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Influence of hemoglobin vesicles, cellular-type artificial oxygen carriers, on human umbilical cord blood hematopoietic progenitor cells *in vitro*

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liposome-encapsulated hemoglobin �hemoglobin-vesicles �hematopoietic progenitor cells �colony assay �biocompatibility

ABSTRACT

Hemoglobin vesicles (HbVs), liposomal oxygen carriers containing human hemoglobin, are candidates for development as clinically useful blood substitutes. Although HbVs are shown to distribute transiently into the bone marrow in animal models, the influence of HbVs on human hematopoietic stem/progenitor cells has not yet been studied. Therefore, we investigated the influence of HbVs at a concentration of up to 3 vol/vol % on the clonogenic activity (in semisolid culture) and proliferative activity (in liquid culture) of human hematopoietic progenitor cells derived from umbilical cord blood (CB) *in vitro*. Continuous exposure

of CB mononuclear cells to HbVs tended to decrease the number and size of mature-committed colonies and most notably reduced the number of colonies of high-proliferative potential colony-forming cells (HPP-CFC). In contrast, exposure to HbVs for 20 h or 3 days, which is more relevant to the clinical setting, had no effect on the number of mature-committed colonies and only modestly decreased the number of HPP-CFC. Continuous exposure (10 days) to HbVs significantly suppressed the cellular proliferation and differentiation of both the erythroid and myeloid lineages in liquid culture. Again, short exposure (20 h or 3 days) did not affect these parameters. Thus, our results show that HbVs, under conditions relevant to the clinical setting, have no adverse effect on human CB hematopoietic progenitor activity *in vitro*. © 2008 Wiley Periodicals, Inc. J Biomed Mater Res 2008

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ARTICLE TEXT

INTRODUCTION

Hemoglobin vesicles (HbVs) or liposome-encapsulated Hbs comprise human hemoglobin encapsulated within a phospholipid bilayer membrane and have been developed as an artificial oxygen carrier.[1-3] Several studies have demonstrated that the HbVs transport oxygen as efficiently as red blood cells,[4-7] making them a promising candidate for clinical trials.

HbVs are injected intravenously, therefore, the biocompatibility of HbVs with blood components is of primary importance to ensure the safety of these materials for clinical use. We have evaluated this biocompatibility by investigating the influence of HbVs on human blood cells as well as plasma *in vitro* and shown that HbVs are highly biocompatible with human blood.[8-10]

It has been clearly demonstrated that intravenously injected liposome products for drug delivery are eventually captured by the reticuloendothelial system (RES), such as Kupffer cells in the liver and macrophages in the spleen and bone marrow.[11] A study in which technetium-99m-labeled HbVs were infused into animals demonstrated that the HbVs were mainly distributed in the liver, spleen and bone marrow,[12] and another histopathological study clarified that the HbVs are promptly metabolized in the RES.[13] Because the clinical utilization of an artificial oxygen carrier as a transfusion alternative would result in the substitution of a large volume of blood, it is important to elucidate the influence of HbVs on subsequent hematopoiesis. There has been concern over whether the HbVs distributed into bone marrow might adversely affect hematopoiesis, because the bone marrow is the major site of hematopoiesis. From this point of view, rats that received an acute 40% exchange-transfusion with HbVs showed complete recovery of the hematocrit within 7 days due to the elevated erythropoietic activity.[14] Furthermore, the number of red blood cells, leukocytes, and platelets remained unchanged for 1 week after the infusion of HbVs at 20% of the whole blood volume.[15] The findings in these animal models strongly suggest the absence of inhibitory activity of HbVs against hematopoiesis. However, the influence of HbVs on the human hematopoietic stem/progenitor cells has not yet been studied.

In vitro models of hematopoiesis, such as colony-forming assays, have been widely used to investigate the proliferation and differentiation of both of pluripotent hematopoietic stem cells and different progenitor cells of blood cell lineages [e.g., burst-forming units of erythrocyte (BFU-E) and colony-forming units of granulocytes/macrophages (CFU-GM)]. These techniques appear to be useful for investigating the pathogenic mechanisms of drug-induced blood disorders and also

for screening the safety of compounds in preclinical testing.[16]

In this study, therefore, we sought to evaluate the influence of HbVs on the clonogenic activity of human umbilical cord blood (CB) hematopoetic cells, which are rich in hematopoietic stem/progenitor cells. In addition, we investigated the effect of HbVs on the proliferation and differentiation of both the erythroid and myeloid lineages of CB hematopoietic cells in liquid culture.

