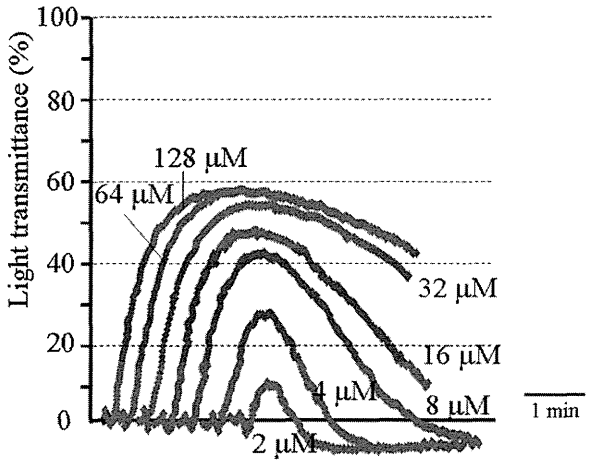

Fig. 3. ADP-induced platelet aggregation. Aggregation measurements using rat PRP (platelet = 2.0×10^5 cells/μL), obtained from F344 male rats, in the presence of several concentrations of ADP solution (final concentration, 2–128 μM). The light transmittance was measured with an aggregometer. The light transmittance of PPP was assumed to be 100%; and that of PRP before the addition of ADP, 0%.

them and thus inhibit platelet aggregation. Fig. 3 shows the concentration dependence of ADP to induce the aggregation of rat PRP, as assessed by using an aggregometer. The transient aggregation of rat PRP increased in an ADP concentration-dependent manner and reached its plateau at 64 μM ADP. Then we examined the inhibitory effect of human-H12 or rat-H12 on platelet aggregation. In this experiment, the concentration of ADP was set at 20 μM in consideration of the sensitivity of aggregation. Both human-H12 and rat-H12 inhibited the aggregation of rat PRP in a dose-dependent manner, and no significant difference in the inhibitory effect was observed between them (Fig. 4): the IC_{50} values of human-H12 and rat-H12 were 0.350 ± 0.148 μM and 0.377 ± 0.155 μM, respectively.

4. Discussion

H12 is a promising peptide for hemostasis or for a probe to deliver hemostatic agents to activated platelets. We previously developed ADP-encapsulated liposomes modified with human-H12 (H12-(ADP)Lipo; Okamura et al., 2005, 2009, 2010a,b). H12-(ADP)Lipo not only accelerates the aggregation of platelets by bridging them, but also further activates the platelets. In fact, H12-modified liposomal ADP, H12-(ADP)Lipo, actually enhances platelet aggregation in human PRP and significantly reduces the bleeding time in thrombocytopenic rat and rabbit models (Okamura et al., 2005, 2009, 2010a,b). Moreover, the binding ability of these H12-modified liposomes toward platelets steeply increases depending on H12 density on the liposomal surface. However, the experiments using human-H12, especially *in vivo* experiments, were done in rats or rabbits, the H12 peptides of which are different from the sequence of human-H12: HHMGGSKQVGDM (rat-H12) and FHMGGAKQAGDV (rabbit-F12). Four and 2 amino acid residues are different from the human-H12 sequence, respectively (Gene Bank, XM_002716891.1; Peter and Verhallen, 1991). In light of these species differences, in this present study we examined whether such differences would affect the ability of these peptides (human-H12 and rat-H12) to bind to rat platelets.

Collagen and ADP are generally used for the activation of platelets, and are known to induce strongly the aggregation of platelets in PRP. However, it is unknown how much of the population of GPIIb/IIIa is in its active form to allow binding to the H12 peptide. In this study, we compared the affinity of human-H12 and rat-H12 for activated GPIIb/IIIa on rat platelets by use of flow cytometry; and, therefore, extremely strong activating agents

