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Production of Soluble Integrin $\alpha2\beta1$ Heterodimer Complex Functionally Active *in Vitro* and *in Vivo*

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Integrin $\alpha2\beta1$, which is a membrane protein consisting of noncovalently bound $\alpha2$ and $\beta1$ chains, mediates cell binding to collagen and plays a role in platelet functions. DNAs encoding the chimeric proteins in which the extracellular domains of each $\alpha2$ and $\beta1$ chain was fused to hinge and Fc regions of human $IgG_1\gamma$ chain were cotransfected into CHO cells. Soluble integrin $\alpha2\beta1$ (s$\alpha2\beta1$) in which $\alpha2$ and $\beta1$ chains were covalently bound by disulfide bonds was recovered from the culture supernatant. s$\alpha2\beta1$ maintained functional characteristics of cell surface $\alpha2\beta1$ as indicated by cation-dependent binding to collagen and conformational changes induced by cations or ligand. Intravenously administered s$\alpha2\beta1$ in rats colocalized with collagen in inflamed microvessels. Moreover, s$\alpha2\beta1$-conjugated liposome administered intravenously reduced bleeding time of the thrombocytopenic mice. These results indicated that s$\alpha2\beta1$ has pharmaceutical utilities as an agent for detecting injured vessels and a component of platelet substitute. © 2002 Elsevier Science

Key Words: **integrin $\alpha2\beta1$; soluble heterodimer complex; ligand binding capacity; *in vivo* function.**

Integrins are adhesion receptors, which participate in cell–cell and cell–extracellular matrix interactions, and are known to play important roles in immune reaction, inflammation, development, morphogenesis, wound healing, thrombosis, and metastasis of cancer (1, 2). The receptors of the integrin superfamily have the heterodimer complex structure in which two different membrane proteins, α and β chains, associate each other (1–3). The heterodimer complex structure is important in binding to ligands, and the combination of the subunits defines the ligand specificities (2, 3).

Integrin $\alpha2\beta1$ is a member of the integrin family and a major collagen receptor in platelets (4). Platelet deficient in integrin $\alpha2\beta1$ failed to adhere to subendothelium of collagen surface (5). Although the biological importance of integrin $\alpha2\beta1$-mediated collagen binding in platelet function is well described (6, 7), the biochemical and structural characterizations of integrin $\alpha2\beta1$ and the effort of using it for research and pharmaceutical purposes are limited because the isolated stable integrin $\alpha2\beta1$ was not available to date.

In general, it is very difficult to isolate fully active membrane bound integrins because the α and β chains easily dissociate during the isolation process and results in loss of functions. It is also difficult for isolated integrins to maintain active state in a various experimental conditions with the same reason. To overcome these problems, the isolated integrins were immobilized on supports like liposomes or plates and shown to be functional *in vitro* (8,9). The other method employed was to keep the α and β chains of integrins $\alpha M\beta2$ and $\alpha8\beta1$ associated utilizing Jun and Fos jip regions (10, 11). However, even in these cases, *in vivo* functions have not been studied.

In the present study, soluble integrin $\alpha2\beta1$ (s$\alpha2\beta1$) in which the extracellular domains of α and β chains were linked by covalent bond utilizing human IgG_1 γ chain was prepared. The s$\alpha2\beta1$ was shown to be structurally stable against the deprivation of metal ions and SDS treatment, and retaining the functional characteristics of the cell surface integrin $\alpha2\beta1$. Moreover, the utility of s$\alpha2\beta1$ for detecting and targeting injured-microvessels and its potential to be a component of platelet substitute were shown.

MATERIALS AND METHODS

Antibodies and peptides. Monoclonal antibodies (mAbs) to integrin $\alpha2$ (P1E6), integrin $\beta1$ (4B4) and integrin $\beta1$-cation and ligand-induced binding sites (CLIBS) (9EG7 and HUTS-4) (12, 13) were purchased from Becton–Dickinson (CA), Coulter (FL), Pharmingen (CA), and Chemicom (CA), respectively. MAb to integrin $\alpha2$ (7E10B) was prepared in our laboratory (14). The hybridoma producing activation mAb to integrin $\beta1$ (TS2/16) (15) was from American Type Culture Collection (ATCC, VA). $F(ab')_2$ fragment of TS2/16 or 7E10B was prepared by pepsin digestion as described (14). Rabbit antibody to rat collagen was from Novotec (France). Peptides; GFOGER-GPP:GPC$(GPP)_5$GFOGER$(GPP)_5$GPC, GFOGER-GAP: GAC$(GAP)_5$-

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GFOGER(GAP)$_5$GAC (16), and DNA oligomers were synthesized in Kurabo (Japan).

