(2200*g*, 10 min, r.t.). The clinical laboratory testing such as prothrombin time (PT), activated partial thromboplastin time (APTT), and fibrinogen antigen (Fbg) was carried out by SRL Inc. (Tokyo).

Statistical analyses A statistical evaluation of various counts of the PRP group vs. the PPP group is shown in Fig. 2. A comparison of the H12-PEG-polyAlb group vs. the PEG-polyAlb or the saline group as shown in Fig. 3 was also carried out using Tukey-Kramer tests. A P-value of less than 0.05 was considered to be statistically significant. Statistical analyses were performed using Stat View software (SAS Institute).

RESULTS

Characterization of H12-PEG-polyAlb We modified the surface of polyAlb particles (diameter of 240 $\pm$ 100 nm) with MALPEG and mPEG, and the number of MALPEG and mPEG molecules that chemically bound to one polyAlb particle was estimated to be approximately 1.2×10^4 and 6.1×10^4 molecules, respectively, by the indirect quantification of free PEG molecules. Furthermore, the number of H12 conjugated per polyAlb was estimated to be approximately 1.0×10^4. The endotoxin concentration in the suspension of H12-PEG-polyAlb at an rHSA concentration of 10 mg mL^{-1} was below 0.25 EU mL^{-1}, and this was acceptable for the *in vivo* study.

Busulphan-induced thrombocytopenic rabbits Rabbits that received busulphan at total doses of 40 mg kg^{-1} and 50 mg kg^{-1} developed profound thrombocytopenia with a decline in platelet count to the half-maximal value on Day 7, and the count reached the lowest value on Day 15 and 14, respectively (Fig. 1). However, the hematocrit and leukocytes of these rabbits also decreased, and the rabbits became anemic and purpuric as shown in Table 1. On the other hand, at a total dose of 30 mg kg^{-1}, thrombocytopenia was just as profound as at the higher doses of busulphan, and the rabbits showed a decline in platelet count to the half-maximal value on Day 7. On Days 13, 14 and 15, the platelet counts were 6.9 $\pm$ 0.1, 3.5 $\pm$ 1.0, and 2.4 $\pm$ 1.3 x 10^4 μL^{-1}, respectively, which were equivalent to 1/15 or 1/20 of the platelet counts of normal rabbits ((37.3 $\pm$ 7.6) x 10^4 μL^{-1}). There was a slight decrease in the leukocyte counts of the busulphan-treated rabbits compared with normal rabbits, and the hematocrits were unchanged, as shown in Table 1. After the platelet counts reached their lowest point on Day 15, the count persisted for at least one day, then gradually started to rise, and recovered to the normal level on Day 20. Consequently, for the *in vivo* experiment we used rabbits having the most severe thrombocytopenia on Day 15 after the busulphan treatment to evaluate the hemostatic ability of H12-PEG-polyAlb.

Platelet transfusion and measurement of ear bleeding time When the PRP with counts of 0.4 x 10^9, 1.2 x 10^9, 2.0 x 10^9, and 4.0 x 10^9 kg^{-1} (1.0 x 10^5, 3.0 x 10^5, 5.0 x 10^5, and 1.0 x 10^6 μL^{-1} at a dose of 4 mL kg^{-1}, respectively) were transfused intravenously into the

thrombocytopenic rabbits ([platelet] = 2.1 ± 0.5 x 10^4 μL^{-1}), the platelet counts gradually increased with time, reaching maximal values 30 min after transfusion, which were 2.2 ± 1.1, 3.2 ± 0.9, 4.0 ± 1.1, and 6.1 ± 0.8 x 10^4 μL^{-1}, respectively (Fig. 2). The mean percentages of recovery at 30 min after transfusion were calculated to be 65.1 ± 14.8, 65.4 ± 22.1, 66.7 ± 15.8, and 76.6 ± 9.2%, respectively. Furthermore, the half-life and survival time of transfused platelets at a dose of 2.0 x 10^9 kg^{-1} were estimated to be 39.1 ± 6.1 hrs and 56.4 ± 5.5 hrs, respectively. On the other hand, the platelet count did not change before and after PPP transfusion.

