used as a template for subsequent amplification. The primers were 5′-CGGGCTCGGGCAGGGCGGGTC-3′ (−215/−195, forward) and 5′-CCGCCCCGCCGCCCGCAC-3′ (−103/−120, reverse), which amplified a 121-bp region of *SDC4* promoter containing putative NF-κB binding sites. PCR products were resolved on a 2% agarose gel that contained 1 μg/ml ethidium bromide.

Immunoprecipitation western blot analysis

We performed immunoprecipitation western blot analysis as described earlier (*19*). In brief, EAhy926 cells (1.5×10^6 cells) were lysed in high-salt lysis buffer [20 mM HEPES pH 7.4, 10% glycerol, 0.35 M NaCl, 1 mM $MgCl_2$, 0.5% Triton X-100, 1 mM DTT, protease inhibitor cocktail and phosphatase inhibitor cocktail (Nakarai Tesque)] for 30 min at 4°C. The lysate was diluted to make a final concentration of 300 mM NaCl, to which anti-p65 antibody or a normal rabbit IgG and Dynabeads Protein A (Invitrogen) were added. After incubation for 4 h at 4°C, beads were washed with wash buffer (10 mM HEPES pH 7.9, 1 mM $MgCl_2$, 300 mM NaCl, 10 mM KCl and 0.2% Triton X-100) and eluted with SDS sample buffer (50 mM Tris-HCl, 2% SDS, 2-mercaptoethanol, 10% glycerol and bromophenol blue). After boiling, the sample was subjected to SDS–PAGE electrophoresis and western blot analysis.

Transfection with small interfering RNA

SMARTpool NF-κB p65 small interfering RNAs (siRNAs) were commercially designed by Dharmacon (Dharmacon, Dallas, TX, USA). The pooled siRNAs contained p65-specific sequences. A non-targeting siRNA#3 (Dharmacon) was used as a negative control. EAhy926 cells were cultured for 24 h and transfected with p65 SMARTpool siRNAs (5 nM) or non-targeting control-pool siRNA (5 nM) using Lipofectamine RNAiMAX (Invitrogen) at 37°C for 6 h. For quantitative RT-PCR assay, cells were further incubated in fresh media for 42 h. For luciferase assay, cells were further incubated in fresh media for 18 h, subsequently transfected with luciferase reporter plasmids for 6 h and then incubated in fresh media for additional 18 h. During the last 3 h in both assays, cells were treated with or without TNF-α.

Statistical analysis

Data are presented as mean ± SD of three independent experiments. Significant differences in the flow cytometry analysis, quantitative RT-PCR and luciferase assays were analysed using Student's *t*-test.

Results

Effects of TNF-α on syndecan-4 expression on the EAhy926 cell surface

We demonstrated that TNF-α treatment induced a 1.6-fold increase in syndecan-4 expression on the EAhy926 cell surface compared with untreated cells by flow cytometry analysis (Fig. 1A). We also showed that TNF-α treatment for 3 h resulted in a 3.1-fold increase in *SDC4* mRNA expression in EAhy926 cells by quantitative RT-PCR (Fig. 1B).

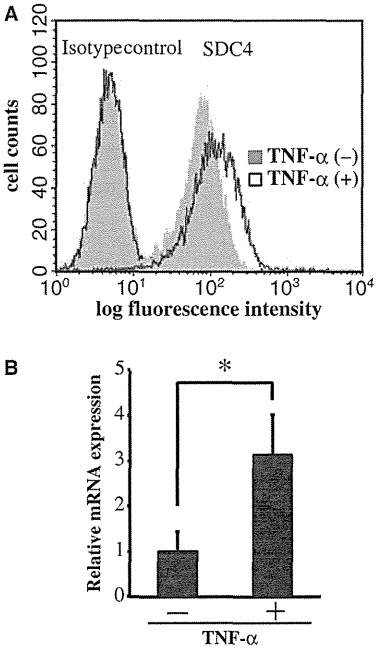

Fig. 1 Effects of TNF-α on syndecan-4 expression in EAhy926 cells. (**A**) Expression of syndecan-4 on the cell surface of EAhy926 cells with or without TNF-α treatment was examined by flow cytometry analysis. The *x*-axis indicates Alexa488 fluorescence intensity, indicative of syndecan-4 staining, and the *y*-axis indicates cell counts. Shaded, untreated; thick line, TNF-α treated. (**B**) Expression of *SDC4* mRNA by EAhy926 cells with or without TNF-α treatment for 3 h was determined by quantitative RT-PCR. The levels of *SDC4* mRNA were normalized with those of *GUSB* mRNA. Results are presented as mean ± SD of three independent experiments. $^*P < 0.05$ by *t*-test compared with untreated cells.

Effects of TNF-α on SDC4 promoter activity

Next, we performed a luciferase assay to investigate critical regions of the *SDC4* promoter involved in upregulation by TNF-α treatment. Four *SDC4* promoter constructs, pSDC4 −1563, −544, −370 and −217, showed markedly enhanced luciferase activity after TNF-α treatment, whereas the pSDC4-151 construct did not (Fig. 2A), suggesting that pSDC4-151 lacked a critical *SDC4* promoter element.

We analysed the *SDC4* promoter using an online data for the putative binding sites of transcription factors, TFSEARCH (http://www.cbrc.jp/research/db/TFSEARCH.html), and found that the *SDC4* promoter had two putative binding sites for the NF-κB subunits p65, p50 or c-Rel at −191/−182 and −137/−127 upstream of the translation start site (with A of the ATG translation start site as +1) as pointed out earlier (*20*). The pSDC4 −1563, −544, −370 and −217 constructs have these two NF-κB binding sites; however, the pSDC4 −151 construct has only downstream one of the sites. To confirm the crucial sites for *SDC4* expression, we generated 2 bp mutations in each putative NF-κB site or both sites of the pSDC4−1563 construct that were designed to block NF-κB binding. The MT −137/−127 and MT-double mutants failed to increase luciferase activity in response to TNF-α

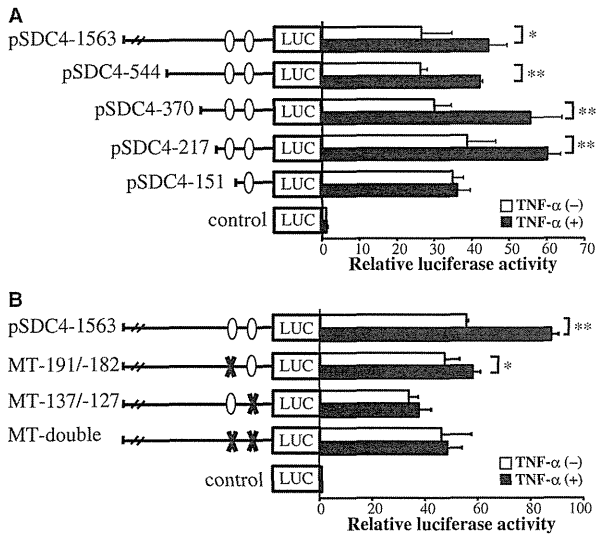

Fig. 2 Measurement of *SDC4* promoter activity by luciferase assay. (**A**) EAhy926 cells were transfected with reporter plasmids (illustrated at left) and treated for 3 h with or without TNF-α (10 ng/ml). Firefly luciferase activity was normalized to co-transfected *Renilla* luciferase activity. Relative luciferase activity was expressed as fold changes in activation compared with control vector. Values represent the mean ± SD of three independent experiments. Ovals indicate putative NF-κB sites in the syndecan-4 promoter region. (**B**) Luciferase reporter constructs with or without mutated NF-κB sites were transfected into EAhy926 cells. Open oval, wild-type NF-κB binding site; cross, mutated NF-κB binding site. $*P<0.05$; $**P<0.01$ by *t*-test compared with untreated cells.

treatment, whereas the MT −191/−182 mutant marginally increased luciferase activity after TNF-α treatment (Fig. 2B). These results suggested that the 5′ sequence including mutated upstream NF-κB site (−191/−182) might have some effects on upregulation of *SDC4* promoter activity by TNF-α through the downstream NF-κB site (−137/−127), and that both the NF-κB sites might be necessary for optimal *SDC4* upregulation by TNF-α but the downstream NF-κB site could be more essential.

NF-κB localization

We performed immunofluorescence staining to identify the localization of NF-κB subunits in response to TNF-α treatment. In quiescent cells, NF-κB localized mostly to the cytoplasm. After TNF-α treatment, p65 and p50 appeared to move into the nucleus (Fig. 3A and B) and c-Rel slightly into the nucleus (Fig. 3C). We went on to examine TNF-α-induced effects on the localization of NF-κB by western blot analysis using nuclear and cytoplasmic protein extracts (Fig. 3D). In quiescent cells, most NF-κB subunits were found in the cytoplasm but not in the nucleus. After TNF-α treatment for 30 min, they appeared in both the cytoplasm and nucleus. These results suggested that TNF-α caused rapid translocation of NF-κB subunits from the cytoplasm to the nucleus of EAhy926 cells.

NF-κB binding to the SDC4 promoter region in vitro

We performed EMSAs to determine whether NF-κB could bind *in vitro* to the sequences of the each putative NF-κB binding site (−137/−127 or −191/−182) in the *SDC4* promoter. We detected three extra bands (1, 2 and 3, Fig. 4A; 5, 6 and 7, Fig. 4B) of the TNF-α-induced nuclear proteins with both the wild-type probes for NF-κB binding sites but not with the mutated probes (Fig. 4A and B). Furthermore, these bands were competed away with 100-fold excess of the same non-labelled probes, indicating that they bound specifically to the NF-κB binding sites of the *SDC4* promoter. Bands 4 and 8 were not affected by TNF-α treatment or by mutations of NF-κB binding sites, but apparently competed with the same, non-labelled probes. These results indicated that the bands 4 and 8 proteins bound to the probes with other than NF-κB binding sites.

To evaluate whether bands 1, 2, 3, 5, 6 or 7 contained NF-κB proteins, we used specific antibodies to generate super shifts or block binding. Using the −198/−172 probe, anti-p65 antibody inhibited binding to band 1 and 3 proteins and anti-c-Rel antibody slightly reduced binding to band 1 protein (Fig. 4A). Using the −149/−123 probe, an anti-p65 antibody generated a super-shifted band, and three bands (5, 6 and 7) disappeared (Fig. 4B). Anti-c-Rel antibody inhibited binding to band 5 protein. Anti-p50 antibody did not appear to show any effect at both regions. These EMSA results suggested that p65 and c-Rel could bind to both the NF-κB binding sites in the *SDC4* promoter region. Next, we performed pull-down assays, and observed that the p65, p50 and c-Rel NF-κB subunits bound to both probes after TNF-α treatment (Fig. 4C). Pull-down assay could be highly sensitive to detect a weak binding of p50, whereas EMSA might not be sufficiently sensitive. The amount of protein bound to the −198/−172 probe was relatively minor compared with those bound to the −149/−123 probe.

Binding of NF-κB to the endogenous SDC4 promoter in EAhy926 cells

To evaluate the possibility that NF-κB regulates *SDC4* expression by directly binding to the *SDC4* promoter, we conducted ChIP assays. This showed that p65, but not p50 and c-Rel, bound to the *SDC4* promoter region after TNF-α treatment (Fig. 5A).

Next, we performed ChIP assays using anti-phosphorylated p65 (on Ser536) antibody and found that phosphorylated p65 was also detected as a TNF-α-induced bound protein to the *SDC4* promoter (Fig. 5B). We further performed immunoprecipitation western blot analysis to detect phosphorylated p65 and found that TNF-α treatment clearly induced the phosphorylation of p65 on Ser536 (Fig. 5C). These data indicated that p65 activation by TNF-α would upregulate syndecan-4 expression through the *ex vivo* binding to the *SDC4* promoter.

The effects of NF-κB inhibition on TNF-α induced SDC4 expression

To investigate the role of NF-κB in syndecan-4 expression, we examined the effects of an inhibitor of NF-κB signalling, BAY11-7082. The inhibitor selectively inhibits TNF-α induced phosphorylation of IκBα and blocks the translocation of NF-κB into the nucleus,

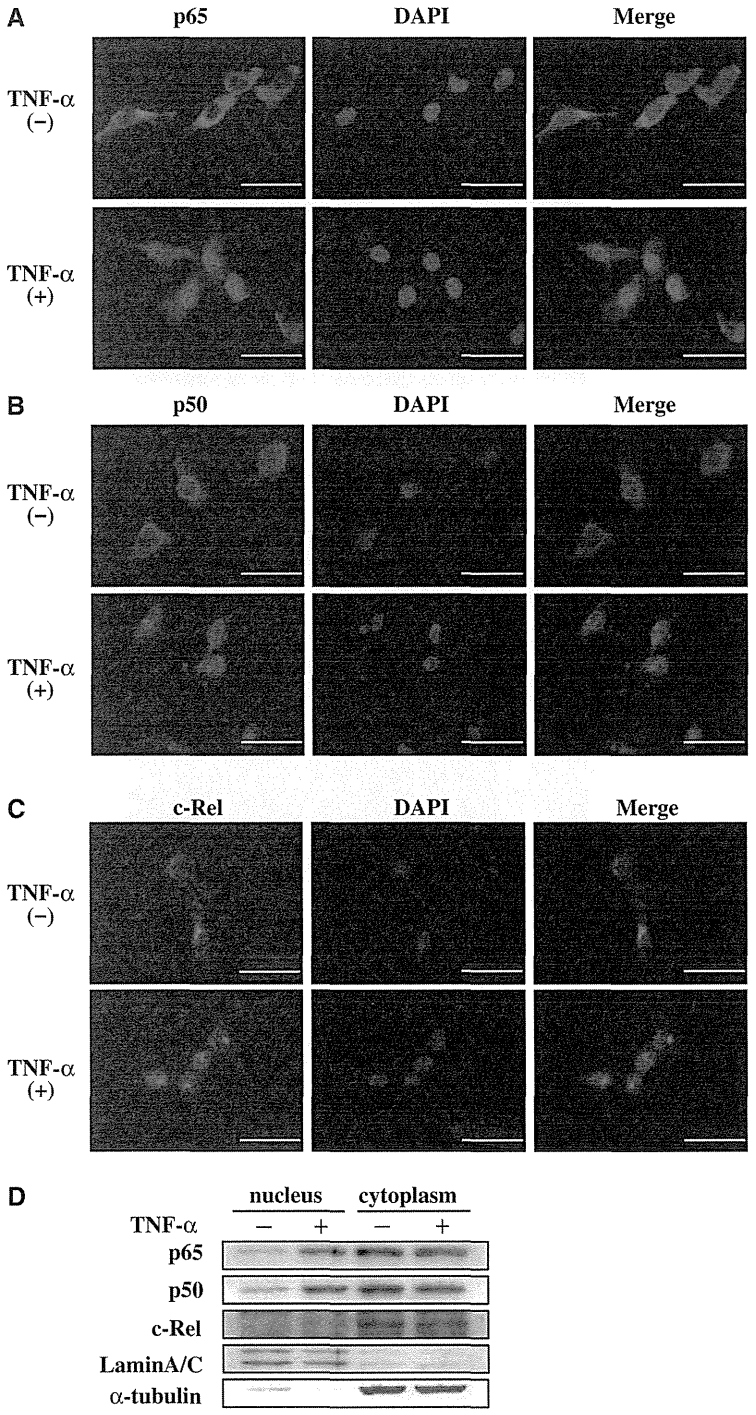

Fig. 3 Localization of NF-κB in EAhy926 cells by immunofluorescence staining and western blot analysis. (A–C) Cells were either treated with TNF-α or fresh medium for 30 min before fixation. Localization of NF-κB was shown with anti-p65 antibody (green) (**A**), anti-p50 antibody (green) (**B**) and anti-c-Rel antibody (green) (**C**). Scale bars denote 50 μm. (**D**) Western blot analysis was performed using nuclear and cytoplasmic proteins isolated from EAhy926 cells treated with or without TNF-α. Lamin A/C and α-tubulin were used as loading controls for the nucleus and cytoplasm, respectively.

thereby preventing its interaction with target gene promoters.