Preparation of CHO clone expressing sα2β1. Human IgG$_1$ γ gene was isolated from the human genomic library (Clontech, CA) based on the sequence (17). The DNA encoding hinge and CH2–CH3 regions was amplified by PCR using primers; 5′-GCGGATCCCG-AGCTGCTGGAAGCAGGCTCAG-3′ (*Bam*HI site underlined), 5′-CCTCTAGACGGCCGTCGCACTCATTTA-3′ (*Xba*I site underlined). The *Bam*HI–*Xba*I fragment of the PCR product was subcloned into the *Bam*HI–*Xba*I site of pBlueScript II SK(+) (Stratagene, CA), then the *Xho*I–*Not*I fragment of the resultant plasmid was introduced into the *Xho*I–*Not*I (modified from *Bam*HI site) sites of pcDLSRα (18). The resultant plasmid was termed IgGSRα. DNA fragment encoding the extracellular domain of integrin β1 (19) was amplified by PCR using template cDNA obtained from human fibroblast cell line MRC-5 (ATCC CCL 171) and primers; 5′-GCGGAAAAGATGAAT-TTACAAC-3′ and 5′-GTGGGATCCTCTGGACCAGTGGGACAC-3′ (*Bam*HI site underlined), and subcloned into the *Sma*I–*Bam*HI sites of pBluescript II KS(+). The *Eco*RI–*Bam*HI fragment of the resultant plasmid was inserted between the *Eco*RI and *Bam*HI sites of IgGSRα to make a plasmid expressing integrin β1 and IgG$_1$ γ chain chimera. DNA fragment encoding the extracellular domain of integrin α2 (20) was amplified as 2 separate fragments by PCR using template cDNA prepared from poly(A)$^+$ RNA of MRC-5 and primers for α2-1 fragment; 5′-GCTCGAGCAAACCCAGCGCAACTACGG-3′ (*Xho*I site underlined), 5′-ATAGTGCCCTGATGACCATTG-3′and for α2-2 fragment; 5′-GATGGCTTTAATGATGTGATTG-3′, 5′-TGTTGG-TACTTCGGCTTTCTC-3′. IgG$_1$SRα was digested with *Bam*HI and the ends were filled with Klenow fragment of DNA polymerase I, then was digested with *Xho*I. The PCR products of integrin α2 were digested with *Xho*I and *Eco*RI for α2-1 and *Eco*RI for α2-2, then both the fragments were inserted into the pretreated IgG$_1$SRα to make a plasmid expressing integrin α2 and IgG$_1$ γ chain chimera.

One microgram each of the expression plasmids was mixed with 0.1 μg each of pSV2dhfr (GIBCO, Grand Island, NY) and pSV2neo (Gibco), and the mixture was introduced into dihydrofolic acid reductase deficient CHO cells (ATCC CRL 9096) using Lipofectin reagent (GIBCO). Then the cells were cultured in the nucleic acid-free α MEM medium containing 10% FBS and 1 mg/ml neomycin (GIBCO), and resistant cells were cloned by the limiting dilution method. The amount of sα2β1 in the culture supernatant of each clone was measured by sandwich-ELISA using anti-integrin α2 mAb (7E10B)-immobilized immunoplate (Nunc, Denmark) and horseradish peroxidase (HRP)-labeled anti-integrin β1 mAb. The clone, which produced the largest amount of sα2β1, was selected. The clone produced approximately 9.8 mg/ml of sα2β1 in the culture supernatant as estimated from the material balance of sα2β1 purification procedure described below.