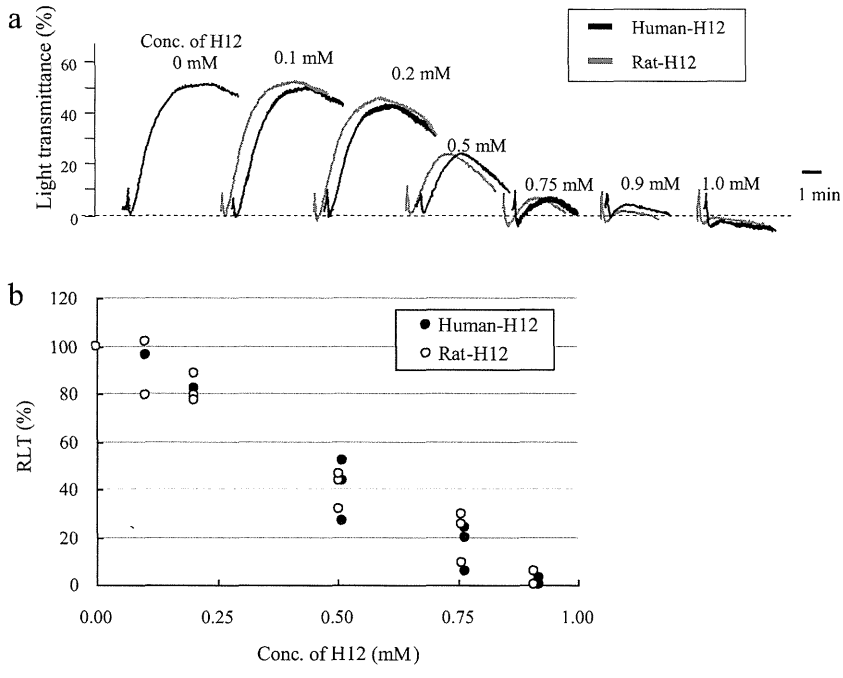

Fig. 4. Inhibitory effect of H12 peptide on the aggregation of rat PRP. (a) Aggregation of rat PRP (2×10^5 platelets/μL) obtained from F344 male rats in the presence of several concentrations of human-H12 (black line) or rat-H12 (gray line) was determined. ADP solution (20 μM as a final concentration) was added to the PRP containing each H12 (0.1–1.0 mM), and light transmittance was measured by using an aggregometer. The light transmittance of PPP was assumed to be 100%, and that of PRP before the addition of H12 and ADP was assumed to be 0%. (b) Effect of H12 on light transmittance change in rat PRP. Relative light transmission (RLT) percentages were obtained from the peak of the transmittance curve: The light transmittance without H12 was assumed to be 100%. The 50% inhibitory concentration of each H12 toward aggregation of rat PRP (IC_{50}) was determined by using the analytical software Graph Pad Prism 5J.

were firstly examined. As a result, PMA was revealed to be the most potent activator of GPIIb/IIIa on rat platelets among the agents tested.

Interestingly, U46619 and ADP did not show any significant activation of GPIIb/IIIa when the binding of FITC-Fbg was monitored. This result suggests that ADP activated only a small population of GPIIb/IIIa molecules, although this population was sufficient for inducing platelet aggregation. In fact, micromolar concentrations of ADP sufficed for platelet activation (Fig. 3).

Next, we used PMA as an activator of GPIIb/IIIa on rat platelets, and determined by flow cytometry the dissociation constant of human-H12 and rat-H12 for dissociation of the H12's from GPIIb/IIIa on rat platelets. The K_d values of human-H12 and rat-H12 were almost equal (Fig. 2). Furthermore, we evaluated the inhibitory effect of each H12 on the aggregation of rat PRP by using an aggregometer. As a result, human-H12 and rat-H12 showed almost equal inhibitory activity (Fig. 4). These results suggest that there was not a big difference in the ability of human-H12 and rat-H12 to bind to activated rat platelets.

Considering the peptide sequence of human-H12 and rat-H12, neither peptide has a proline residue which might have distorted the peptide structure in these sequences. On the other hand, each peptide has glycine residues, which break the secondary structure of the peptide, in the same positions. Therefore, it may be assumed that the molecular mobility of both peptides is almost the same. Moreover, the difference in the electric charge between the 2 peptides is only a little, because both peptides have the charged amino acid residues at the same positions. The hydrophobicity of these peptides was calculated based on the partition coefficient between water and octanol and the distribution energy to the lipoidal barrier (Wimley et al., 1996; Wimley and White, 1996). As a result, the ΔG (kcal/mol)/residue of membrane/octanol of human-H12 and rat-H12 were calculated to be 0.38/1.12 and 0.37/1.07, respectively. This result suggests that human-H12 and rat-H12 have similar characteristics, such as molecular mobility, electric charge, and hydrophilicity, even though 4 out of the 12 amino acid residues are different. These results may explain the similar ability of human-H12 and rat-H12 to bind to GPIIb/IIIa on rat platelets.

On the other hand, rabbit-H12 has 83% similarity to human-H12 at the amino acid sequence level. The calculated ΔG (kcal/mol)/residue of membrane/octanol of rabbit-H12, FHMG-GAKQAGDV, was 0.25/0.95. Therefore, the hydrophilicity of rabbit-H12 may be considered to be almost the same as that of human-H12, as in the case of rat-H12. It is known that human-H12 binds to GPIIb at the positions of GPIIb from 294 to 314, TLGAVEILDSYYQRLHRLRGE (Poncz et al., 1987), on platelets. The sequences of GPIIb of rat and rabbit at the position corresponding to the human-H12 binding site are TLGAVEILDSYYQTLHRLHGE (Gene Bank, XM_0010815132) and TMGAVEILDSYFYRLHRLQGE (Gene Bank, AAD51954.1), respectively. We calculated the ΔG (kcal/mol)/residue of membrane/octanol of human-GPIIb, rat-GPIIb, and rabbit-GPIIb to be 0.25/0.71, 0.23/0.66, and 0.18/0.57, respectively. Therefore, in terms of hydrophobicity, the GPIIb of these 3 species is also very similar.

In conclusion, our data suggest that there was not much species difference, at least among the reactivity of human-H12 and rat-H12 to rat platelets. Also, the results of the rat experiments allow us to predict the practical use of H12 as a platelet-aggregation enhancer in humans, such as H12-(ADP)Lipo.

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