Western blot analysis showed that BAY11-7082 pretreatment inhibited the nuclear translocation of NF-κB subunits by TNF-α (Fig. 6A). The level of *SDC4* mRNA was significantly increased by TNF-α treatment, whereas cells pretreated with BAY11-7082 caused decrease in this induction, although incompletely (Fig. 6B). BAY11-7082 also completely blocked TNF-α-induced *SDC4* promoter activity (Fig. 6C). These data suggested that NF-κB could have an important role in mediating the effects of TNF-α on *SDC4* promoter activity, but additional key molecules other than NF-κB may also regulate *SDC4* mRNA expression.

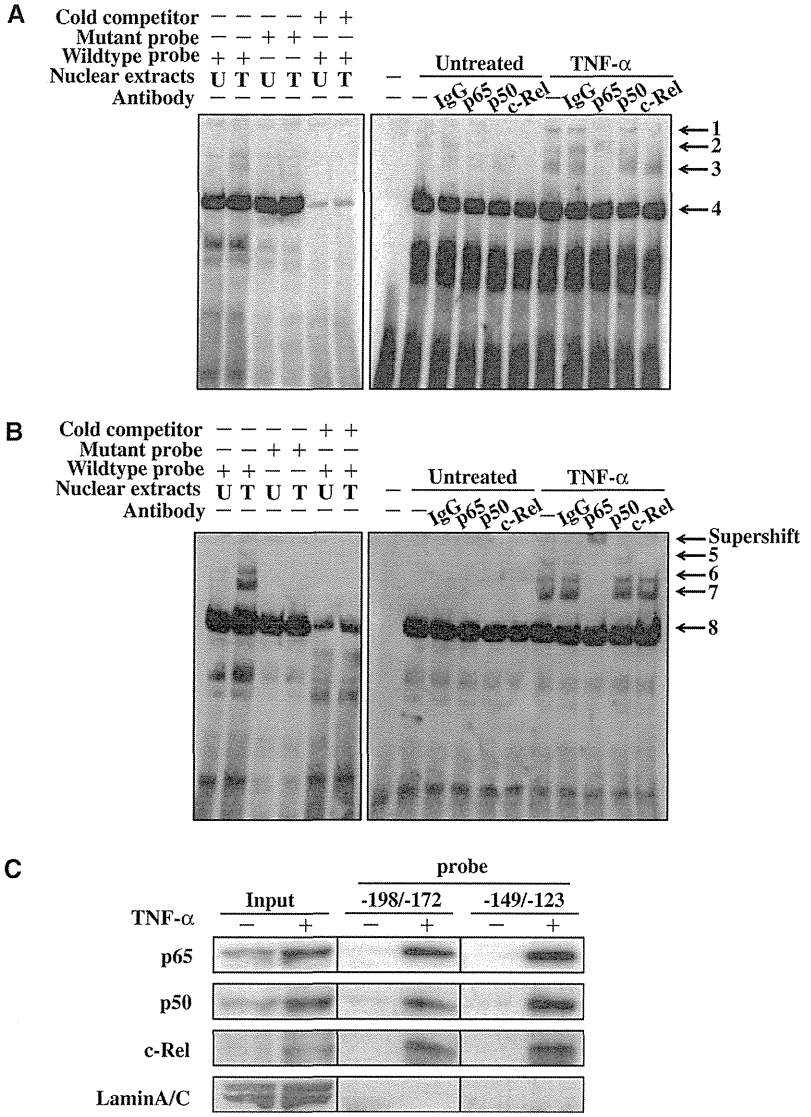

Fig. 4 Binding of NF-κB to *SDC4* promoter region *in vitro*. (**A** and **B**) Biotin-labelled probes covering the NF-κB binding site (−191/−182 and −137/−127) in the *SDC4* promoter region were used for EMSAs. Unlabelled competitors and mutant oligonucleotides were also used to confirm protein-binding specificity. Supershift assays are shown in the right panel. Nuclear proteins and biotinylated probes were mixed with specific anti-p65, anti-p50 or anti-c-Rel antibody, or normal IgG (as a negative control), and EMSAs were performed. The probes correspond to the sequences of the *SDC4* promoter between −198 and −172 (**A**) and between −149 and −123 (**B**), respectively. U, untreated; T, treated with TNF-α. (**C**) For pull-down assays, the nuclear extracts from EAhy926 cells treated with or without TNF-α were incubated with −198/−172 or −149/−123 probe. Proteins specifically bound to respective probes were subjected to western blot analysis using specific antibodies (anti-p65, anti-p50, anti-c-Rel and anti-lamin A/C).

The effects of p65 knockdown on TNF-α-induced SDC4 expression

To evaluate the role of p65 in the *SDC4* upregulation by TNF-α, we conducted RNA interference experiments. Western blot analysis showed that p65 expression in the p65 siRNA (p65si) transfected cells was decreased after 24 h and was completely inhibited after 48 h, whereas control siRNA had no effect on p65 expression (Fig. 7A). Knockdown of p65 significantly, although incompletely, suppressed the upregulation of *SDC4* mRNA expression by TNF-α (Fig. 7B) and completely impaired the *SDC4* promoter activity induced by TNF-α (Fig. 7C). In quantitative RT-PCR, the level of *SDC4* mRNA was determined by measuring amounts of mRNA transcribed from the genomic *SDC4* locus in cells. All transcriptional processes, such as transcriptional initiation, elongation and termination, affect quantitative RT-PCR measurement, but only changes in transcription initiation could affect the luciferase reporter assay. Therefore, these data suggested again that NF-κB could have a critical role for TNF-α effects on *SDC4* promoter activity, but additional mechanisms other than NF-κB activity may also regulate *SDC4* mRNA expression.

Inhibitory effects of transcriptional elongation by DRB on SDC4 upregulation by TNF-α

Next, we examined the effects of transcriptional elongation on TNF-α-induced *SDC4* mRNA expression using DRB as an inhibitor of transcriptional

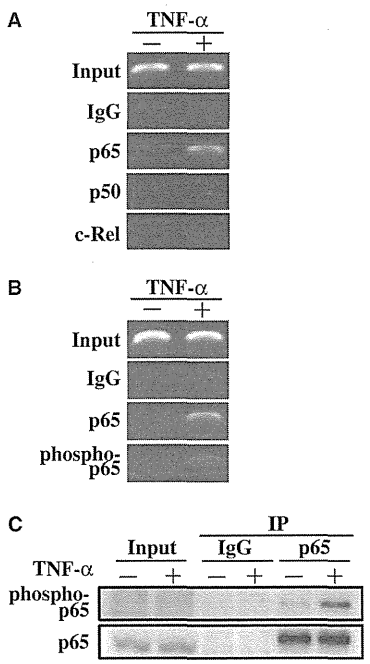

Fig. 5 Binding of NF-κB to the *SDC4* promoter region in EAhy926 cells. (**A**) ChIP assays of the *SDC4* promoter region. Protein–DNA complexes were immunoprecipitated with antibodies (normal IgG, anti-p65, anti-p50 and anti-c-Rel). Protein-bound DNA fragments were isolated, purified and used as templates for PCR amplification of the *SDC4* promoter region (from −215 to −103), including the putative NF-κB binding sites. An aliquot of non-immunoprecipitated chromatin was used as an input control. (**B**) Phosphorylation of p65 was analysed by ChIP assay. EAhy926 cells were treated with or without TNF-α, and phosphorylated p65 was immunoprecipitated with anti-phospho-p65 (on Ser536) antibody. ChIP assays were performed as described earlier. (**C**) Phosphorylation of p65 was also analysed by immunoprecipitation western blot analysis. The cell lysate from EAhy926 cells treated with or without TNF-α was immunoprecipitated using anti-p65 antibody or normal IgG. The immunoprecipitated materials were analysed by western blot analysis using anti-phosphorylated p65 or anti-p65 antibody.

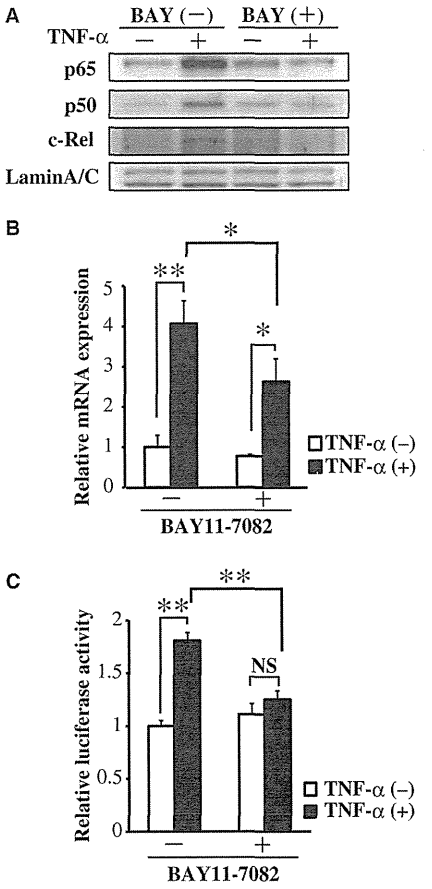

Fig. 6 Effects of NF-κB inhibition on *SDC4* expression. (A–C) Cells were pretreated with or without BAY11-7082 (10 μM) for 1 h and treated with TNF-α for 30 min (**A**) or 3 h (B, C) in the continued presence or absence of BAY11-7082. (A) Inhibition of nuclear NF-κB translocation was determined by western blot analysis. Lamin A/C was used as a loading control. (**B**) Expression of *SDC4* mRNA was measured by quantitative RT-PCR. (**C**) To measure *SDC4* promoter activity, a luciferase assay was performed using the pSDC4-1563 construct. Values represented mean ± SD of three independent experiments. $*P<0.05$; $**P<0.01$ by *t*-test compared with untreated cells. NS, not significant.

elongation. The results showed that increased amounts of *SDC4* mRNA expression induced by TNF-α were significantly suppressed by DRB treatment (Fig. 8). Meanwhile, treatment with DRB had no effect on the basal levels of *SDC4* mRNA. These results suggested that TNF-α-induced syndecan-4 expression was also regulated in the process of the transcriptional elongation.

Discussion

Syndecan-4 is a cell-surface heparan sulfate proteoglycan that has been recently suggested to play important roles in many pathologies, including vascular wall injury, skin wounds, arthritis and myocardial infarction (*7*, *8*, *21–24*). During the tissue injury and repair process, cytokines, chemokines and growth factors are released from various cell types. TNF-α, one of the most potent pro-inflammatory cytokines, is known to upregulate syndecan-4 expression in endothelial cells (*13*), but the detailed molecular mechanisms involved remain unclear.

To better understand the molecular regulatory mechanisms involved in syndecan-4 expression by TNF-α, we investigated the *SDC4* promoter regions and transcription factors responsible for the effects of TNF-α on human endothelium-like EAhy926 cells. In this study, we report the novel findings that the two critical NF-κB p65 binding sites in the *SDC4* promoter are responsible for the effects of TNF-α, and the other NF-κB-p65 independent pathways might also be involved in the response to TNF-α through effects on transcriptional elongation.

Zhang *et al.* reported that TNF-α upregulated *SDC4* expression at least in part through the NF-κB-dependent pathway in mice (*13*). NF-κB is a family of evolutionary conserved dimeric proteins encoded by five gene members: RelA/p65, RelB, c-Rel, NF-κB1/p50 and NF-κB2/p52. NF-κB proteins exist as homo- or hetero-dimers that function as positive regulators of transcription (*25*). An online search using TFSEARCH (http://mbs.cbrc.jp/research/db/

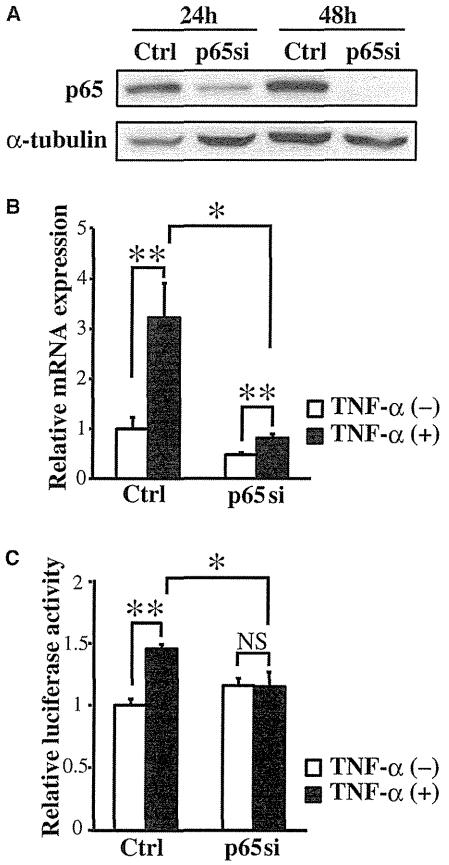

Fig. 7 Effects of p65 knockdown on TNF-α-induced *SDC4* mRNA upregulation. (**A**) To knockdown p65 by siRNA, EAhy926 cells were transfected with p65 siRNA (p65si) or control siRNA (Ctrl) at 5 nM and incubated for the indicated times. Expression of p65 was determined by western blot analysis. (**B**) To measure the effects of p65 knockdown on *SDC4* mRNA expression, the expression of *SDC4* mRNA in the EAhy926 cells transfected with siRNA (5 nM for 48 h) was measured by quantitative RT-PCR. Cells transfected with siRNA were treated with or without TNF-α for 3 h before isolation of mRNA. (**C**) Effects of p65 knockdown on *SDC4* promoter activity were measured by transfecting EAhy926 cells with the pSDC4-1563 construct after transfection of siRNA (5 nM for 24 h). *SDC4* promoter activity was assessed using the luciferase assay. Values represent mean ± SD of three independent experiments. $^{*}P<0.05$; $^{**}P<0.01$ by *t*-test compared with untreated cells. NS, not significant.