Purification of sα2β1 and immunoprecipitation. The sα2β1 producing CHO clone was cultured using EX-CELL 301 media (JRH Bioscience, Lenexa, KS) without serum. The culture supernatant was collected and concentrated by ultrafiltration. sα2β1 was purified by collagen Sepharose affinity column chromatography by the method described already (21). The elutes by 20 mM Tris–HCl (pH 7.5) containing 10 mM EDTA and 150 mM NaCl were further purified by gel filtration chromatography (TSKGel3000SW, TOSO, Japan). Cell membrane associated wild type integrin α2β1 (wtα2β1) was isolated from the lysate of K562 by the same method described above. The purity of the obtained sα2β1 and wtα2β1 was greater than 95% and 90%, respectively by SDS–PAGE and Coomassie blue staining. Protein assay was done using BCA protein assay kit (Pierce, Rockford, IL) using bovine serum albumin (BSA) as a standard. sα2β1 was detected by the sandwich-ELISA method as described above.

Labeling of the purified sα2β1 with ^{125}I using IODO-BEADS (Pierce), and the conjugation of the mAbs with Affigel-10 beads (Bio-Rad, Hercules, CA) followed the manufacturer's instruction. Immunoprecipitation was done basically as described (22). After incubating ^{125}I-labeled sα2β1 with mAb-conjugated beads at 4°C for 16 h, the beads were washed with 200 mM Tris–HCl (pH 8.0) containing 0.5 M NaCl, 0.1% NP-40 and 1 mM $MgCl_2$ or 10 mM EDTA. The bound proteins were separated by SDS–PAGE and detected by autoradiography as described (23). Sequential immunoprecipitation of ^{125}I labeled sα2β1 with anti-integrin mAb followed the method described already (23). After the first immunoprecipitation, the reacted beads were washed in 25 mM Tris–HCl (pH 7.4) containing 150 mM NaCl (TBS buffer) and the proteins were recovered by boiling at 100°C for 5 min with 2% SDS. The recovered proteins were diluted in TBS buffer, and subjected for the second immunoprecipitation.

Binding of sα2β1 to ligand. Wells of a 96-well immunoplate were coated with collagen (pig, rat or mouse Type I, 0.1 μg/ml) and were treated with PBS containing 1% heat denatured BSA for 3 h at 4°C. sα2β1 and wtα2β1 were biotinylated according to the manufacturer's instruction by mixing the protein and sulfo-*N*-hydroxysuccinimidobiotin (Pierce) at 1:1 ratio (w/w). Biotinylated sα2β1 or wtα2β1 was added to each well of the collagen coated plate in TBS buffer containing 0.1% BSA and either cation (Mg^{2+}, Mn^{2+}, Ca^{2+}), anti-integrin mAbs or EDTA, and the plate was incubated for 3 h at 30°C. Unbound sα2β1 or wtα2β1 was removed from the plate by washing with the same TBS buffer used for the binding reaction. Then, extraavidin peroxidase (Sigma) in TBS buffer containing 1 mM $MnCl_2$ was added to each well. After washing, bound sα2β1 or wtα2β1 was detected by *ortho*-phenylenediamine and the absorbance at 490 nm. Apparent K_d value was calculated using the method reported (24).

Binding assay of sα2β1 to anti-CLIBS mAbs. Biotinylated sα2β1 was incubated with cation, or cation and ligand peptide in TBS buffer for 10 min at room temperature. The incubated mixture was added to the immunoplate immobilized with anti-integrin β1-CLIBS mAb (0.5 μg/ml for 9EG7, or 1 μg/ml for HUTS-4) and incubated for 30 min at room temperature. Bound biotinylated sα2β1 was detected as above.