The ear bleeding times of the normal rabbits ([platelet] = 37.3 ± 7.6 x 10^4 μL^{-1}) and the thrombocytopenic rabbits ([platelet] = 2.4 ± 1.3 x 10^4 μL^{-1}) after the saline injection were 112 ± 24 and 1695 ± 197 s, respectively (Fig. 3). The bleeding time of the thrombocytopenic rabbits was approximately 15 times longer than that of the normal rabbits. The bleeding time at 30 min after PPP transfusion was 1473 ± 442 s, which was almost comparable to that obtained with the control thrombocytopenic rabbits injected with saline. At doses of 0.4 x 10^9, 1.2 x 10^9, 2.0 x 10^9, and 4.0 x 10^9 kg^{-1}, there was a dose-dependent reduction in the bleeding time of the PRP, and the bleeding time was significantly reduced to 1505 ± 410, 863 ± 540, 867 ± 440, and 505 ± 257 s, respectively (Fig. 2).

Hemostatic effects of the H12-PEG-polyAlb When the H12-PEG-polyAlb at the dose of 40 mg kg^{-1} was injected into the thrombocytopenic rabbits, the bleeding time was not reduced

(1416 ± 533 s) and was comparable to the control PEG-polyAlb at a dose of 40 mg kg^{-1} (1431 ± 402 s), as shown in Fig. 3. However, intravenous administration of the H12-PEG-polyAlb at a dose of 80 mg kg^{-1} significantly reduced the bleeding time to 834 ± 266 s from compared to administration of saline (1695 ± 197 s) or the control PEG-polyAlb (1592 ± 286 s) at a dose of 80 mg kg^{-1}. At a dose of 120 mg kg^{-1}, the H12-PEG-polyAlb also significantly reduced the bleeding time to 990 ± 294 s compared to the control PEG-polyAlb (1772 ± 49 s), and the reduction effect was comparable to the response to the H12-PEG-polyAlb at a dose of 80 mg kg^{-1}.

Measurement of blood coagulation parameters Three blood coagulation parameters: PT, APTT, and Fbg antigen were evaluated for the rabbit blood approximately 60 min after the measurement of bleeding time, as listed in Table 2. No significant difference was seen between the sample groups (H12-PEG-polyAlb and PEG-polyAlb) and the control saline group, indicating no influence of the H12-PEG-polyAlb particles on the endogenous and exogenous coagulation activities.

DISCUSSION

We previously succeeded in prolonging the *in vivo* blood residence time of H12-conjugated polyAlb by PEG modification (H12-PEG-polyAlb), and we confirmed that the H12-PEG-polyAlb maintained an ability to specifically bind activated platelets (Okamura

et al., 2007). Furthermore, the H12-PEG-polyAlb dose-dependently shortened the tail bleeding time of rats with moderate thrombocytopenia (Okamura *et al.*, 2007). In this study, we have evaluated the hemostatic effects of H12-PEG-polyAlb as a platelet substitute using larger animals with severe thrombocytopenia (thrombocytopenic rabbits).

We conjugated H12 to the surface of polyAlb particles modified with PEG chains, estimating the conjugation density on the polyAlb surface as approximately 46 x 10^3 molecules μm^{-2}. We previously confirmed that the conjugation density of H12 over 46 x 10^3 molecules μm^{-2} need to maintain binding ability toward activated platelets, based on the flow cytometric analyses (Okamura *et al.*, 2005; 2007). The density was similar to that of the H12-PEG-polyAlb in our previous studies, which was enhanced thrombus formation under the *in vitro* flow conditions, and significantly reduced the tail bleeding time of thrombocytopenic rats. On the other hand, the number of GPIIb/IIIa on one platelet is approximately 80 x 10^3 molecules, of which density is calculated to be approximately 4.1 x 10^3 μm^{-2} based on the diameter of typical platelet (approximately 2.5 μm), referring to a report by Wagner *et al.* (1996). Consequently, the conjugation number of H12 on the surface of the polyAlb particle was 10-fold level in comparison with that of GPIIb/IIIa.