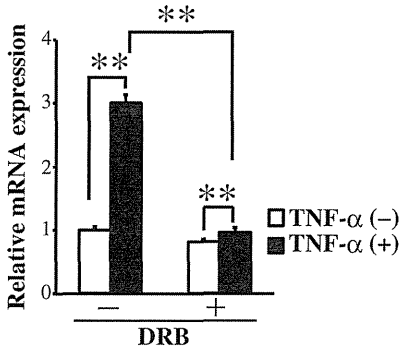

Fig. 8 Effects of DRB on TNF-α-induced *SDC4* mRNA upregulation. EAhy926 cells were pretreated with or without DRB (50 μM), a transcriptional elongation inhibitor, for 1 h and subsequently treated with TNF-α for 3 h. *SDC4* mRNA expression was measured by quantitative RT-PCR. Values represented mean ± SD of three independent experiments. $^{**}P<0.01$ by *t*-test compared with untreated cells. NS, not significant.

TFSEARCHJ.html) in the human *SDC4* promoter showed two putative NF-κB binding sites (−191/−182 and −137/−127) in the proximal region with relative high scores for c-Rel/c-Rel, p65/p65 and p50/p65 dimer binding probabilities.

Recently, Wang *et al.* reported that one of the NF-κB subunits, p65, was important for TNF-α-induced upregulation of *SDC4* promoter activity, but they focused on only the downstream NF-κB binding site (−137/−127) (*26*). In contrast, we demonstrated here that both the putative NF-κB binding sites that bound p65 in *in vitro* EMSA and pull-down assays were important for optimal TNF-α-induced upregulation of *SDC4* promoter activity. We also showed that p65 bound to the *SDC4* promoter region containing both NF-κB binding sites using ChIP assays *ex vivo*. Meanwhile, c-Rel and p50 binding to the *SDC4* promoter were detected by EMSA and pull-down assays, but not by a ChIP assay, suggesting that they could bind to the DNA sequence *in vitro* but not *ex vivo*. After TNF-α treatment, the putative NF-κB binding sites of the *SDC4* promoter region may be occupied by other nuclear proteins, such as p65, or have difficulty binding to c-Rel and p50 in the cellular chromatin environment *ex vivo*.

We also investigated the effects of BAY11-7082, an NF-κB inhibitor and p65 siRNA on TNF-α-induced *SDC4* upregulation. We observed that both completely suppressed luciferase reporter activity of the *SDC4* promoter construct, but only partially reduced *SDC4* mRNA upregulation, measured by quantitative RT-PCR. These results suggested that TNF-α-induced *SDC4* mRNA upregulation occurred through NF-κB dependent and other mechanisms. Indeed, the upregulation effect of TNF-α on *SDC4* mRNA expression was partially abrogated by DRB, a pharmacological inhibitor of cyclin-dependent kinase 9 (CDK9). Previous studies indicated that NF-κB-target genes that were upregulated following stimulation with TNF-α included the positive transcription elongation factor b (P-TEFb), a CDK9/cyclin T complex (*27*, *28*). Another study examined NF-κB interactions with P-TEFb to stimulate transcriptional elongation (*29*). Our results from the p65 siRNA and DRB experiments suggested that TNF-α-induced *SDC4* mRNA upregulation was also enhanced by transcriptional elongation in the absence of p65, but p65 may be required for complete transcriptional activation.

It has been reported that p65 is crucial for the expression of inflammatory cytokine genes in dendritic cells, whereas p50 and c-Rel controlled the expression of genes important for dendritic cell-induced T-cell responses. This dichotomy suggests that there are fundamentally important differences in the biological functions of genes regulated by different NF-κB subunits (*30*). Here, we demonstrated that NF-κB p65, but not p50 or c-Rel, is a key mediator of syndecan-4 upregulation by TNF-α. Additional detailed elucidation of the detailed regulatory mechanisms involved

in syndecan-4 upregulation by TNF-α might further the understanding of the processes of inflammation and tissue repair, and could potentially lead to the identification of therapeutic targets.

In summary, we demonstrated that TNF-α-induced syndecan-4 upregulation mainly depended on transcriptional regulatory mechanisms, such as transcriptional initiation and mRNA elongation, in human vascular endothelium-like EAhy926 cells. Therefore, we concluded that the NF-κB p65 subunit could be a key mediator of syndecan-4 upregulation by TNF-α through two binding sites in the *SDC4* promoter, and the other NF-κB-p65 independent pathway might also be involved through effects on transcriptional elongation.

Acknowledgements

We would like to thank Dr Cora-Jean S. Edgell for providing EAhy926 cells and Enago for translation services. This study was partially supported by grants-in-aid from the Japanese Ministry of Education, Culture, Sports, Science, and Technology (22590524) and from the Japanese Ministry of Health, Labour and Welfare (Research on Measures for Intractable Diseases).

Conflict of Interest
None declared.

References

1. Kojima, T., Leone, C.W., Marchildon, G.A., Marcum, J.A., and Rosenberg, R.D. (1992) Isolation and characterization of heparan sulfate proteoglycans produced by cloned rat microvascular endothelial cells. *J. Biol. Chem.* **267**, 4859–4869
2. Kojima, T., Shworak, N.W., and Rosenberg, R.D. (1992) Molecular cloning and expression of two distinct cDNA-encoding heparan sulfate proteoglycan core proteins from a rat endothelial cell line. *J. Biol. Chem.* **267**, 4870–4877
3. Kojima, T., Katsumi, A., Yamazaki, T., Muramatsu, T., Nagasaka, T., Ohsumi, K., and Saito, H. (1996) Human ryudocan from endothelium-like cells binds basic fibroblast growth factor, midkine, and tissue factor pathway inhibitor. *J. Biol. Chem.* **271**, 5914–5920
4. Carey, D.J. (1997) Syndecans: multifunctional cell-surface co-receptors. *Biochem. J.* **327**, 1–16
5. Ishiguro, K., Kadomatsu, K., Kojima, T., Muramatsu, H., Iwase, M., Yoshikai, Y., Yanada, M., Yamamoto, K., Matsushita, T., Nishimura, M., Kusugami, K., Saito, H., and Muramatsu, T. (2001) Syndecan-4 deficiency leads to high mortality of lipopolysaccharide-injected mice. *J. Biol. Chem.* **276**, 47483–47488
6. Tanino, Y., Chang, M.Y., Wang, X., Gill, S.E., Skerrett, S., McGuire, J.K., Sato, S., Nikaido, T., Kojima, T., Munakata, M., Mongovin, S., Parks, W.C., Martin, T.R., Wight, T.N., and Frevert, C.W. (2012) Syndecan-4 regulates early neutrophil migration and pulmonary inflammation in response to lipopolysaccharide. *Am. J. Respir. Cell Mol. Biol.* **47**, 196–202
7. Matsui, Y., Ikesue, M., Danzaki, K., Morimoto, J., Sato, M., Tanaka, S., Kojima, T., Tsutsui, H., and Uede, T. (2011) Syndecan-4 prevents cardiac rupture and dysfunction after myocardial infarction. *Circ. Res.* **108**, 1328–1339
8. Ikesue, M., Matsui, Y., Ohta, D., Danzaki, K., Ito, K., Kanayama, M., Kurotaki, D., Morimoto, J., Kojima, T., Tsutsui, H., and Uede, T. (2011) Syndecan-4 deficiency limits neointimal formation after vascular injury by regulating vascular smooth muscle cell proliferation and vascular progenitor cell mobilization. *Arterioscler. Thromb. Vasc. Biol.* **31**, 1066–1074
9. Zhou, Z., Connell, M.C., and MacEwan, D.J. (2007) TNFR1-induced NF-kappaB, but not ERK, p38MAPK or JNK activation, mediates TNF-induced ICAM-1 and VCAM-1 expression on endothelial cells. *Cell. Signal.* **19**, 1238–1248
10. Baldwin, A.S. (1996) The NF-kappa B and I kappa B proteins: new discoveries and insights. *Annu. Rev. Immunol.* **14**, 649–683
11. Pahl, H.L. (1999) Activators and target genes of Rel/NF-kappaB transcription factors. *Oncogene* **18**, 6853–6866
12. Chen, Z.J. (2005) Ubiquitin signalling in the NF-[kappa]B pathway. *Nat. Cell Biol.* **7**, 758–765
13. Zhang, Y., Pasparakis, M., Kollias, G., and Simons, M. (1999) Myocyte-dependent regulation of endothelial cell syndecan-4 expression. *J. Biol. Chem.* **274**, 14786–14790
14. Smith, M.F., Novotny, J., Carl, V.S., and Comeau, L.D. (2006) *Helicobacter pylori* and toll-like receptor agonists induce syndecan-4 expression in an NF-kappaB-dependent manner. *Glycobiology* **16**, 221–229
15. Magalhães, A., Marcos, N.T., Carvalho, A.S., David, L., Figueiredo, C., Bastos, J., David, G., and Reis, C.A. (2009) *Helicobacter pylori* cag pathogenicity island-positive strains induce syndecan-4 expression in gastric epithelial cells. *FEMS Immunol. Med. Microbiol.* **56**, 223–232
16. Suzuki, A., Nakashima, D., Miyawaki, Y., Fujita, J., Maki, A., Fujimori, Y., Takagi, A., Murate, T., Teranishi, M., Matsushita, T., Saito, H., and Kojima, T. (2012) A novel ENG mutation causing impaired co-translational processing of endoglin associated with hereditary hemorrhagic telangiectasia. *Thromb. Res.* **129**, e200–e208
17. Suzuki, T., Fujisawa, J.I., Toita, M., and Yoshida, M. (1993) The trans-activator tax of human T-cell leukemia virus type 1 (HTLV-1) interacts with cAMP-responsive element (CRE) binding and CRE modulator proteins that bind to the 21-base-pair enhancer of HTLV-1. *Proc. Natl. Acad. Sci. U.S.A.* **90**, 610–614
18. Suzuki, A., Sanda, N., Miyawaki, Y., Fujimori, Y., Yamada, T., Takagi, A., Murate, T., Saito, H., and Kojima, T. (2010) Down-regulation of PROS1 gene expression by 17beta-estradiol via estrogen receptor alpha (ERalpha)-Sp1 interaction recruiting receptor-interacting protein 140 and the corepressor-HDAC3 complex. *J. Biol. Chem.* **285**, 13444–13453
19. Lin, C., Smith, E.R., Takahashi, H., Lai, K.C., Martin-Brown, S., Florens, L., Washburn, M.P., Conaway, J.W., Conaway, R.C., and Shilatifard, A. (2010) AFF4, a component of the ELL/P-TEFb elongation complex and a shared subunit of MLL chimeras, can link transcription elongation to leukemia. *Mol. Cell* **37**, 429–437
20. Takagi, A., Kojima, T., Tsuzuki, S., Katsumi, A., Yamazaki, T., Sugiura, I., Hamaguchi, M., and Saito, H. (1996) Structural organization and promoter activity of the human ryudocan gene. *J. Biochem.* **119**, 979–984
21. Li, L., Couse, T.L., Deleon, H., Xu, C.P., Wilcox, J.N., and Chaikof, E.L. (2002) Regulation of syndecan-4 expression with mechanical stress during the development of angioplasty-induced intimal thickening. *J. Vasc. Surg.* **36**, 361–370

22. Gallo, R.L., Ono, M., Povsic, T., Page, C., Eriksson, E., Klagsbrun, M., and Bernfield, M. (1994) Syndecans, cell surface heparan sulfate proteoglycans, are induced by a proline-rich antimicrobial peptide from wounds. *Proc. Natl. Acad. Sci. U.S.A.* **91**, 11035–11039
23. Echtermeyer, F., Bertrand, J., Dreier, R., Meinecke, I., Neugebauer, K., Fuerst, M., Lee, Y.J., Song, Y.W., Herzog, C., Theilmeier, G., and Pap, T. (2009) Syndecan-4 regulates ADAMTS-5 activation and cartilage breakdown in osteoarthritis. *Nat. Med.* **15**, 1072–1076
24. Kojima, T., Takagi, A., Maeda, M., Segawa, T., Shimizu, A., Yamamoto, K., Matsushita, T., and Saito, H. (2001) Plasma levels of syndecan-4 (ryudocan) are elevated in patients with acute myocardial infarction. *Thromb. Haemost.* **85**, 793–799
25. Bakkar, N. and Guttridge, D.C. (2010) NF-κB signaling: a tale of two pathways in skeletal myogenesis. *Physiol. Rev.* **90**, 495–511
26. Wang, J., Markova, D., Anderson, D.G., Zheng, Z., Shapiro, I.M., and Risbud, M.V. (2011) TNF-α and IL-1β promote a disintegrin-like and metalloprotease with thrombospondin type I motif-5-mediated aggrecan degradation through syndecan-4 in intervertebral disc. *J. Biol. Chem.* **286**, 39738–39749
27. Nowak, D.E., Tian, B., Jamaluddin, M., Boldogh, I., Vergara, L.A., Choudhary, S., and Brasier, A.R. (2008) RelA Ser276 phosphorylation Is required for activation of a subset of NF-kappaB-dependent genes by recruiting cyclin-dependent kinase 9/cyclin T1 complexes. *Mol. Cell. Biol.* **28**, 3623–3638
28. Shan, B., Zhuo, Y., Chin, D., Morris, C.A., Morris, G.F., and Lasky, J.A. (2005) Cyclin-dependent kinase 9 is required for tumor necrosis factor-alpha-stimulated matrix metalloproteinase-9 expression in human lung adenocarcinoma cells. *J. Biol. Chem.* **280**, 1103–1111
29. Barboric, M., Nissen, R.M., Kanazawa, S., Jabrane-Ferrat, N., and Peterlin, B.M. (2001) NF-kappaB binds P-TEFb to stimulate transcriptional elongation by RNA polymerase II. *Mol. Cell* **8**, 327–337
30. Wang, J., Wang, X., Hussain, S., Zheng, Y., Sanjabi, S., Ouaaz, F., and Beg, A.A. (2007) Distinct roles of different NF-kappa B subunits in regulating inflammatory and T cell stimulatory gene expression in dendritic cells. *J. Immunol.* **178**, 6777–6788