In vivo study. Six hours after the administration of 1 mg/kg of LPS (*Salmonella typhosa,* Sigma) to Wistar male rats (350 g, CRJ, Japan), 1 mg/kg of sα2β1 was intravenously administered. Twenty-four hours later, the rats were anesthetized and sacrificed by exsanguination and the femoral vein was cannulated with a polyethylene catheter (Atom, Tokyo, Japan) for infusion. The mesentery was perfused with PBS containing of 2 mM $MgCl_2$, 1% (wt/vol) BSA (PBS-BSA) for 10 min, subsequently with 0.5 mg/kg of FITC (Alexa 488 green, Molecular Probes, Eugene, OR)-conjugated rabbit anti-rat collagen antibody and rhodamine (Alexa 568 red)-conjugated anti-human integrin α2 mAb (7E10B) according to the manufacturer's instruction for 15 min. The mesentery was reperfused with PBS–BSA for 10 min, fixed in 4% (wt/vol) paraformaldehyde. The ileocecal portion of the mesenteric microvessels was gently exposed and mounted glass coverslips. Postcapillay venules 25–40 μm in diameter were selected, were viewed with a Zeiss LSM 410 confocal microscope to acquire three-dimensional images. The width of the confocal plane along the z-axis was set to 0.5 μm.

Determination of bleeding time. Mice (CRL Japan, balb/c, 7-week-old, female) were exposed to total-body irradiation at dose of 400 rad using an X-ray apertures (MBR-1520; Hitachi, Japan). Seven days after the irradiation, the platelet in the peripheral blood of the mice was counted, and the mice whose platelet count was decreased (thrombocytopenic mice) were selected to be used. Following the method described in previous report (25), sα2β1-conjugated liposome or human IgG-conjugated liposome (negative control) was prepared by carrying sα2β1 or human IgG, respectively, to the surface of liposome, and were intravenously administered to the mice 10 min before the bleeding time measurement. The tail in horizontal position was warmed to 37°C, and amputated 5 mm from the tip. Blood was blotted onto the filter paper every 5 s. sα2β1-conjugated liposome binding to mouse collagen (0.1 μg/ml) *in vitro* was evaluated as the percentage of collagen surface coverage by the method described already (7).

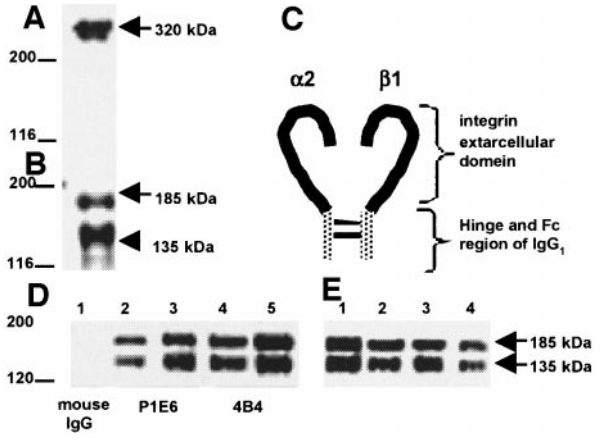

FIG. 1. SDS–PAGE, schematic structure of s$\alpha2\beta1$, and stable association of $\alpha2$ and $\beta1$ chains in the s$\alpha2\beta1$ complex. s$\alpha2\beta1$ was purified from the culture supernatant of transfected CHO cells. The purified protein was separated by 7% SDS–PAGE under nonreducing (A) or reducing (B) conditions. Molecular masses are shown on the left (in kDa). Schematic drawing of the soluble integrin $\alpha2\beta1$ heterodimer was shown in C. (D) The effect of EDTA wash on the association of the $\alpha2$ and $\beta1$ chains in the s$\alpha2\beta1$ complex. ^{125}I-labeled s$\alpha2\beta1$ was immunoprecipitated by anti-integrin $\alpha2$ (P1E6) or $\beta1$ (4B4) or control mouse IgG mAb-conjugated beads. The reacted beads were washed with the buffer containing 1 mM $MgCl_2$ (Lanes 1, 2, and 4) or 10 mM EDTA (Lanes 3 and 5). The bound proteins were separated by 7% SDS–PAGE under reducing conditions and detected by autoradiography. (E) Sequential immunoprecipitation of the s$\alpha2\beta1$ complex by anti-integrin $\alpha2$ and $\beta1$ mAbs. The combination of the 1st and 2nd mAbs are as follows: anti-$\beta1$ and anti-$\beta1$ (Lane 1), anti-$\beta1$ and anti-$\alpha2$ (Lane 2), anti-$\alpha2$ and anti-$\beta1$ (Lane 3), and anti-$\alpha2$ and anti-$\alpha2$ (Lane 4).