Next, we induced severe thrombocytopenia in the rabbits using busulphan in order to evaluate the hemostatic ability of the H12-PEG-polyAlb *in vivo*. We obtained a platelet extinction curve similar to that seen in previous studies (Kuter *et al.*, 1995). Sola *et al.* previously reported that a low hematocrit resulted in a significant prolongation in the bleeding

time. In fact, the bleeding times on Day 15 of all rabbits that received busulphan at total doses of 40 or 50 mg kg^{-1} were not measurable, since the bleeding did not stop for more than 30 min (data not shown). From the data in Table 1 showing hematologic indices, we evaluated conditions producing a low platelet count but maintaining a constant hematocrit value, and we determined that the appropriate busulphan dose was 30 mg kg^{-1} for the rabbits. Furthermore, the bleeding time of the thrombocytopenic rabbits on Day 15 was significantly extended in comparison with that of the normal rabbits and the thrombocytopenic rabbits on Day 13 or 14 (data not shown). We decided that the ear incision was to be made on Day 15, and prepared the severely thrombocytopenic rabbits.

We judged that there are specific hemostatic effects when H12-PEG-polyAlb was significantly reduced the ear bleeding time of thrombocytopenic rabbits in comparison with saline and control PEG-polyAlb (H12 non-conjugaton) groups. Using these rabbits, we confirmed the significant hemostatic effect of the H12-PEG-polyAlb at the doses of 80 mg kg^{-1} and 120 mg kg^{-1}, although a dose-dependent reduction was not observed, as shown in Fig. 3. On the other hand, the H12-PEG-polyAlb at a dose of 40 mg kg^{-1} did not produce this effect. We previously reported a significant hemostatic effect of H12-PEG-polyAlb at a dose of 40 mg kg^{-1} using rats with moderately thrombocytopenia (Okamura *et al.*, 2007). The present results indicate that a higher dose of the H12-PEG-polyAlb was necessary to assist platelet hemostasis in the severe thrombocytopenia model. Based on our previous studies of the H12-particles under flow conditions *in vitro* (Takeoka *et al.*, 2003), we hypothesized that

the H12-PEG-polyAlb would work at the vascular injury by the following mechanisms: (1) adhesion of the H12-PEG-polyAlb could be initiated by the activated platelets, which had already adhered on the surface of the exposed collagen at the vascular injury, (2) the H12-PEG-polyAlb adhering to the surface of the platelet could provide additional binding sites for activated platelets, and (3) the H12-PEG-polyAlb could accelerate thrombus formation by enhancing aggregation of the flowing platelets. However, in the case of severe thrombocytopenia, adhesion of the H12-PEG-polyAlb decreased because the fewer platelets adhered on the collagen. It was suggested that higher doses of the H12-PEG-polyAlb would be necessary to assist platelet hemostasis by recruiting the flowing platelets and filling up the vascular injury site using the volume of the H12-PEG-polyAlb particles. However, considering the dose-independent reduction of the particles, it was also suggested that there was a limited time window for the H12-PEG-polyAlb to accumulate and fill up the vascular injury site, and that additional processes such as amplification of platelet aggregation and blood coagulation would be necessary to further promote platelet hemostasis.

Furthermore, we also confirmed that hematologic indices (data not shown) and coagulation parameters as shown in Table 2 did not change before and after the infusion of the H12-PEG-polyAlb, suggesting that the polyAlb was a safe particle with a minimal likelihood of causing side effects after injection.

We used a platelet transfusion model as a positive control for the platelet substitutes. The survival time of the transfused platelets was comparable to that obtained from the ^{51}Cr

and ^{111}In-labeled platelets evaluated in previous reports (Packham *et al.*, 1992; Franco *et al.*, 1994), whereas the mean percentage of recovery was similar to the previous reports (Rand *et al.*, 2002; Packham *et al.*, 1992; Franco *et al.*, 1994). Furthermore, confirmation of a dose-dependent reduction in the bleeding time after administering PRP to severe thrombocytopenic rabbits established a positive control for comparison with platelet substitutes. We proposed an equation (1) relating the transfused platelet counts to the bleeding time as follows:

$$y = -252.03\ x + 1425.6\ (r^2 = 0.85) \qquad \text{(equation 1)}$$

where the x value gives the PRP (10^9 kg^{-1}), and y value gives the ear bleeding time (s). We estimated the hemostatic capacity of H12-PEG-polyAlb using equation (1). The bleeding time (834 $\pm$ 266 s) of the H12-PEG-polyAlb at a dose of 80 mg kg^{-1} (approximately 7.4 x 10^{13} particles kg^{-1}) was similar to that following platelet transfusion at a dose of 2.4 x 10^9 kg^{-1}. Similarly, in the case of 120 mg kg^{-1} (approximately 1.1 x 10^{14} particles kg^{-1}), the bleeding time of the H12-PEG-polyAlb was similar to that of platelet transfusion at 1.7 x 10^9 kg^{-1}. These results indicate the hemostatic capacity of the H12-PEG-polyAlb at the particle number 3.1 x 10^4 to 6.5 x 10^4 would correspond to that of one platelet. We also evaluated the hemostatic capacity based on the total injected particle volume, because the particle diameter of the H12-PEG-polyAlb (240 $\pm$ 100 nm) is 10-fold smaller than that of a typical platelet (2~3 μm). We calculated that the volume of the particles required for hemostatic support was 31- to 65-fold less than that of platelets that would produce a similar effect.

In conclusion, the H12-PEG-polyAlb significantly shortened the ear bleeding time of severely thrombocytopenic rabbits. We assessed the hemostatic capacity of the H12-PEG-polyAlb based on comparisons with platelet transfusions and calculated that the hemostatic capacity of the H12-PEG-polyAlb was approximately 31 or 65-fold greater than that of a similar volume of platelets. Thus, the H12-PEG-polyAlb may be a suitable candidate for an alternative to human platelet concentrates infused to treat bleeding in patients with severe thrombocytopenia. In the future, we plan to assess the hemostatic ability of the H12-PEG-polyAlb to treat animals with severe thrombocytopenia resulting from blood loss during surgery.

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FIGURE CAPTIONS

Fig. 1 Dose-response effect of busulphan on rabbits. rabbits were treated with busulphan at a total dose of 30 mg kg^{-1} (○), 40 mg kg^{-1} (△), or 50 mg kg^{-1} (□). Ear vein blood was collected at defined intervals and platelet counts were measured. Arrows indicate the injection day of busulphan (N = 3).

Fig. 2 Effects of platelet transfusion on ear bleeding time (○). The transfused amount of platelets were 0.4, 1.2, 2.0, and 4.0×10^9 kg^{-1}. ●; platelet count in the rabbits at 30 min after platelet transfusion (N = 6).

Fig. 3 Hemostatic effects of the H12-PEG-polyAlb on ear bleeding time (●). The administered amount of H12-PEG-polyAlb was 40, 80, and 120 mg kg^{-1} equivalent of rHSA. ○; platelet count in the rabbits (N = 5~6). *$P<0.05$ vs. saline group, and $^{\dagger}P<0.05$ vs. PEG-polyAlb group at the same dose.

Table 1 Hematological parameters on Day 15 after busulphan injection.

	HCT (%)	WBC (x10^3 /μL)	PLT (x10^4 /μL)
normal	33.5 ± 4.2	3.1 ± 1.2	37.3 ± 7.6
busulphan 30 mg/kg	31.3 ± 2.1	3.0 ± 1.8	2.4 ± 1.3
40 mg/kg	27.7 ± 4.0	2.7 ± 1.0	0.4 ± 0.1
50 mg/kg	26.6 ± 3.1	1.7 ± 1.5	1.0 ± 0.3

Table 2 Blood coagulation parameters after administration of H12-PEG-polyAlb at a dose of 80 mg/kg

	PT (s)	APTT (s)	Fbg (g/dL)
saline (normal)	8.1 ± 0.2	26.1 ± 1.2	256.6 ± 15.3
saline (thrombocytopenia)	8.3 ± 0.6	23.6 ± 5.3	259.7 ± 15.2
PEG-polyAlb	8.5 ± 0.5	26.1 ± 2.0	264.2 ± 26.6
H12-PEG-polyAlb	8.4 ± 0.4	24.7 ± 3.8	267.8 ± 10.3