Thrombosis Research 131 (2013) 444–449

Contents lists available at SciVerse ScienceDirect

Thrombosis Research

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

Regular Article

Overexpression of factor VII ameliorates bleeding diathesis of factor VIII-deficient mice with inhibitors

Atsushi Yasumoto [a,b,1], Seiji Madoiwa [a,1], Yuji Kashiwakura [a,1], Akira Ishiwata [a,1], Tsukasa Ohmori [a], Hiroaki Mizukami [c], Keiya Ozawa [c], Yoichi Sakata [a], Jun Mimuro [a,*]

[a] Division of Cell and Molecular Medicine, Center for Molecular Medicine, Jichi Medical University, Shimotsuke, Tochigi-ken 329–0498, Japan
[b] Department of Hematology, Hokkaido University Graduate School of Medicine, Sapporo, Hokkaido 060–8638, Japan
[c] Division of Genetic Therapeutics, Center for Molecular Medicine, Jichi Medical University, Shimotsuke, Tochigi-ken 329–0498, Japan

ARTICLE INFO

Article history:
Received 21 November 2012
Received in revised form 26 February 2013
Accepted 12 March 2013
Available online 6 April 2013

Keywords:
Factor VII
Adeno-associated virus vector
Hemophilia A
Gene therapy

ABSTRACT

Introduction: Factor VIII (FVIII) treatment for hemophilia A has difficulties in correcting bleeding diathesis in the presence of inhibitors.
Materials and Methods: An adeno-associated virus type 8 (AAV8) vector containing the factor VII (FVII) gene or the activated factor VII (FVIIa) gene was used to investigate the therapeutic effect of FVII or FVIIa overexpression in FVIII-deficient mice with inhibitors.
Results: Following repeated human FVIII injection, FVIII-deficient mice developed anti-human FVIII antibodies that cross-reacted with mouse FVIII. High transgene expression of murine FVII or murine FVIIa was achieved using the AAV8 vector and resulted in increased blood FVII activity greater than 800% of normal murine FVII levels in vector-injected FVIII-deficient mice. Thromboelastography analysis showed significant improvements in clotting time, clot formation time, α angle, and mean clot firmness in AAV8 vector-injected FVIII-deficient mice with inhibitors. Overexpression of FVIIa ameliorated the bleeding phenotype of FVIII-deficient mice with inhibitors and significantly increased the survival rate after tail clipping. In addition, overexpression of FVII increased the survival rate of FVIII-deficient mice with inhibitors after tail clipping though it was not as efficient as FVIIa overexpression.
Conclusions: These data suggest that FVII overexpression is an alternative strategy for the treatment of hemophilia A with inhibitors.

Introduction

Hemophilia A is an inherited X-linked bleeding disorder caused by abnormalities of the coagulation factor VIII (FVIII) gene. The current standard therapy for hemophilia A is intravenous injection of recombinant FVIII or plasma-derived FVIII concentrates. Prophylactic administration of FVIII concentrates is effective in preventing harmful bleeding. However, severe hemophilia A patients develop antibodies against FVIII (inhibitors) upon frequent infusion of FVIII concentrates. Gene therapy enables the prevention of life-threatening bleeding in the brain and harmful bleeding in joints by sustained elevation of coagulation factor levels and provides next generation therapy for hemophilia [1,2]. Indeed, clinical trials for hemophilia A and B have been conducted with a variety of gene therapy vectors [3–7]. The current strategy of gene and cell therapy for hemophilia A is the transfer of the normal FVIII gene *in vivo* or transplantation of cells expressing FVIII. However, this strategy may not work for hemophilia A with inhibitors. Thus, an alternative gene transfer approach for hemophilia A with inhibitors could be the overexpression of activated factor VII (FVIIa) [8], which is effective for reducing bleeding diathesis of hemophilia B mice and hemophilia A and B dogs [8,9]. In addition, ectopic expression of FVIIa in platelets reduced bleeding in hemophilia A mice [10]. In the current study, we investigated the forced expression of the mouse FVIIa gene or the FVII gene by an adeno-associated virus type 8 (AAV8) vector in FVIII-deficient (F8KO) mice in the presence of inhibitors against mouse FVIII to determine the therapeutic effect of overexpression of FVII using a genetic approach for hemophilia A with inhibitors.

Materials and Methods

Vector Construction

The characteristics and activity of the liver specific chimeric enhancer/promoter complex, consisting of an enhancer element of the hepatic

Abbreviations: FVII, Factor VII; FVIII, Factor VIII; AAV, adeno-associated virus; F8KO, Factor VIII knock out; HCR, hepatic control region; HAAT, human alpha-1 antitrypsin; CMV, cytomegalovirus; SV40, simian virus 40; PCR, polymerase chain reaction; PT, prothrombin time; APCC, activated prothrombin complex concentrate; FX, Factor X; FIX, Factor IX; FXII, Factor XII.

* Corresponding author at: Division of Cell and Molecular Medicine, Center for Molecular Medicine, Jichi Medical University, 3311-1 Yakushiji, Shimotsuke, 329–0498, Japan. Tel.: +81 285 58 7398; fax: +80 285 44 7817.
E-mail address: mimuro-j@jichi.ac.jp (J. Mimuro).

[1] These authors contributed equally to this work.

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http://dx.doi.org/10.1016/j.thromres.2013.03.007

control region (HCR, +1– + 325) of the human *Apo E/C-I* gene and the 5' flanking region of the human *α1-antitrypsin* (HAAT, −275– + 25) gene, (HCRHAAT promoter), were described previously [11,12]. The mouse FVII gene (cDNA) and mouse FVIIa gene were cloned in our laboratory as reported previously [10]. A DNA fragment encoding the murine FVII gene (cDNA) or murine FVIIa gene (cDNA) was inserted downstream of the HCRHAAT promoter of p1.1HCRHAAT [11,12] to produce p1.1HCRHAAT-mFVII or p1.1HCRHAAT-mFVIIa. A DNA fragment spanning the cytomegalovirus (CMV) promoter, the LacZ gene, and the polyadenylation signal sequence of the pAAV2 CMV-Lac Z plasmid (Strategene, La Jolla, CA, USA) was replaced by a DNA fragment spanning the HCRHAAT promoter, the FVII gene and the simian virus 40 (SV40) polyadenylation signal sequences of p1.1HCRHAAT-mFVII to produce pAAV2-HCRHAAT-mFVII. The gene transfer plasmid vector pAAV2-HCRHAAT-mFVIIa was constructed as for p1.1HCRHAAT-mFVII.

AAV Vector Production

The vector production system was kindly supplied by Avigen Inc. (San Diego, CA, USA). AAV vectors were packaged with the AAV8 capsid by pseudotyping [11,12]. The chimeric packaging plasmid for AAV8 capsid pseudotyping was synthesized as described previously [12]. DNA fragments harboring the mouse FVII gene or the mouse FVIIa gene located downstream of the HCRHAAT promoter and flanked by AAV2 ITRs were packaged by triple plasmid transfection of human embryonic kidney 293 (HEK 293) cells, kindly supplied by Avigen Inc., with the chimeric packaging plasmid (AAV2 rep/AAV8 cap), and the adenovirus helper plasmid pHelper (Stratagene, La Jolla, CA, USA), as described previously [11–13]. For virus vector purification, DNase (Benzonase, Merck Japan, Tokyo, Japan)-treated viral particle-containing samples were 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 16 °C, as described previously [11,12]. Titration of recombinant AAV vectors was performed by quantitative polymerase chain reaction (PCR) using a real time PCR system (StepOnePlus, Applied Biosystems, Tokyo, Japan).

Animal Experiments

C57BL/6 wild-type mice were purchased from Japan SLC Inc. (Hamamatsu, Japan). FVIII-deficient (F8 knock out, F8KO) mice with a targeted destruction of exon 16 of mouse *F8* were reported previously by Bi et al. [14], and generously provided by Dr. H. H. Kazazian Jr. (University of Pennsylvania, Philadelphia, PA, USA) [11,13]. Mice were maintained under standard lighting conditions in a clean room. All surgical procedures were carried out in accordance with the guidelines of the institutional Animal Care and Concern Committee of Jichi Medical University.

Inhibitor Generation in FVIII-deficient Mice

F8KO mice were repeatedly injected with human FVIII concentrates (Kogenate FS, Bayer Yakuhin Ltd, Tokyo, Japan) according to the method described by Madoiwa [15] with modifications. Briefly, F8KO mice (16-weeks-old) were injected with 0.05 U/g of human FVIII concentrates (Kogenate) once a week, 4 times and blood was drawn after immunization. Mouse plasma samples were subjected to the FVIII inhibitor assay (Bethesda method) using human FVIII-deficient plasma (Thrombocheck Factor VIII, Sysmex, Kobe, Japan) [15]. Cross-reactivity of FVIII inhibitors raised against human FVIII to mouse FVIII was determined with normal mouse plasma and mouse FVIII-deficient plasma obtained from untreated F8KO mice. Briefly, plasma obtained from F8KO mice was mixed with plasma from wild-type mice (C57/B6) for 2 hours and remaining FVIII activity was quantified using FVIII-deficient plasma.

Determination of Mouse FVII Activity in Mice

The AAV8 vector (5×10^{13} vg/kg) carrying either the mouse FVII gene (AAV8-mFVII) or the mouse FVIIa gene (AAV8-mFVIIa) was injected to the cervical vein plexus of 20-week-old F8KO mice under anesthesia. Blood was drawn from the cervical vein plexus and mixed with 1/10 volume of 3.8% sodium citrate 4 weeks after the vector injection. Platelet-poor plasma was prepared and FVII levels in mouse plasma were quantified by the prothrombin time (PT) method using prothrombin time reagent (Thrombocheck PT, Sysmex, Kobe, Japan) and FVII-deficient plasma (Sysmex, Kobe, Japan) and standardized to normal mouse plasma. Since the plasma of hemophilia A mice has normal mouse FVII activity, the baseline FVII activity of the plasma obtained was determined before vector injection. The plasma obtained from vector-injected mice was diluted and subjected to measurement of FVII activity.

Thromboelastography Analysis

Thromboelastography analysis of mouse blood was performed using a ROTEM apparatus (Pentapharm GmbH) as previously described [10]. Briefly, blood samples containing 0.38% sodium citrate were prepared 4 weeks after vector injection and analyzed using a ROTEM apparatus with the star-TEM reagent (Pentapharm GmbH) according to the manufacturer's instructions.

Tail Clipping Test

Mice were subjected to tail clipping under anesthesia 7 weeks after vector injection. Tails of mice were excised with surgical scalpels 2 cm proximate from the tail ends. Then mice were observed under standard conditions for 24 hours to determine the rate of mortality.

Statistical Analysis

Student's *t*-test, Welch's *t*-test and Chi-square test were used for statistical analysis using software Statcel Ver.3 for Excel. *P* values less than 0.05 were considered statistically significant.

Results

Inhibitor Development in F8KO Mice After Human FVIII Injection

Human FVIII is immunogenic in mice and repeated injection of human FVIII concentrates results in antibody development. As shown in Fig. 1, F8KO mice receiving repeated injection of human FVIII concentrates developed antibodies that cross-reacted with mouse FVIII. The inhibitory titer of the antibody against mouse FVIII (inhibitors) was sufficiently high to inhibit mouse FVIII. There was no significant difference between inhibitor titers in AAV8-mFVII-injected and AAV8-mFVIIa-injected F8KO mice. Thus, these mice could be used to study genetic approaches for hemophilia A with inhibitors.

Expression of Mouse FVII and Mouse FVIIa in F8KO Mice with Inhibitors

The AAV8 vector (5×10^{13} vg/kg) carrying either the mouse FVII gene or the mouse FVIIa gene was injected to F8KO mice with inhibitors against mouse FVIII, to investigate whether the vector could express high levels of FVII or FVIIa in mice. The mean level of FVII activity increased to more than 800% of the baseline in F8KO mice receiving AAV8-mFVII (Fig. 2). The levels of FVII activity in AAV8-mFVIIa injected mouse plasma were similar to those for AAV8-mFVII-injected F8KO mice. There was no significant difference between the FVII activity levels of AAV8-mFVII-injected F8KO mice and AAV8-mFVIIa-injected F8KO mice.

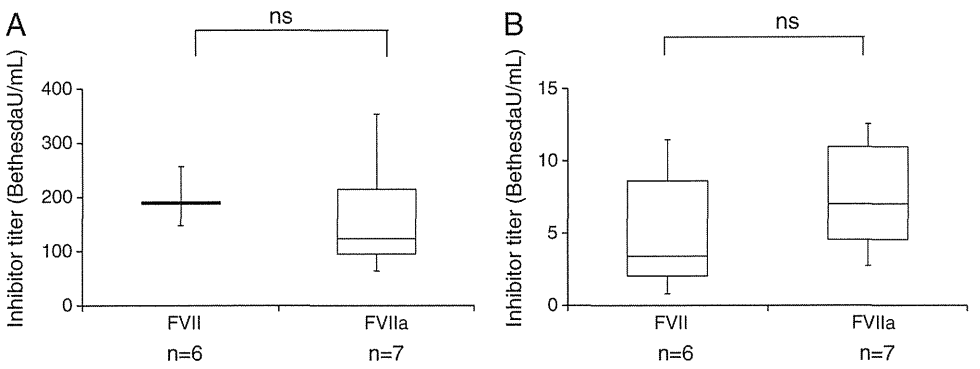

Fig. 1. Inhibitory titers of anti-FVIII antibodies. Inhibitory activity against human FVIII (A) and mouse FVIII (B) in F8KO mice are shown. Plasma samples were diluted in buffer, incubated with normal human plasma or wild-type mouse plasma for 2 hours and subjected to the APTT assay using FVIII-deficient plasma. Inhibitory titers were calculated according to the Bethesda method. Values are shown as Whisker Box plots. There were no significant differences between the FVII levels of AAV8-mFVII-injected mice (n = 6) and AAV8-mFVIIa-injected mice (n = 7) (Student's *t*-test). ns: not significant.