RESULTS AND DISCUSSION

Expression of s$\alpha2\beta1$

DNA encoding the extracellular domain of integrin $\alpha2$ chain fused to the hinge and Fc regions of human IgG_1 γ chain was placed under the control of SRα promoter. Similar construct was also prepared for the integrin $\beta1$ chain. Both of the expression plasmids were cotransfected into CHO cells and the clones producing s$\alpha2\beta1$, which was detected as immunoreactive protein to both anti-integrin $\alpha2$ and anti-integrin $\beta1$ mAbs in their culture supernatant were selected.

s$\alpha2\beta1$ was purified from the culture supernatant of the transfectant by the combination of collagen Sepharose and gel filtration column chromatographies. SDS–PAGE of the purified s$\alpha2\beta1$ gave one band of approximately 320 kDa under nonreducing condition (Fig. 1A), and 2 bands of 185 and 135 kDa under reducing condition (Fig. 1B). The sizes of bands were closed to the calculated molecular weight of 150 kDa for $\alpha2$-hinge-Fc chimera and 110 kDa for $\beta1$-hinge-Fc chimera, respectively (17, 19, 20). Generation of 2 bands under reducing condition instead of one band in nonreducing condition suggested that the heterodimer complex structure was maintained by disulfide bonds probably through the hinge region of the chimeras (schematic structure shown in: Fig. 1C).

Association of the integrin $\alpha2$-hinge-Fc and $\beta1$-hinge-Fc chimeras in the purified s$\alpha2\beta1$ was confirmed by immunoprecipitation. Immunoprecipitation of s$\alpha2\beta1$ by anti-integrin $\alpha2$ or $\beta1$ mAb showed 2 bands in SDS–PAGE under reducing condition (Fig. 1D). In addition, substitution of 1 mM $MgCl_2$ with 10 mM EDTA in the washing buffer did not affect the immunoprecipitation pattern by the mAbs (Fig. 1D). Sequential immunoprecipitations with four different combinations of mAbs (first/second: $\alpha2/\alpha2$, $\alpha2/\beta1$, $\beta1/\alpha2$, $\beta1/\beta1$) gave the same pattern in SDS–PAGE (Fig. 1E). Taken together, these results indicated that the purified s$\alpha2\beta1$ contained integrin $\alpha2$ and $\beta1$ chains in one molecule. In addition, the heterodimer structure was stable with regard to its independence to cation which contrast with the case of wild type integrin $\alpha2\beta1$ (wt$\alpha2\beta1$) (26) and its resistance to SDS treatment shown in the sequential immunoprecipitation.

Functional Characteristics of s$\alpha2\beta1$

To know whether s$\alpha2\beta1$ retained functional characteristics of the cell surface wt$\alpha2\beta1$, its binding to immobilized collagen was examined. s$\alpha2\beta1$ showed significant binding to immobilized collagen and the binding was inhibited by the presence of anti-integrin $\alpha2$ and $\beta1$ mAbs (Fig. 2A), suggesting both chains contributed its function as was reported for cell surface $\alpha2\beta1$ (2, 7, 27). The binding was also inhibited by the presence of 5 mM EDTA (Fig. 2B), indicating the requirement of cations for the function of s$\alpha2\beta1$ as for isolated or cell surface $\alpha2\beta1$ (28, 29). The results showed that s$\alpha2\beta1$ required cations for the ligand binding but not for the heterodimer formation.