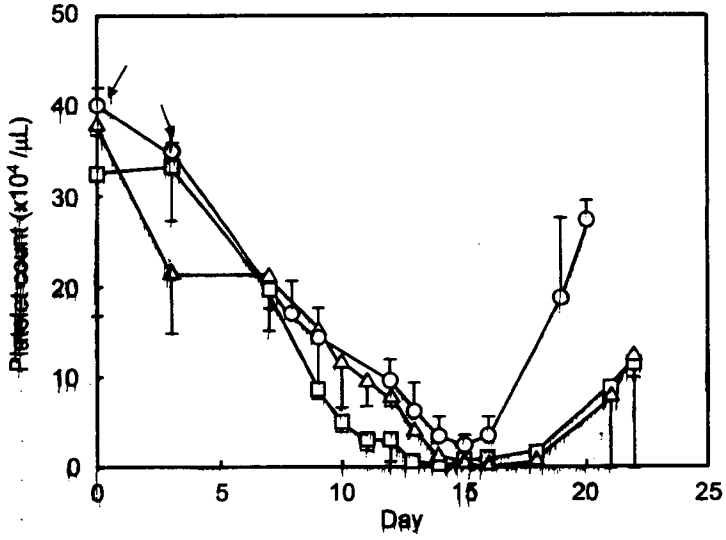

Fig. 1 Okamura et al.

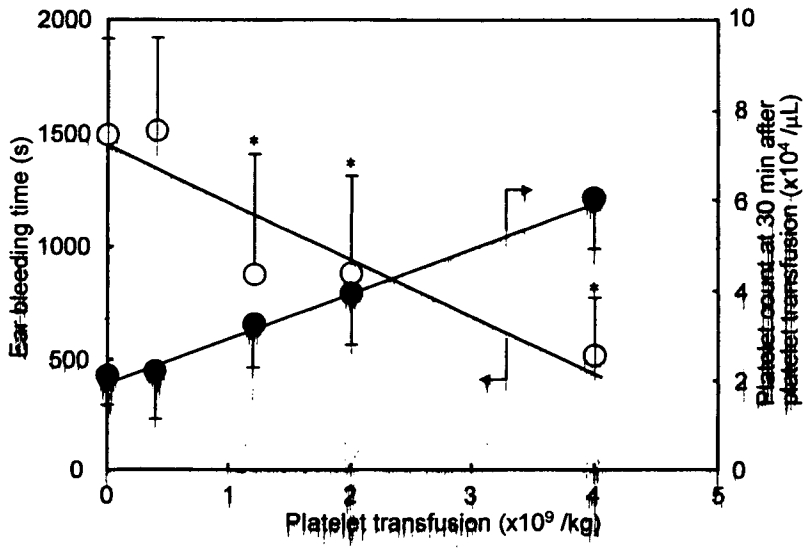

Fig. 2 Okamura et al.

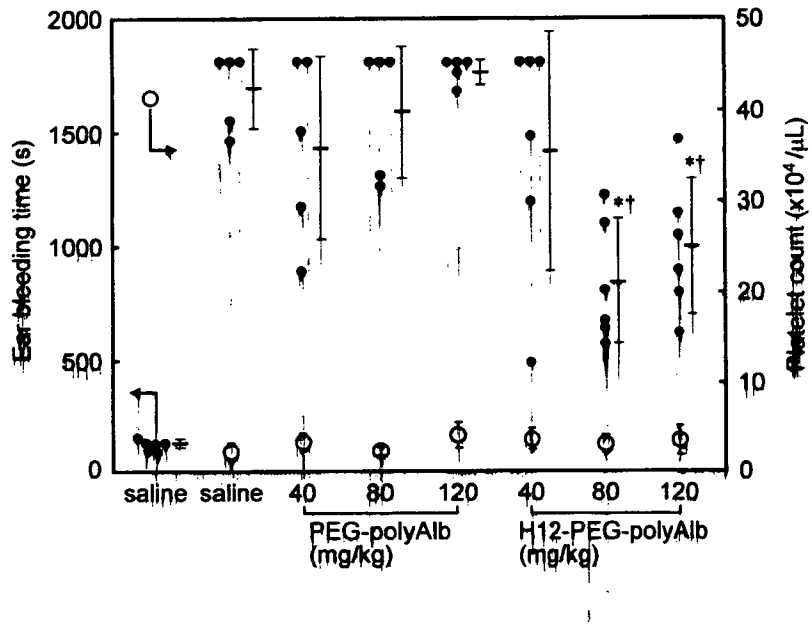

Fig. 3 Okamura et al.