Treatment Efficacy of Overexpressed FVII or FVIIa

Treatment efficacy of overexpressed FVII and FVIIa by the respective AAV8 vectors was studied in F8KO mice with inhibitors by thromboelastography analysis and a tail-clipping test (Figs. 3, 4 and Table 1). The representative thromboelastograms of AAV8-mFVIIa-injected mice were comparable to wild-type mice except for the clotting time (Fig. 3). Thromboelastography analysis of blood from AAV8-mFVII-injected mice showed that the parameters and thromboelastogram improved but that the changes were to a lesser extent compared with AAV8-mFVIIa-injected mice. Thromboelastography analysis showed that the clot formation time, maximum clot firmness and α angle improved in F8KO mice with AAV8-mFVIIa injection and were similar to those of wild-type mice. All thromboelastography parameters except for the clotting time of FVIIa-overexpressed F8KO mice were similar to those of wild-type mice. Although the clotting time was shortened in FVIIa overexpressed F8KO mice, it was still prolonged relative to wild-type mice. The thromboelastography parameters of FVII-overexpressed F8KO mice were inferior to those of FVIIa-overexpressed mice. The improvement of the thromboelastography parameters from FVII-overexpressed F8KO mice was apparent. However, the *in vivo* effect of FVII or FVIIa overexpression might be less than expected from the thromboelastography analysis. Therefore, these mice were subjected to tail clipping challenge 7 weeks after vector injection to investigate whether overexpression of FVII or FVIIa could ameliorate the bleeding diathesis of F8KO mice. As reported previously, all F8KO mice that did not receive vector injection (n = 7) died within 24 hours of tail clipping regardless of the presence of inhibitors against mouse FVIII. The survival rate of AAV8-mFVIIa-injected mice (n = 7) was 85.7% and 50.0% for AAV8-mFVII-injected mice (n = 6), suggesting that overexpression of FVII and of FVIIa significantly ameliorated bleeding diathesis of F8KO mice with inhibitors.

The FVII activities in AAV8-mFVII-injected F8KO mice and AAV8-mFVIIa-injected mice were 515% (18 weeks after vector injection) and 488% (29 weeks after vector injection), respectively.

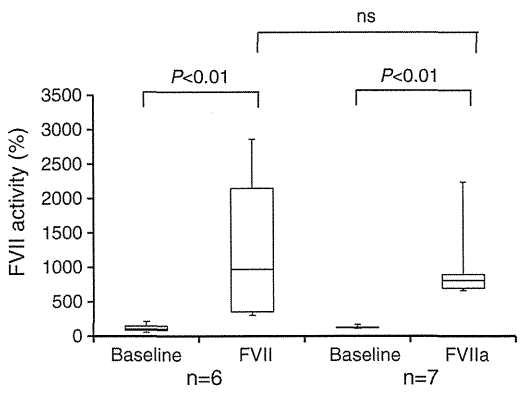

Fig. 2. Mouse FVII and mouse FVIIa expression in F8KO mice after injection of AAV8 vectors. FVII activity levels in plasma from F8KO mice 4 weeks after vector injection (FVII, AAV8-mFVII-injected mice (n = 6); FVIIa, AAV8-mFVIIa-injected mice (n = 7) were quantified. The baseline FVII activity levels (baseline) of these mice were also measured simultaneously. Values are shown as Whisker Box plots. The differences between values were analyzed by Student's *t*-test and *p* values are shown. ns: not significant.

Discussion

Inhibitor (antibody against infused FVIII) development in hemophilia A patients interferes with FVIII treatment. Thus, the next generation therapy for hemophilia A using a genetic approach to force expression of the normal FVIII gene or transplanting cells expressing FVIII may be applicable to hemophilia A patients without inhibitors, but may not be effective in correcting bleeding diathesis in hemophilia A patients with inhibitors. The incidence of inhibitor development in hemophilia A is much higher than for hemophilia B, suggesting that inhibitor development in hemophilia A patients is a serious problem that interferes with therapy [16]. In the medical practice setting, bleeding events of hemophilia A patients with inhibitors can be treated with activated prothrombin complex concentrate (APCC) or recombinant FVIIa. Therefore, forced expression of FVIIa is an alternative approach for hemophilia gene therapy when the recipients have inhibitors as described previously [8]. Treatment with overexpression of FVIIa in hemophilia B mice using an AAV2 vector was effective for ameliorating the bleeding phenotype of hemophilia B mice [8]. This was also shown in hemophilia A and B dogs treated with AAV8 vectors carrying the canine FVIIa gene [9]. In the current study, we explored the possibility of ameliorating bleeding diathesis of hemophilia A mice with inhibitors by overexpressing FVII or FVIIa using an AAV8 vector carrying the respective gene.

Inhibitors against human FVIII develop by repeated infusion of human FVIII concentrates to FVIII-deficient mice. This inhibitor is thought to be an antibody against the xenoantigen (human FVIII) but it also cross-reacts with mouse FVIII (Supplementary Fig. S1). Therefore, it could act as an alloantibody against mouse FVIII in F8KO (hemophilia A) mice. Titers of the inhibitor against mouse FVIII were approximately 1/40–1/20 of that against human FVIII by the Bethesda assay but high enough to inhibit mouse FVIII activity. Thereby, hemophilia A mice with inhibitors generated by repeated injection of human FVIII concentrates could be used as a model of hemophilia A with inhibitors.

We overexpressed FVII or FVIIa in hemophilia A mice with inhibitors against mouse FVIII, using an AAV8 vector. Levels of FVIIa in mice are thought to be comparable to the therapeutic levels of

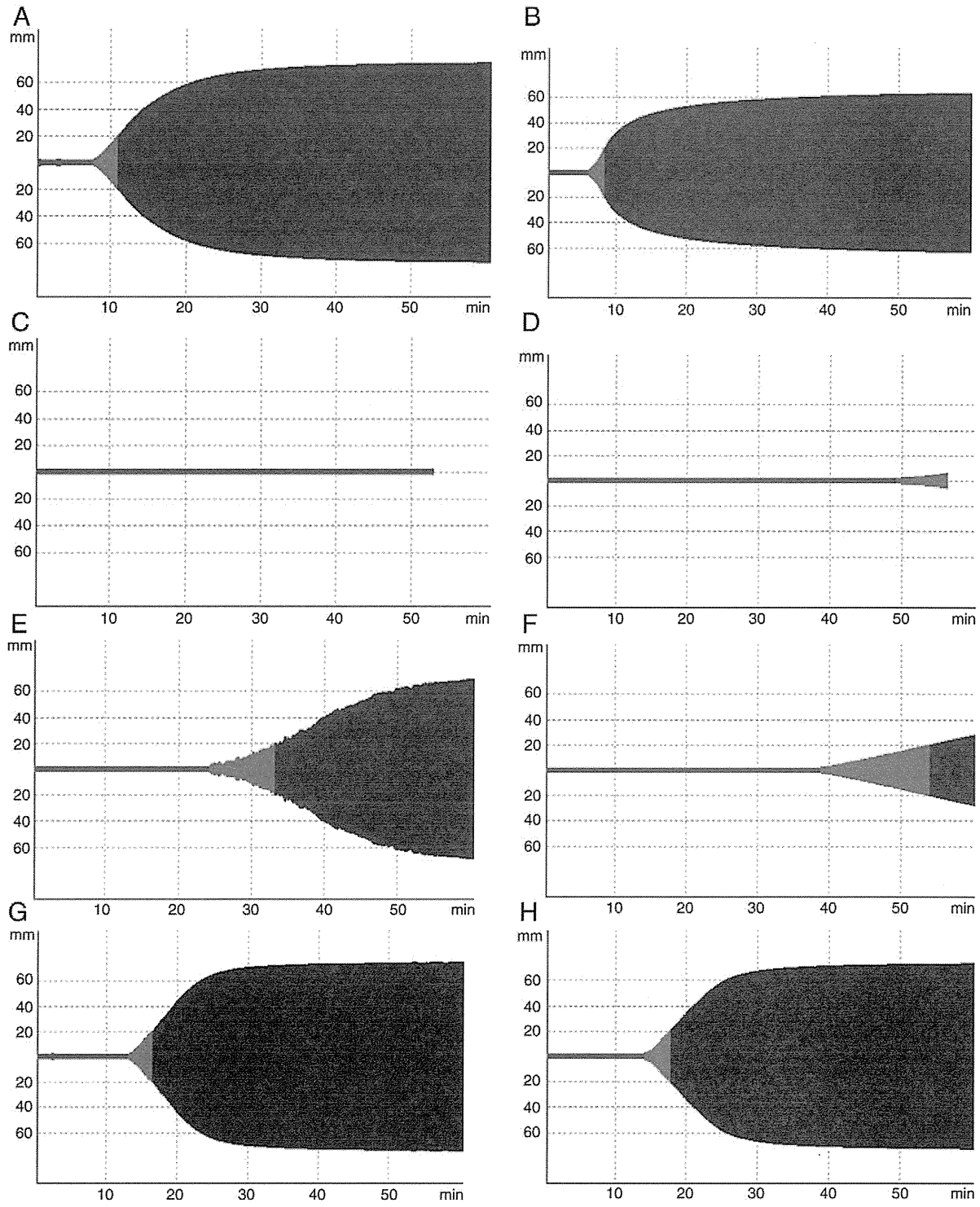

Fig. 3. Thromboelastography analysis. Mouse blood obtained from wild-type mice (A, B), F8KO mice (C, D), AAV8-mFVII-injected F8KO mice with inhibitors (E, F), or AAV8-mFVIIa-injected mice with inhibitors (G, H) was analyzed using a ROTEM delta. The representative thromboelastograms of these mice are shown.

recombinant human FVIIa concentrates in hemophilia A patients with inhibitors to stop bleeding. The therapeutic effect of overexpression of FVII or FVIIa shown in the thromboelastography analysis was confirmed by tail clipping challenge *in vivo*. The overexpression of FVIIa by an AAV8 vector significantly increased the survival rate of hemophilia A mice with inhibitors. The therapeutic effect of FVII overexpression was also significant in the tail clipping challenge in hemophilia A mice with inhibitors. The survival rate of FVII-overexpressed F8KO mice after tail clipping was higher than untreated F8KO mice and lower than FVIIa-overexpressed F8KO mice. Although the survival rate was lower than that for mice with FVIIa overexpression, the difference between these survival rates was not statistically significant.

When hemophilia patients were administered the anti-virus drug ribavirin for the treatment of hepatitis C, these patients bled less frequently [17]. Increased levels of FVII in these patients might account for reduced bleeding diathesis [18], suggesting that high expression levels of FVII in hemophilia patients with or without inhibitors

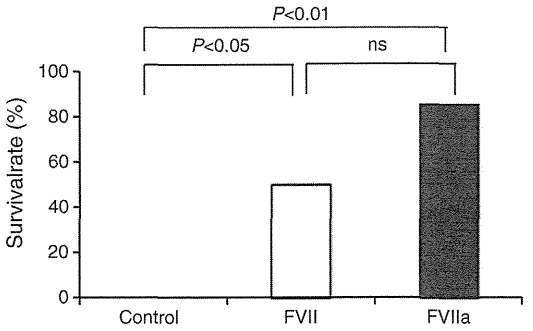

Fig. 4. Survival rates of F8KO mice after tail clipping. The survival rates of F8KO mice are shown. All wild-type mice survived challenge by tail clipping (data not shown), while all F8KO mice (n = 7) died after tail clipping regardless of the presence of inhibitors against FVIII. Significant numbers of mouse FVII overexpressing F8KO mice with inhibitors (n = 6) and mouse FVIIa overexpressing F8KO mice with inhibitors (n = 7) survived challenge by tail clipping. The differences between values were analyzed by Chi-square test and *p* values are shown. ns: not significant.

might ameliorate bleeding. Our results were consistent with this previous report. The therapeutic effect of forced FVII expression using an AAV vector was previously studied in FVII deficient (F7KO) mice [19]. In this animal model, forced FVII expression protected postnatal hemorrhage and improved the survival of vector-injected F7KO mice. The mechanism of therapeutic effect of forced FVII expression in F7KO mice was similar to that of forced FVIII expression in F8KO mice. The current study showed a therapeutic effect of FVII overexpression on bleeding diathesis in F8KO mice.

The hypothetical mechanism of ameliorating bleeding diathesis of F8KO mice by FVII overexpression could be explained as follows. FVII is converted to FVIIa mainly by activated factor X (FXa), and possibly by activated factor IX (FIXa), activated factor XII (FXIIa), thrombin, and FVIIa [20–23]. The physiological role of FVII activating protease in blood coagulation is still unknown [24]. When bleeding occurs, FVIIa binds to tissue factor and activates FX, FIX, and possibly FVII [25]. When FVII is present at a high concentration due to AAV8 vector-induced FVII overexpression, subsequently generated FXa, FIXa, and FVIIa activate FVII and produce a large amount of FVIIa that could in turn bind to tissue factor at the bleeding site and accelerate coagulation. However, this may be less efficient than the amplification of the coagulation cascade by the FIXa/FVIIIa pathway. Enhanced hemostasis with FVII overexpression might differ from FVIIa overexpression. When FVIIa is overexpressed, the FVIIa concentration in the circulation is high and FVIIa in complex with tissue factor activates FX and FIX. Compared with FVIIa overexpression, the FVIIa concentration in the circulation may not be such high when FVII is overexpressed, but the local FVIIa concentration at the bleeding site might be high due to amplification effects as described above. This might account for the increased survival of F8KO mice with FVII overexpression after tail clipping although the improvement of ROTEM parameters with FVII was modest.

Studies to test the safety of overexpression of FVIIa demonstrated it was related to the early death of mice [26,27]. This was apparent in mice with overexpression of an FVIIa variant that had a higher coagulation activity [26]. This adverse event was attributed to the thrombogenicity of the overexpressed FVIIa or its variant. Therefore, continuous overexpression of FVIIa might have a higher risk of adverse events than intermittent administration of FVIIa. The safety of overexpression of FVIIa in dogs was previously shown using molecular markers [9]. However, no pathological examination was conducted, so the thrombogenicity of FVIIa overexpression in the canine model is not clear. Overexpression of human FVII with an AAV8 vector was studied in monkeys and human FVII overexpression continued up to 28 weeks after vector injection without toxicity [19]. Although further studies are required to determine the safety of continuous FVII overexpression regarding thrombogenicity, we demonstrated a therapeutic effect of FVII overexpression in a mouse model of hemophilia A with inhibitors. Our future studies will investigate the safety of FVII overexpression in hemophilia mice and wild type mice in comparison with FVIIa overexpression. In addition to the genetic approach, FVII variants with a longer plasma half-life or drugs that enhance FVII gene expression might be an alternative therapeutic approach for hemophilia patients with inhibitors in clinical settings.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.thromres.2013.03.007.