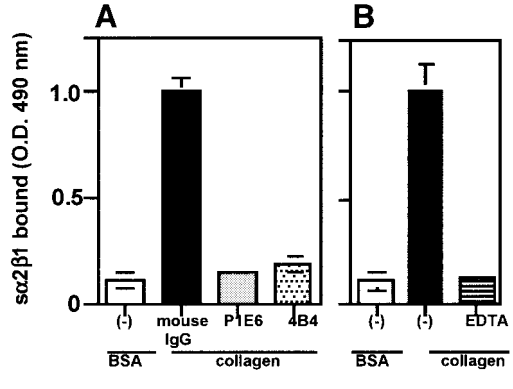

FIG. 2. Binding of s$\alpha2\beta1$ to solid-phase collagen. (A) Inhibition by anti-integrin $\alpha2$ or $\beta1$ mAb. Biotinylated s$\alpha2\beta1$ was allowed to bind to the collagen-coated microtiter plate with 1 mM $MgCl_2$ in the presence of 20 μg/ml of anti-integrin $\alpha2$ or $\beta1$ mAb or control mouse IgG. Basal binding of s$\alpha2\beta1$ was estimated using BSA-coated plate. (B) Dependence on cation. Biotinylated s$\alpha2\beta1$ was allowed to bind to the collagen-coated microtiter plate in the presence of 1 mM $MgCl_2$ or 5 mM EDTA. Data were expressed as the mean of triplicate wells. Error bars represented SE.

TABLE 1

Apparent Affinities of Soluble sα2β1 to Solid-Phase Collagen

Conditions	K_d values (nM)		
	Mn^{2+}	Mg^{2+}	Ca^{2+} Mg^{2+}
sα2β1	0.25 ± 0.02	1.61 ± 0.19	3.41 ± 0.40
wtα2β1	0.52 ± 0.06	6.39 ± 0.90	39.18 ± 4.73

Note. Binding of soluble sα2β1 to solid-phase collagen was determined in TBS buffer containing 1 mM $MnCl_2$ or 2 mM $MgCl_2$ or 1 mM $CaCl_2$ + 1 mM $MgCl_2$. Apparent dissociation constants were calculated using the following equation: $A = A_{max}([s]/(K_d + [r]))$, where A indicated the absorbance at 490 nm, A_{max} indicated the maximum absorbance at saturation and was treated as a variable parameter, [s] indicated the molar concentration of free sα2β1, and K_d indicated the apparent dissociation constant. K_d values were derived by curve-fitting using a computer-based nonlinear regression analysis program (Sigma Plot Version 6). Scatchard analyses of the binding data were linear, showing that binding was noncooperative. All binding assays and titration curves were repeated at least three times in the independent experiment.

The affinity of sα2β1 and cell surface wtα2β1 isolated from K562 for immobilized collagen was compared in the presence of various cations. K_d value of sα2β1 in the presence of Mn^{2+} was 6.4 times and 13.6 times smaller than that in the presence of Mg^{2+} and Ca^{2+}, respectively (Table 1), showing that sα2β1 had higher affinity to collagen in the presence of Mn^{2+}. This tendency is the same in the isolated wtα2β1 (Table 1) and the already reported cell surface integrin α2β1 (28, 29). The ligand binding affinity of sα2β1 was higher than that of wtα2β1 (Table 1) when it was compared under the same cation conditions. Since heterodimer structure of sα2β1 is assumed to be more stable than wtα2β1 in solution because of disulfide bonding between α2 and β1 chains, the stability is likely attributed to the higher binding affinity. It should be noted that the binding affinity of sα2β1 is comparable to that of cell surface α2β1 (30) even though sα2β1 is used in solution without solid support. Moreover, sα2β1 showed binding to rat or mouse collagen (K_d value: 0.67 ± 0.29, 6.32 ± 0.31 nM in the presence of Mg^{2+}).

Expression of CLIBS Epitope in sα2β1

It is reported that ligand or cation binding to cell surface integrin α2β1 induces conformational changes, which is detected by the specific anti-integrin β1 cation and ligand-induced binding site (CLIBS) mAbs (12, 13, 31). To know whether sα2β1 undergoes similar conformational changes, binding study to immobilized anti-CLIBS mAbs in the presence of cations or ligands were performed. Binding of sα2β1 to anti-CLIBS mAb (HUTS-4) (12) was the highest in the presence of 1 mM Mn^{2+} and anti-integrin β1 activation mAb (15), a little lower in 1 mM Mg^{2+} and about half of the maximum binding in Ca^{2+} (Fig. 3A). The binding to anti-integrin β1 mAb was not changed in conditions and was comparable to the maximum binding level to anti-CLIBS mAbs (Fig. 3A).