Table 1
Changes in thromboelastography parameters following the over expression of mFVII and mFVIIa.

	CT	CFT	MCF	α angle
Wild type	255 ± 91.8 (n = 4)	175.5 ± 25.1 (n = 4)	64.3 ± 1.0 (n = 4)	60.5 ± 2.1 (n = 4)
F8KO	2726 ± 409.7 (n = 8)	ND	ND	ND
FVII	1698 ± 403.0 (n = 4)	1132 ± 572.0 (n = 5)	33 ± 35.9 (n = 5)	28.5 ± 10.1 (n = 4)
FVIIa	751.6 ± 139.4 (n = 5)	183 ± 36.0 (n = 5)	74.4 ± 1.7 (n = 5)	59.2 ± 4.6 (n = 5)

ND: values were not determined because CFT, α angle, and MCF could not be measured in most control F8KO mouse blood samples.
CT: clotting time, CFT: clot formation time, MCF: maximum clot firmness.
Wild-type: wild-type mice, F8KO: F8 knock out (FVIII-deficient) mice, FVII: FVII over expressing F8KO mice, FVIIa: FVIIa over expressing F8KO mice.

Acknowledgements

This work was supported by Grants-in-Aid for Scientific Research from the Japanese Ministry of Education, Culture, Sports, Science and Technology; Health and Labour Science Research Grants for Research from the Japanese Ministry of Health, Labour and Welfare; and the Support Program for Strategic Research Platform from the Japanese Ministry of Education, Culture, Sports, Science and Technology.

Conflict of Interest Statement

The authors confirm no conflicts of interest to declare.

References

[1] Mannucci PM, Tuddenham EG. The hemophilias–from royal genes to gene therapy. N Engl J Med 2001;344:1773–9.
[2] Hasbrouck NC, High KA. AAV-mediated gene transfer for the treatment of hemophilia B: problems and prospects. Gene Ther 2008;15:870–5.
[3] Chuah MK, Collen D, VandenDriessche T. Clinical gene transfer studies for hemophilia A. Semin Thromb Hemost 2004;30:249–56.
[4] Kay MA, Manno CS, Ragni MV, Larson PJ, Couto LB, McClelland A, et al. Evidence for gene transfer and expression of factor IX in haemophilia B patients treated with an AAV vector. Nat Genet 2000;24:257–61.
[5] 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.
[6] 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.
[7] Nathwani AC, Tuddenham EG, Rangarajan S, Rosales C, McIntosh J, Linch DC, et al. Adenovirus-associated virus vector-mediated gene transfer in hemophilia B. N Engl J Med 2011;365:2357–65.
[8] Margaritis P, Arruda VR, Aljamali M, Camire RM, Schlachterman A, High KA. Novel therapeutic approach for hemophilia using gene delivery of an engineered secreted activated Factor VII. J Clin Invest 2004;113:1025–31.
[9] Margaritis P, Roy E, Aljamali MN, Downey HD, Giger U, Zhou S, et al. Successful treatment of canine hemophilia by continuous expression of canine FVIIa. Blood 2009;113:3682–9.
[10] Ohmori T, Ishiwata A, Kashiwakura Y, Madoiwa S, Mitomo K, Suzuki H, et al. Phenotypic correction of hemophilia A by ectopic expression of activated factor VII in platelets. Mol Ther 2008;16:1359–65.

[11] 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.
[12] Ishiwata A, Mimuro J, Mizukami H, Kashiwakura Y, Yasumoto A, Sakata A, et al. Mutant macaque factor IX T262A: a tool for hemophilia B gene therapy studies in macaques. Thromb Res 2010;125:533–7.
[13] 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.
[14] Bi L, Lawler AM, Antonarakis SE, High KA, Gearhart JD, Kazazian Jr HH. Targeted disruption of the mouse factor VIII gene produces a model of haemophilia A. Nat Genet 1995;10:119–21.
[15] Madoiwa S, Yamauchi T, Hakamata Y, Kobayashi E, Arai M, Sugo T, et al. Induction of immune tolerance by neonatal intravenous injection of human factor VIII in murine hemophilia A. J Thromb Haemost 2004;2:754–62.
[16] Darby SC, Keeling DM, Spooner RJ, Wan Kan S, Giangrande PL, Collins PW, et al. The incidence of factor VIII and factor IX inhibitors in the hemophilia population of the UK and their effect on subsequent mortality, 1977–99. J Thromb Haemost 2004;2:1047–54.
[17] Honda T, Toyoda H, Hayashi K, Katano Y, Yano M, Nakano I, et al. Ribavirin and use of clotting factors in patients with hemophilia and chronic hepatitis C. JAMA 2005;293:1190–2.
[18] Yamamoto K, Honda T, Matsushita T, Kojima T, Takamatsu J. Anti-HCV agent, ribavirin, elevates the activity of clotting factor VII in patients with hemophilia: a possible mechanism of decreased events of bleeding in patients with hemophilia by ribavirin. J Thromb Haemost 2006;4:469–70.
[19] Binny C, McIntosh J, Della Peruta M, Kymalainen H, Tuddenham EG, Buckley SM, et al. AAV-mediated gene transfer in the perinatal period results in expression of FVII at levels that protect against fatal spontaneous hemorrhage. Blood 2012;119:957–66.
[20] Radcliffe R, Nemerson Y. Mechanism of activation of bovine factor VII. Products of cleavage by factor Xa. J Biol Chem 1976;251:4749–802.
[21] Kisiel W, Fujikawa K, Davie EW. Activation of bovine factor VII (proconvertin) by factor XIIa (activated Hageman factor). Biochemistry 1977;16:4189–94.
[22] Bajaj SP, Rapaport SI, Brown SF. Isolation and characterization of human factor VII. Activation of factor VII by factor Xa. J Biol Chem 1981;256:253–9.
[23] Masys DR, Bajaj SP, Rapaport SI. Activation of human factor VII by activated factors IX and X. Blood 1982;60:1143–50.
[24] Stavenuiter F, Dienava-Verdoold I, Boon-Spijker MG, Brinkman HJ, Meijer AB, Mertens K. Factor seven activating protease (FSAP): does it activate factor VII? J Thromb Haemost 2012;10:859–66.
[25] Komiyama Y, Pedersen AH, Kisiel W. Proteolytic activation of human factors IX and X by recombinant human factor VIIa: effects of calcium, phospholipids, and tissue factor. Biochemistry 1990;29:9418–25.
[26] Margaritis P, Roy E, Faella A, Downey HD, Ivanciu L, Pavani G, et al. Catalytic domain modification and viral gene delivery of activated factor VII confers hemostasis at reduced expression levels and vector doses in vivo. Blood 2011;117:3974–82.
[27] Aljamali MN, Margaritis P, Schlachterman A, Tai SJ, Roy E, Bunte R, et al. Long-term expression of murine activated factor VII is safe, but elevated levels cause premature mortality. J Clin Invest 2008;118:1825–34.

The Journal of Arthroplasty 29 (2014) 569–573

Contents lists available at ScienceDirect

The Journal of Arthroplasty

journal homepage: www.arthroplastyjournal.org

Changes in Blood Coagulation–Fibrinolysis Markers By Pneumatic Tourniquet During Total Knee Joint Arthroplasty With Venous Thromboembolism

Hideaki Watanabe, MD [a], Ichiro Kikkawa, MD [a], Seiji Madoiwa, MD [b], Hitoshi Sekiya, MD [a], Shinya Hayasaka, MD [c], Yoichi Sakata, MD [b]

[a] *Department of Orthopedic Surgery, Jichi Medical University, Tochigi, Japan*
[b] *Research Division of Cell and Molecular Medicine, Center for Molecular Medicine, Jichi Medical University, Tochigi, Japan*
[c] *Department of Health Science, Daito Bunka University, Higashimatsuyama, Japan*

ARTICLE INFO

Article history:
Received 19 June 2013
Accepted 10 August 2013

Keywords:
cross-linked fibrin degradation products by leukocyte elastase (e-XDP)
D-dimer
soluble fibrin monomer complex
plasminogen activator inhibitor type 1
total knee arthroplasty
venous thromboembolism

ABSTRACT

This study investigated changes in blood coagulation–fibrinolysis markers during total knee arthroplasty (TKA). Preoperative 16-row multidetector row computed tomography (MDCT) revealed no asymptomatic venous thromboembolism (VTE) in the 42 patients recruited. Using MDCT postoperatively, patients were divided into thrombus (asymptomatic VTE, 19 patients) and no-thrombus (23 patients) groups. Blood taken at intervals before and after pneumatic tourniquet release revealed increased plasminogen activator inhibitor type-1 (PAI-1) at 30 s for both groups and at 90 s (both $P = 0.01$) in the thrombus group. D-dimer levels were highest at 30 and 90 s for both groups ($P = 0.01$). PAI-1 and D-dimer levels were strongly correlated at both time points in the thrombus group. Inactivating fibrinolysis due to PAI-1 may lead to asymptomatic VTE after TKA.

In orthopedic surgery, it is extremely important to prevent the development of postoperative venous thromboembolism (VTE), particularly symptomatic, fatal pulmonary embolism (PE), after total knee arthroplasty (TKA) [1]. Antithrombotic therapies using agents such as unfractionated or low-molecular-weight heparin have been administered to patients after surgery. Despite the implementation of aggressive antithrombotic protocols, however, the incidence of fatal PE remains at 0.15% [2] and that of symptomatic PE remains at 0.41% [3], with no changes in mortality rates since the 1990s [4]. Furthermore, in a cohort in Korea, the presence of asymptomatic VTE was 35.7% after TKA, as determined using multidetector row computed tomography (MDCT) [5]. Although it is thought that prophylactic antithrombotic treatments are necessary to prevent postoperative fatal and symptomatic PE, previous reports have found no difference in the incidence of these two entities or of asymptomatic VTE, regardless of whether prophylactic antithrombotic therapy was given [2–7]. In addition, reports indicate that the infection rate in prophylactically treated patients is increased owing to hematoma caused by hemorrhage [8–10] and coagulation abnormalities [11] associated with the therapy early after surgery. It is important for orthopedic surgeons to avoid these complications because such infections can last a lifetime. Even if patients achieve remission, they are prone to infection relapse. The routine administration of prophylactic antithrombotic treatment is not recommended in East Asia [12]. Based on these observations, to reduce postoperative infections associated with the overuse of antithrombotic treatment in low-risk patients, we have considered it clinically important to be able to detect early asymptomatic VTE that may cause fatal or symptomatic PE after surgery in patients who are not administered prophylactic antithrombotic treatments. Also, we start antithrombotic therapy only in those patients who need it [6,13]. There are currently no blood coagulation–fibrinolysis markers available for early detection of postoperative asymptomatic VTE following TKA.

Since 2005, some studies have indicated that VTE is affected by the use of the pneumatic tourniquet, causing particular postoperative changes in coagulation–fibrinolysis pathways [14–17]. Therefore, we hypothesized that detecting changes in blood coagulation–fibrinolysis markers in patients with asymptomatic VTE immediately after the pneumatic tourniquet is released might be used to indicate whether patients require antithrombotic therapy. This information could help prevent postoperative bleeding after administering antithrombotics to patients who were at low risk of developing VTE. The purpose of this study was to investigate the changes of blood coagulation–fibrinolysis markers in asymptomatic VTE immediately after release of the pneumatic tourniquet during surgery.

The Conflict of Interest statement associated with this article can be found at http://dx.doi.org/10.1016/j.arth.2013.08.011.

Reprint requests: Hideaki Watanabe, MD, Department of Orthopedic Surgery, Jichi Medical University, 3311-1 Yakushiji, Shimotsuke, Tochigi 3290498, Japan.

0883-5403/2903-0024$36.00/0 – see front matter © 2014 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.arth.2013.08.011

Materials and Methods

Patients

The study protocol was approved by the Ethics Review Board of our university. This prospective, single-center study enrolled patients who underwent TKA at our institution between April 2007 and March 2009 and gave consent to participate in the study. As exclusion criteria, patients with a past history of symptomatic VTE, cerebral hemorrhage, cerebral infarction, cardiac infarction, or drug allergy to a contrast medium were excluded from the study. In addition, patients with liver disease, renal disease, and/or congenital clotting factor deficiencies and those undergoing antithrombotic therapy or hemodialysis were excluded from the study. Patients with asymptomatic VTE by preoperative MDCT were also excluded.

We enrolled 42 patients who underwent TKA for osteoarthritis (30 knees) or rheumatoid arthritis (12 knees). The cohort comprised 1 male and 41 female patients, with a mean age of 71 years (range 49–84 years). TKA was performed under general anesthesia in all patients, and a pneumatic tourniquet was used. Its pressure was raised before surgery while the leg was exsanguinated and lowered about 90 min later. The tourniquet was used only one time. The patients wore an elastic stocking on the unaffected leg during surgery. Later, they wore them on both affected and unaffected legs and used an intermittent pneumatic compression device until walking training was initiated, in accordance with the Japanese Guidelines for Prevention of Venous Thromboembolism [18]. No postoperative prophylactic antithrombotic therapy was administered. If the patients developed symptomatic VTE and/or if VTE was detected by MDCT, aggressive antithrombotic therapy was initiated.

MDCT

For diagnosis of VTE, 16-row MDCT was performed on the 4th day before surgery and then the 4th day after surgery. These time points mark the interval at which the incidences of PE and VTE are reported to be high [19]. The latter is the earliest point at which patients could comfortably undergo MDCT during the postoperative period.

The MDCT slice thicknesses were 2 mm in the thoracic region and 5 mm from the abdomen to the lower limbs. The window levels were 40–60 and 40–50, and the window widths were 400–500 and 200–400, respectively. A single radiologist (M.D.) evaluated the MDCT images in a blinded manner before and after the surgery. The incidence of postoperative new asymptomatic VTE was calculated.

Preoperative MDCT revealed no asymptomatic VTE in any of the 42 patients included in the study. The patients were classified postoperatively via MDCT into two groups. The thrombus group was defined as patients with a new asymptomatic VTE, and the no-thrombus group was defined as those without asymptomatic VTE.

Blood Coagulation–Fibrinolysis Markers

Blood samples were taken to measure the plasma levels of plasminogen activator inhibitor-1 (PAI-1), soluble fibrin monomer complex (SFMC), D-dimer, and cross-linked fibrin degradation products by leukocyte elastase (e-XDP) immediately before and after release of the pneumatic tourniquet and then at 30, 90, and 180 s after release of the pneumatic tourniquet (Fig. 1). Citrated plasma samples were stored at −80 °C until analysis. The plasma PAI-1 levels were measured by a latex photometric immunoassay (Mitsubishi Chemical Medience Corporation, Tokyo, Japan) using the polyclonal antibody F(ab') fragment [20]. Plasma SFMC, D-dimer, and e-XDP levels were measured by latex immunoagglutination assays (Mitsubishi Chemical Medience Corporation) using the monoclonal antibodies IF-43 and JIF-23, respectively [21,22]. The plasma e-XDP levels were measured by a latex immunoagglutination assay (Mitsubishi Chemical Medience Corporation) using the monoclonal antibody IF-123 [23].

Statistical Analysis

Statistical analyses were performed using SPSS for Windows version 11.0 software (SPSS, Chicago, IL, USA). PAI-1, SFMC, D-dimer, and e-XDP levels were analyzed by the Shapiro–Wilk test if they did not fit a normal distribution. The PAI-1, SFMC, D-dimer, and e-XDP levels were compared between the thrombus and no-thrombus groups before release using the Mann–Whitney U-test. The PAI-1, SFMC, D-dimer and e-XDP levels were compared between immediately, at 30, 90, 180 s after release, respectively, and immediately before release using the Friedman test. If a significant difference was noted, the data were compared using the Wilcoxon signed rank test and corrected using Bonferroni's inequality. Spearman's rank correlation was used to determine whether blood coagulation–fibrinolysis markers that differed significantly were affected by each other. The gender and disorder distributions were compared between the thrombus and no-thrombus groups using Fisher's exact test. Age, volume of intraoperative hemorrhage, and operation time were compared using an unpaired *t*-test. The level of statistical significance was set at $P < 0.05$ for all tests.

Results

No patients developed symptomatic VTE during or after TKA in this study. Postoperative MDCT revealed asymptomatic VTE in 19 (45.2%) patients (thrombus group) and no VTE in 23 patients (54.7%) (no-thrombus group). Aggressive antithrombotic therapy was initiated in the 19 patients in whom new asymptomatic VTE was detected following postoperative MDCT (Table).

Changes in Operative Blood Coagulation–Fibrinolysis Markers Before Release of the Pneumatic Tourniquet

There were no significant differences in the preoperative PAI-1, SFMC, D-dimer, or e-XDP levels between the thrombus and no-thrombus groups ($P = 0.23$, $P = 0.23$, $P = 0.39$, and $P = 0.89$, respectively) (Fig. 2).

Operative Blood Coagulation–Fibrinolysis Markers After Release of the Pneumatic Tourniquet

The PAI-1 level showed the most significant increases at 30 s (median 27.3 ng/ml, $P = 0.01$) and 90 s (median 28.5 ng/ml, $P = 0.01$) after release of the pneumatic tourniquet in the thrombus groups and at 30 s (median 38.7 ng/ml, $P = 0.01$) after release in the no-thrombus group (Fig. 2).

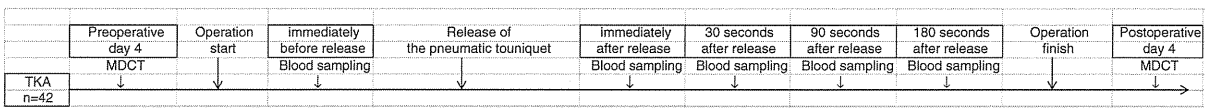

Fig. 1. Study protocol.

Table
Study Demographics.

		Thrombus Group (n = 19)	No-Thrombus Group (n = 23)	P	95% Confidence Interval
Gender	Male:Female	0:19	1:22	0.55[a]	
Ages	Years	72 (60–82)	71 (49–84)	0.76[b]	−5 to 6
Disorder distribution	Osteoarthritis:Rheumatoid arthritis	13:6	17:6	0.48[a]	
Volume of intraoperative hemorrhage	ml	46 (0–120)	32 (0–260)	0.43[b]	−22 to 49
Operation time	min	155 (122–199)	148 (106–186)	0.31[b]	−7 to 21

[a] Fisher's exact test.
[b] Unpaired t-test.

The D-dimer level showed the most significant increases at 30 s (median 0.84 μg/ml, $P = 0.01$) and 90 s (median, 0.86 μg/ml, $P = 0.01$) after tourniquet release in the thrombus groups and at 30 s (median 0.73 μg/ml, $P = 0.01$) and 90 s (median 0.7 μg/ml, $P = 0.01$) after tourniquet release in the no-thrombus group (Fig. 2).

The SFMC and e-XDP levels did not differ significantly among the various time points before and after tourniquet release in the thrombus and no-thrombus groups (Fig. 2).

Spearman's rank correlation showed that the PAI-1 levels were strongly correlated with the D-dimer levels at 30 s ($\gamma = 0.57$, $P = 0.01$) and 90 s ($\gamma = 0.6$, $P = 0.01$) after tourniquet release in the thrombus group and were not correlated with the D-dimer levels at 30 ($P = 1.00$) and 90 s ($P = 1.00$) after tourniquet release in the no-thrombus group (Fig. 3).

There was no significant difference in gender, age, volume of intraoperative hemorrhage, or operation time between the thrombus and no-thrombus groups (Table 1).

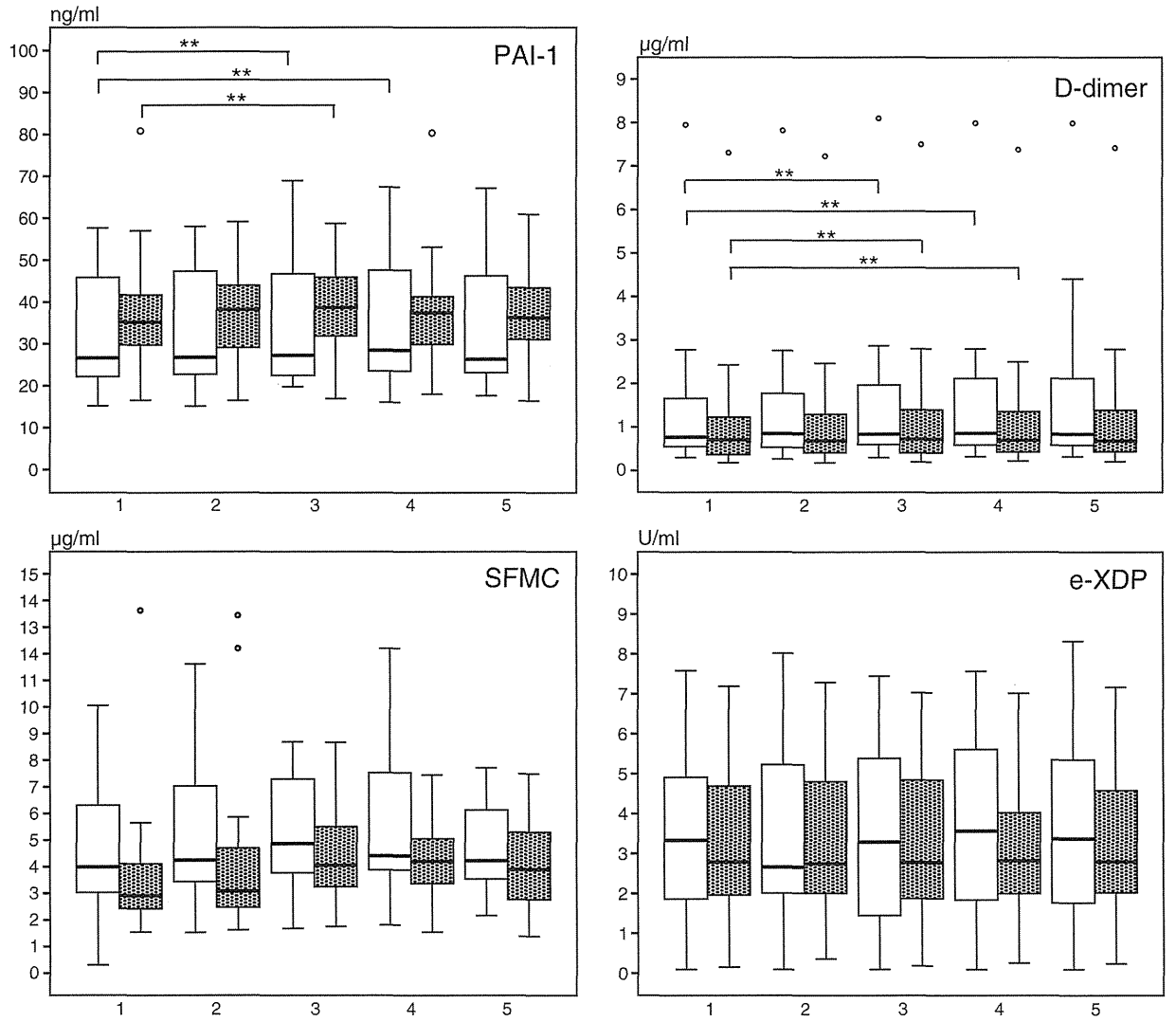

Fig. 2. Changes in the PAI-1 and SFMC, D-dimer, e-XDP levels before and after release of the pneumatic tourniquet. On the x-axis, the numbers correspond to the following: 1: immediately before release of the pneumatic tourniquet; 2: during; 3: at 30 s; 4: at 90 s; 5: at 180 s after release of the pneumatic tourniquet. White boxes, thrombus group; dot boxes, no-thrombus group. °Outlier. $^{**}P < 0.05$ versus the preoperative level in the thrombus and the no-thrombus group by the Wilcoxon signed-rank test with correction by Bonferroni's inequality. PAI-1, plasminogen activator inhibitor-1; SFMC, soluble fibrin monomer complex; e-XDP, cross-linked fibrin degradation products by leukocyte elastase.

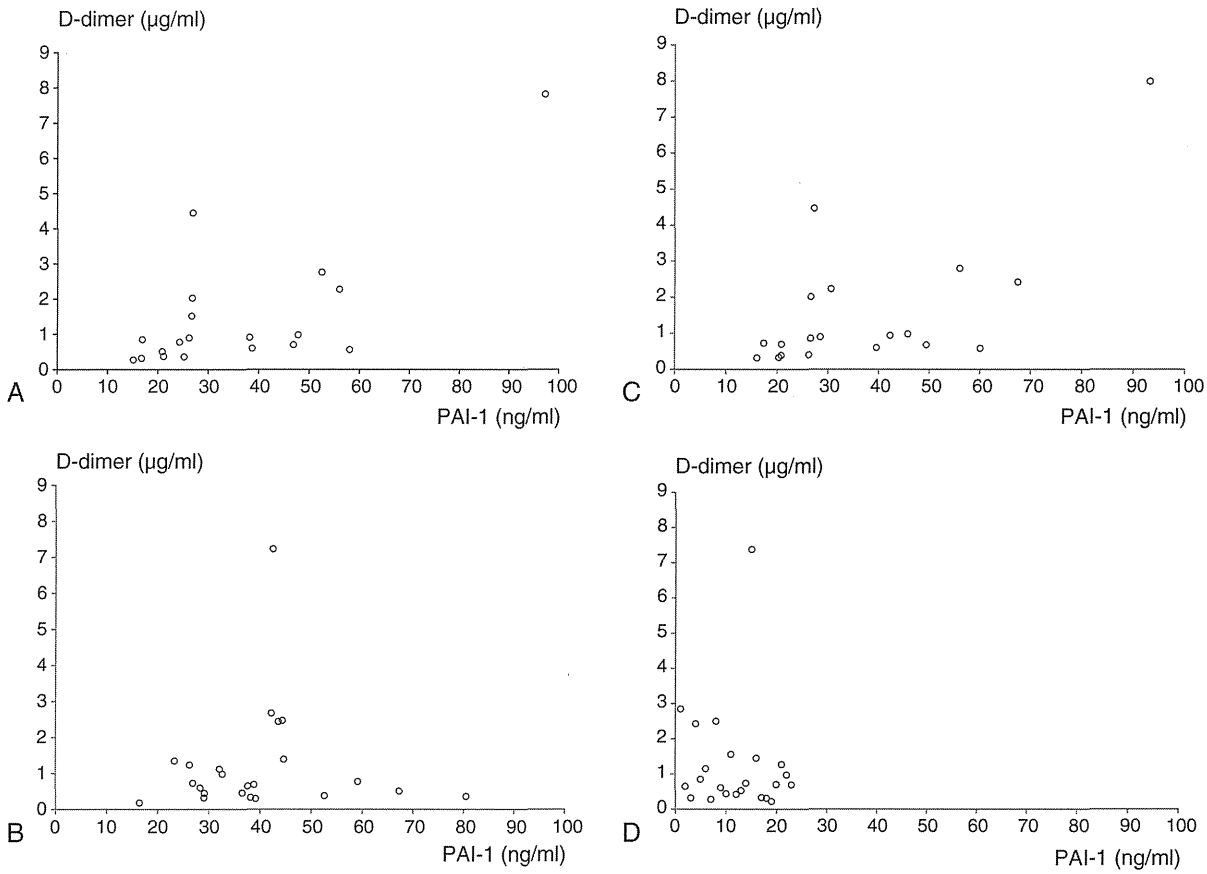

Fig. 3. Correlation between the PAI-1 and D-dimer levels at different time points. (A) At 30 s after release of the pneumatic tourniquet in the thrombus group ($P = 0.01$. $\gamma = 0.57$). (B) At 30 s after release of the pneumatic tourniquet in the no-thrombus group ($P = 1.00$). (C) At 90 s after release of the pneumatic tourniquet in the thrombus group ($P = 0.01$. $\gamma = 0.6$). (D) At 90 s after release of the pneumatic tourniquet in the no-thrombus group ($P = 1.00$). PAI-1, plasminogen activator inhibitor-1.

Discussion

Coagulation–fibrinolysis markers that may be predictive of postoperative asymptomatic DVT and VTE after TKA have been identified. Bounameaux et al [24] performed venography and D-dimer measurements on day 3 after TKA. They found that the D-dimer level was significantly elevated in patients with asymptomatic DVT and that the sensitivity and specificity were 58.8% and 73.5%, respectively, at a cutoff level of 3000 μg/ml. In 2000, Rever et al [25] performed venography after TKA and reported that the SFMC level was significantly elevated in patients with asymptomatic DVT on postoperative days 3 and 6. In 2012, Watanabe et al [26] performed MDCT after TKA and reported that the e-XDP level on postoperative day 1 and the D-dimer level on postoperative day 4 were significantly elevated in patients with asymptomatic VTE. They also noted that the sensitivity and specificity were 75% and 75%, respectively, for e-XDP levels and 59% and 63%, respectively, for D-dimer levels, with cutoff levels of 8.2 U/ml and 7.5 μg/ml, respectively.