In addition, the binding of sα2β1 to immobilized anti-CLIBS mAb (9EG7) (13) and anti-integrin β1 mAb was comparable when 0.1 mg/ml of ligand peptide GFOGER-GPP (16) was added to the reaction mixture (Fig. 3B). However the binding was much lower with the control non-ligand peptide GFOGER-GAP, although the binding to anti-integrin β1 mAb stayed the same level (Fig. 3B).

Taken together, these results suggested that sα2β1 underwent conformational changes seen in cell surface α2β1 by cations and ligand (12, 29).

Ligand Binding of sα2β1 in Vivo

There was no report on the *in vivo* function of the isolated integrin molecules so far. To examine whether sα2β1 is functional *in vivo*, rats treated by LPS, which was reported to induce inflammation on vessels and expose the collagen layer just beneath the endothelial cells (32), was intravenously administrated sα2β1. Then, several parts of mesenteric microvessels were stained by anti-collagen antibody and anti-integrin α2 mAb. The sites stained by both antibodies colocalized along the vessels (Fig. 4A), suggesting that the administered sα2β1 bound collagen layer exposed by the inflammation.

Cell surface α2β1 on inactive platelet is in the quiescent form, thus platelets barely adhere to collagen in

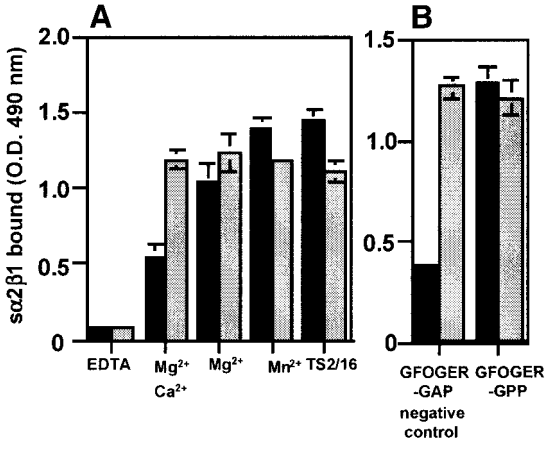

FIG. 3. Expression of the cation and ligand-induced site (CLIBS) on sα2β1. Anti-CLIBS mAb (9EG7, or HUTS-4; solid bars) or anti-integrin β1 mAb (4B4; gray bars) was immobilized on a microtiter plate and the binding of sα2β1 was compared. (A) Effect of cation. The mAb-coated wells were incubated with biotinylated sα2β1 in the presence of either 1 mM cation (Ca^{2+}, Mg^{2+}, Mn^{2+}) or 5 mM EDTA. (B) Effect of ligand binding. Biotinylated sα2β1 was allowed to bind with peptide GFOGER-GPP or GFOGER-GAP (negative control peptide) in the presence of 1 mM $CaCl_2$ and 1 mM $MgCl_2$. Data were expressed as the mean of triplicate wells. Error bars represented SE.

the presence of plasma *in vitro* (7, 29). Activation of the platelet results in the transformation of $\alpha 2\beta 1$ into active form, then leading to the platelet adhesion to collagen (7, 29). On the other hand, s$\alpha 2\beta 1$ described in this study was already active in collagen binding (Table 1), therefore it could bind to collagen *in vivo.*

Hemostatic Activity of s$\alpha 2\beta 1$-Conjugated Liposome

Binding of s$\alpha 2\beta 1$ to collagen layer in injured vessels *in vivo* hinted at its usefulness in hemostasis. Liposome carrying s$\alpha 2\beta 1$ on its surface was prepared, bound to mouse collagen specifically *in vitro* (Fig. 5A) and its hemostatic activity was examined *in vivo.* The bleeding time of the thrombocytopenic mice was remarkably prolonged compared to that of the normal mice, and was shortened when s$\alpha 2\beta 1$-conjugated liposome was administered (Fig. 5B). In addition, pretreatment of liposome by the anti-integrin $\alpha 2$ mAb (100 μg/ml) neutralized the effect of s$\alpha 2\beta 1$-conjugated liposome (Fig. 5B), showing s$\alpha 2\beta 1$ itself contributed the primary hemostatic activity.