Despite this work, there have been no reports of blood coagulation–fibrinolysis markers for predicting postoperative asymptomatic VTE in patients undergoing TKA. In the present study, the PAI-1 level at 90 s after release of the pneumatic tourniquet was significantly higher in the thrombus group than in the no-thrombus group. Furthermore, Spearman's rank correlation showed that PAI-1 was strongly correlated with D-dimer at 90 s ($\gamma = 0.6$, $P = 0.01$) after release in thrombus group and was not correlated with D-dimer at 90 s ($P = 1.00$) after release in the no-thrombus group. As there were no significant differences in gender, age, disorder distribution, volume of intraoperative hemorrhage, or operation time between the thrombus and no-thrombus groups, we can surmise that the PAI-1 level at 90 s after release of the pneumatic tourniquet may be associated with asymptomatic VTE after TKA and is a dependent marker for D-dimer. D-dimer is produced by fibrin and the presence of a thrombus. An elevation in the D-dimer level indicates fibrinolysis of the thrombus. PAI-1 inactivates fibrinolysis by acting on the plasmin in the plasminogen activator–plasmin system. Therefore, we believe that increased inactivation of fibrinolysis leads to the development of asymptomatic VTE after TKA. A recent study has also demonstrated an association between PAI-1 levels and VTE after total hip arthroplasty [27]. If PAI-1 causes asymptomatic VTE and subsequent symptomatic, fatal PE, inactivation of PAI-1 may prevent the development of symptomatic, fatal PE. This study is a single-center study. If the results of future multicenter studies are similar, it may be stated that PAI-1 is likely to cause asymptomatic VTE and, subsequently, symptomatic, fatal PE.

The D-dimer levels at 30 and 90 s and PAI-1 levels at 30 s after release of the pneumatic tourniquet were significantly elevated in both groups compared with the values before tourniquet release. Katsumata et al [14] and Nishiguchi et al [15] measured changes in coagulation–fibrinolysis markers between patients with and without the pneumatic tourniquet after TKA. They identified significantly higher D-dimer levels immediately after surgery and on the first day with the pneumatic tourniquet. In a similar study, Reikeras et al [17] also found elevated D-dimer levels immediately after release of the pneumatic tourniquet. From these clinical studies and our study, we consider that the use of the pneumatic tourniquet during TKA affects the concentrations of blood coagulation–fibrinolysis markers and may cause thrombus formation. Thus, the pneumatic tourniquet should be used at little as possible during TKA.

The PAI-1 level was strongly correlated with the D-dimer level at 30 s ($R = 0.57$, $P = 0.01$) after release in thrombus group and was

not correlated with the D-dimer level at 30 s ($P = 1.00$) after release in no-thrombus groups. The PAI-1-like products that are not associated with fibrinolysis may be in the bone marrow and may be released into the bloodstream at an early time point after release of the pneumatic tourniquet.

One limitation of our study is that we do not know whether early detection of asymptomatic VTE prevents symptomatic, fatal PE. Therefore, we have continued to follow these patients in daily clinics after completion of this study. So far, none of the patients has suffered from symptomatic, fatal PE. Another limitation of our study is that MDCT was performed 4 days preoperatively and postoperatively, and the results therefore reflect the state of asymptomatic VTE at these time points. This is because the incidence of PE after TKA was reported to be high at postoperative days 3 or 4 [16]. Furthermore, day 4 was the earliest point during the postoperative period at which the patients had less pain and could comfortably undergo MDCT. However, because MDCT was not performed between the day of surgery and postoperative day 3, it can be assumed that not all asymptomatic VTEs were detected during the perisurgical period. Thus, the incidence of postsurgical asymptomatic VTE may be underestimated, and larger studies are required to verify the changes in coagulation–fibrinolysis markers in patients with asymptomatic VTE during surgery.

In summary, we investigated changes in blood coagulation–fibrinolysis markers during TKA in thrombus and no-thrombus groups using MDCT. PAI-1 levels were highest at 30 s in both groups and at 90 s in the thrombus group. D-dimer levels were highest at 30 and 90 s in both groups. PAI-1 and D-dimer levels were strongly correlated at both time points in the thrombus group, whereas they were not correlated in the no-thrombus group. Inactivating fibrinolysis due to PAI-1 may lead to the development of asymptomatic VTE and, subsequently, to symptomatic, fatal PE after TKA. Inactivation of PAI-1 may prevent the development of symptomatic, fatal PE after TKA.

Acknowledgments

The authors wish to thank Drs. Masao Naito, Naoya Sugimoto, Masato Sakaguchi, Kenzo Takatoku, Hisashi Takada and Hiroshi Tamura for their assistance during this study.

References

1. Berend KR, Lombardi Jr AV. Multimodal venous thromboembolic disease prevention for patients undergoing primary or revision total joint arthroplasty: the role of aspirin. Am J Orthop 2006;35:24.
2. Howie C, Hughes H, Watts AC. Venous thromboembolism associated with hip and knee replacement over a ten-year period: a population study. J Bone Joint Surg Br 2005;87:1675.
3. Soohoo NF, Zingmond DS, Lieberman JR, et al. Primary total knee arthroplasty in California 1991 to 2001: does hospital volume affect outcomes? J Arthroplasty 2006;21:199.
4. Geerts WH, Heit JA, Clagett GP, et al. Prevention of venous thromboembolism. Chest 2001;119:132S.
5. Park KH, Cheon SH, Lee JH, et al. Incidence of venous thromboembolism using 64 channel multidetector row computed tomography indirect venography and anticoagulation therapy after total knee arthroplasty in Korea. Knee Surg Relat Res 2012;24:19.
6. Watanabe H, Sekiya H, Kariya Y, et al. The incidence of venous thromboembolism before and after total knee arthroplasty using 16-row multidetector computed tomography. J Arthroplasty 2011;26:1488.
7. Sharrock NE, Gonzalez Della Valle A, Go G, et al. Potent anticoagulants are associated with a higher all-cause mortality rate after hip and knee arthroplasty. Clin Orthop Relat Res 2008;466:714.
8. Saleh K, Olson M, Resig S, et al. Predictors of wound infection in hip and knee joint replacement: results from a 20 year surveillance program. J Orthop Res 2002;20:506.
9. Parvizi J, Ghanem E, Joshi A, et al. Does "excessive" anticoagulation predispose to periprosthetic infection? J Arthroplasty 2007;22:24.
10. Burnett RS, Clohisy JC, Wright RW, et al. Failure of the American College of Chest Physicians-1A protocol for lovenox in clinical outcomes for thromboembolic prophylaxis. J Arthroplasty 2007;22:317.
11. Saxena A, Baratz M, Austin MS, et al. Periprosthetic joint infection can cause abnormal systemic coagulation. J Arthroplasty 2011;26:50.
12. Cho KY, Kim KI, Khurana S, et al. Is routine chemoprophylaxis necessary for prevention of venous thromboembolism following knee arthroplasty in a low incidence population? Arch Orthop Trauma Surg 2013;133:551.
13. Abunasser J, Tejada JP, Foley RJ. The diagnosis and management of pulmonary embolism. Conn Med 2012;76:5.
14. Katsumata S, Nagashima M, Kato K, et al. Changes in coagulation–fibrinolysis marker and neutrophil elastase following the use of tourniquet during total knee arthroplasty and the influence of neutrophil elastase on thromboembolism. Acta Anaesthesiol Scand 2005;49:510.
15. Nishiguchi M, Takamura N, Abe Y, et al. Pilot study on the use of tourniquet: a risk factor for pulmonary thromboembolism after total knee arthroplasty? Thromb Res 2005;115:271.
16. Kageyama K, Nakajima Y, Shibasaki M, et al. Increased platelet, leukocyte, and endothelial cell activity are associated with increased coagulability in patients after total knee arthroplasty. J Thromb Haemost 2007;5:738.
17. Reikeras O, Clementsen T. Time course of the thrombosis and fibrinolysis in total knee arthroplasty with tourniquet application. Local versus systemic activations. J Thromb Thrombolysis 2009;28:425.
18. Editorial Committee on Japanese guideline for prevention of venous thromboembolism: digest. 2nd ed. Medical Front International Limited, Tokyo, 2004; p. 15–6.
19. Warwick D, Friedman RJ, Agnelli G, et al. Insufficient duration of venous thromboembolism prophylaxis after total hip or knee replacement when compared with the time course of thromboembolic events: findings from the Global Orthopaedic Registry. J Bone Joint Surg Br 2007;89:799.
20. Ono T, Sogabe M, Ogura M, et al. Automated latex photometric immunoassay for total plasminogen activator inhibitor-1 in plasma. Clin Chem 2003;49:987.
21. Soe G, Kohno I, Inuzuka K, et al. A monoclonal antibody that recognizes a neoantigen exposed in the E domain of fibrin monomer complexed with fibrinogen or its derivatives: its application to the measurement of soluble fibrin in plasma. Blood 1996;88:2109.
22. Matsuda M, Terukina S, Yamazumi K, Maekawa H, Soe G. A monoclonal antibody that recognizes the NH2-terminal conformation of fragment D. p. 43–8. In: Fibrinogen 4, current basic and clinical aspects: proceedings of the International Fibrinogen Workshop, Kyoto, Japan, 27–28 August 1989. Amsterdam: Excerpta Medica; 1990: 43–8.
23. Kohno I, Inuzuka K, Itoh Y, et al. A monoclonal antibody specific to the granulocyte-derived elastase-fragment D species of human fibrinogen and fibrin: its application to the measurement of granulocyte-derived elastase digests in plasma. Blood 2000;95:1721.
24. Bounameaux H, Miron MJ, Blanchard J, et al. Measurement of plasma D-dimer is not useful in the prediction or diagnosis of postoperative deep vein thrombosis in patients undergoing total knee arthroplasty. Blood Coagul Fibrinolysis 1998;9:749.
25. Rever G, Blanchard J, Bounameaux H, et al. Inability of serial fibrin monomer measurements to predict or exclude deep venous thrombosis in asymptomatic patients undergoing total knee arthroplasty. Blood Coagul Fibrinolysis 2000;11:305.
26. Watanabe H, Madoiwa S, Sekiya S, et al. Predictive blood coagulation markers for early diagnosis of venous thromboembolism after total knee joint replacement. Thromb Res 2011;128:e137.
27. Yukizawa Y, Inaba Y, Watanabe S, et al. Association between venous thromboembolism and plasma levels of both soluble fibrin and plasminogen-activator inhibitor 1 in 170 patients undergoing total hip arthroplasty. Acta Orthop 2012;83:14.

The Prevalence of Neutralizing Antibodies Against Adeno-Associated Virus Capsids Is Reduced in Young Japanese Individuals

Jun Mimuro,[1]* Hiroaki Mizukami,[2] Midori Shima,[3] Tadashi Matsushita,[4] Masashi Taki,[5] Shinji Muto,[6] Satoshi Higasa,[7] Michio Sakai,[8] Tsukasa Ohmori,[1] Seiji Madoiwa,[1] Keiya Ozawa,[2] and Yoichi Sakata[1]

[1]*Division of Cell and Molecular Medicine, Center for Molecular Medicine, Jichi Medical University, Shimotsuke, Japan*
[2]*Division of Genetic Therapeutics, Center for Molecular Medicine, Jichi Medical University, Shimotsuke, Japan*
[3]*Department of Pediatrics, Nara Medical University, Kashihara, Japan*
[4]*Department of Blood Transfusion, Nagoya University Hospital, Nagoya, Japan*
[5]*Department of Pediatrics, St. Marianna University School of Medicine, Yokohama City Seibu Hospital, Yokohama, Japan*
[6]*Department of Pediatrics, St. Marianna University School of Medicine, Kawasaki, Japan*
[7]*Division of Hematology, Department of Internal Medicine, Hyogo College of Medicine, Nishinomiya, Japan*
[8]*Department of Pediatrics, School of Medicine, University of Occupational and Environmental Health, Kitakyushu, Japan*

Pre-existing antibodies against adeno-associated virus (AAV), caused by natural AAV infections, interfere with recombinant AAV vector-mediated gene transfer. We studied the prevalence of neutralizing antibodies against AAV serotypes 1, 2, 5, 8, and 9 in healthy subjects (n = 85) and hemophilia patients (n = 59) in a Japanese population. For healthy subjects, the prevalence of neutralizing antibodies against AAV serotypes 1, 2, 5, 8, and 9 was 36.5%, 35.3%, 37.6%, 32.9%, and 36.5%, respectively, while that in hemophilia patients was 39.7%, 28.8%, 35.6%, 32.9%, and 27.4%, respectively. There was no difference in the prevalence of neutralizing antibody against each AAV serotype between the healthy subjects and the hemophilia patients. The prevalence of neutralizing antibodies against all AAV serotypes increased with age in both healthy subjects and hemophilia patients. High titers of neutralizing antibodies against AAV2 (≥1:224) and AAV8 (≥1:224) were more evident in older individuals (≥42 years old). Approximately 50% of all screened individuals were seronegative for neutralizing antibodies against each AAV tested, while approximately 25% of individuals were seropositive for each AAV serotype tested. The prevalence of seronegativity for all AAV serotypes was 67.0% (healthy subjects, 68.6%; hemophilia patients, 65.0%) and 18.6% (healthy subjects, 20.5%; hemophilia patients, 15.7%) in young (<42 years old) and older subjects (≥42 years old), respectively. The findings from this study suggested that young subjects are more likely to be eligible for gene therapy based on AAV vectors delivered via an intravascular route because of the low prevalence of antibodies to AAV capsids. ***J. Med. Virol.*** © 2013 Wiley Periodicals, Inc.

KEY WORDS: AAV vector; gene therapy; epidemiology

Grant sponsor: Grants-in-Aid for Scientific Research; Grant numbers: 20591155; 21591249; 21790920; Grant sponsor: Support Program for Strategic Research Infrastructure from the Japanese Ministry of Education, Culture, Sports, Science and Technology; Grant sponsor: Health, Labour and Science Research Grants for Research on HIV/AIDS; Grant sponsor: Research on Intractable Diseases from the Japanese Ministry of Health, Labour and Welfare

The authors declare no conflicts of interest.

*Correspondence to: Jun Mimuro, MD, PhD, Division of Cell and Molecular Medicine, Center for Molecular Medicine, Jichi Medical University, 3311-1 Yakushiji, Shimotsuke 329-0498, Japan. E-mail: cell.mol.bio.j@gmail.com

Accepted 20 September 2013

DOI 10.1002/jmv.23818
Published online in Wiley Online Library (wileyonlinelibrary.com).