Previously reported components of platelet substitute having hemostatic activity *in vivo* are the platelet "aggregation" related proteins, which are derived from blood component (33). However, the present study showed that s$\alpha 2\beta 1$, platelet "adhesion" related protein, has the similar potential for the first time. Ongoing *in vitro* study also shows the reconstitution of adhesion properties of human platelets in liposome carrying both s$\alpha 2\beta 1$ and recombinant GPIb fragment under flow conditions (Nishiya, T., Kainoh, M., Murata, M., Handa, M., and Ikeda, Y., submitted for publication).

In the present study, soluble integrin $\alpha 2\beta 1$ (s$\alpha 2\beta 1$) in which the extracellular domains of integrin $\alpha 2$ and $\beta 1$ chains were linked by disulfide bonds by utilizing IgG Fc region, was prepared. The produced s$\alpha 2\beta 1$ maintained the functions and characteristics of cell surface integrin $\alpha 2\beta 1$, although it was not immobilized on material surface or cell membrane but was used as soluble state. Moreover, s$\alpha 2\beta 1$ had collagen binding activity, and s$\alpha 2\beta 1$-conjugated liposome had hemostatic activity *in vivo.* Thus, s$\alpha 2\beta 1$ is the potential candidate of the component of platelet substitute.

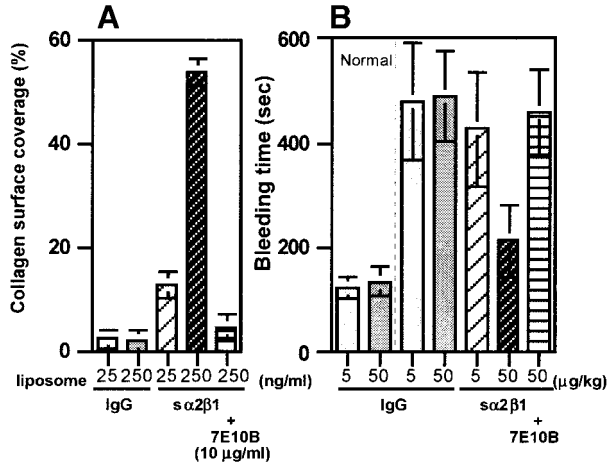

FIG. 5. Effect of the s$\alpha 2\beta 1$-conjugated liposome on bleeding time. (A) Binding of s$\alpha 2\beta 1$-conjugated liposome to collagen. Binding capacity of the s$\alpha 2\beta 1$ or IgG-conjugated liposome to mouse collagen was analyzed as described under Materials and Methods. (B) s$\alpha 2\beta 1$ or human IgG-conjugated liposome (11, 110 molecules/particle, mean particle size: 220 nm) was administrated intravenously (5, 50 μg/kg) and tail vein bleeding time was determined as described under Materials and Methods. Data represent mean ± SE (n = 9–11) and were generated on three separated trials. The mean platelet count in the X-ray irradiated mice was about 21.3×10^4 cells/μl, which was one-fifth of normal value.

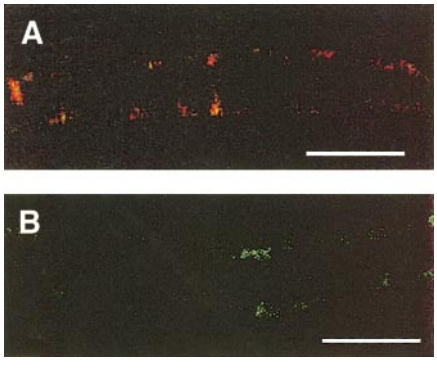

FIG. 4. Binding of intravenously injected s$\alpha 2\beta 1$ to rat mesenteric vessels. Rats were administrated LPS and 6 h later, s$\alpha 2\beta 1$ (A) or human IgG (negative control) (B) was intravenously injected. Microvessels were incubated with FITC-conjugated anti-collagen antibody and rhodamine-conjugated anti-integrin $\alpha 2$ mAb. Samples were examined by confocal microscopy. s$\alpha 2\beta 1$ binding was displayed in red, and distribution of collagen was displayed in green. Bars, 40 μm.

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