MATERIAL AND METHODS

HbVs

HbVs were prepared under sterile conditions, as described previously.[17][18] The Hb was purified from outdated donated blood provided by the Japanese Red Cross Society (Tokyo, Japan). The encapsulated Hb solution (38 g/dL) contained 14.7 mmol/L pyridoxal 5′-phosphate (PLP) as an allosteric effector at a molar ratio of [PLP]/[Hb] of 2.5. The lipid bilayer was composed of 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine, cholesterol, 1,5-*O*-dihexadecyl-*N*-succinyl-L-glutamate (Nippon Fine Chemical, Osaka, Japan), and 1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine-*N*-[poly(ethylene glycol) (5,000)] (NOF, Tokyo, Japan) at a molar ratio of 5:5:1:0.033. In some experiments, empty liposomes, which have the same constituents as HbVs, except for the absence of Hb, were used. The concentration of lipopolysaccharide, measured by a modified Limulus test, was less than 0.4 EU/mL.[19] The physicochemical parameters were P_{50}, 27 Torr; 262 ア 77-nm particle diameter; and MetHb content <3%. The concentration of Hb in the HbVs dispersion was adjusted to 10 g/dL. The concentration of HbVs in this study was set at about 3 vol/vol %, based on the following rationale. Intravenously injected HbVs are eventually captured by phagocytes in the RES, including the spleen, liver, and bone marrow. The half-life of HbVs in the circulation in humans has been estimated to be 66 h by the study of circulation kinetics using rats and rabbits,[12] and the percent infused dose of HbVs of bone marrow in humans was estimated to be 6.4% at 48 h after 25% top loading of HbVs, in studies of the organ distribution of HbVs in rats and rabbits.[12] Based on this estimation, the distribution of HbVs in the human bone marrow at 48 h after infusion at 25 vol/vol % (1225 mL of HbVs) of the blood volume (4.9 L, 70 mL/kg, body weight) in a 70-kg individual may be expected to be 78.4 mL (6.4 vol/vol % of the infused dose of HbVs). The volume of the bone marrow space has been estimated as 2.6-4 L in an average-sized human (70 kg).[20] From these values, the amount of HbVs in the human bone marrow can be calculated to be about 2-3 vol/vol %.

Preparation of human CB

Use of human umbilical CB for the experiments was approved by the Committee of Hokkaido CB Bank. CB was obtained during normal full-term deliveries. CB CD34+ cells were prepared as described previously.[21] In brief, after sedimentation of red blood cells by incubating the CB samples with the same volume of 6% (w/v) hydroxyethyl starch dissolved in Ringer's solution (Veen-D, Nikken Chemical, Tokyo, Japan) at room temperature for 30 min, the low-density (<1.077 g/mL) mononuclear cells were collected with Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden). For some experiments, the cells were further enriched with CD34+ cells using a MACS CD34 Progenitor Isolation Kit (Milenyi Biotech, Bergish-Gladbach, Germany) according to the manufacturer's instructions. In all experiments, the purity of the CD34+ cells was >85%.

Clonal cell culture

The methylcellulose clonal culture was performed in 35-mm suspension culture dishes (Nippon

Becton Dickinson [BD], Tokyo, Japan). The population of CD34+ cells among the mononuclear cells was determined by flow cytometry, and the CB-derived mononuclear cells were seeded at 300 CD34+ cells/dish. A complete methylcellulose medium for human clonal culture assays (Methocult GFH4434V; StemCell Technologies, Vancouver BC, Canada) was used. The presence of up to 3% HbVs did not interfere with the microscopic detection of the colonies formed.

After 14 days incubation at 37-C in a humidified atmosphere containing 5% CO_2, the BFU-E, CFU-GM, CFU-Mix, and colony-forming units in culture (CFU-C) were scored under an inverted microscope. Densely packed colonies that reached >1 mm in size were scored as high proliferative potential colony-forming cells (HPP-CFC) after 28 days incubation. In some experiments, the CB-derived mononuclear cells were suspended to obtain 1500 CD34+ cells/mL in Iscove's modified Dulbecco's medium (IMDM, Gibco BRL, Rockville, MD) containing 30% FCS (Equitech Bio, Igram, TX), 1% bovine serum albumin (BSA; Sigma Chemical, St Louis, MO), 10 ng/mL human interleukin-3 (IL-3), 10 ng/mL human stem cell factor (SCF, provided by Kirin Brewery, Tokyo, Japan), 10 ng/mL granulocyte colony-stimulating factor (G-CSF, a gift from Chugai Pharmaceutical, Tokyo, Japan), and 50 U/mL granulocyte-macrophage colony-stimulating factor (GM-CSF; Schering Research, Bloomfield, NJ). Then, different concentrations of HbVs were added to the cell suspension. The cells were incubated either for 20 h or for 3 days. Subsequently, they were recovered, washed to remove the HbVs, and resuspended in 5 mL of MethoCult GF. One milliliter of the resultant cell suspension (by adjusting CD34+ cells to 300 cells/dish) was seeded into a 35-mm dish for the clonal assay.

Liquid culture

CD34+ cells enriched from CB-derived mononuclear cells were suspended in 4 mL of the following culture media and seeded in 12.5-cm^2 flasks (Nippon BD, Tokyo, Japan). The culture medium for the erythroid lineage was IMDM-containing 30% FBS, 1% BSA, 10 ng/mL human IL-3, 10 ng/mL human SCF, and 2 U/mL human erythropoietin (provided by Chugai Pharmaceutical). The culture medium for the myeloid lineage was IMDM-containing 30% FBS, 1% BSA, 50 μM β-mercaptoethanol, 10 ng/mL human IL-3, 10 ng/mL human SCF, 10 ng/mL G-CSF, and 50 U/mL GM-CSF. These combinations of cytokines have been shown to promote proliferation and differentiation of CD34+ cells toward mature erythroid and myeloid lineage cells, respectively.[22][23] Various concentrations of HbVs were added to the medium containing the cells. After 10 days incubation at 37-C in a humidified atmosphere containing 5% CO_2, the total cell counts were determined. CD235a+ (glycophorin A) cells for the erythroid lineage and CD15+ cells for the myeloid lineage, respectively, were analyzed by flow cytometry. For determining the effects of short-term exposure, the cells were incubated with HbVs for either 20 h or 3 days, washed to remove the HbVs, and then incubated for a total of 10 days.

Flow-cytometric analysis

Aliquots of cells were stained with monoclonal antibodies in PBS/0.1% BSA at 4-C for 30 min. The analysis was performed using a BD LSR flow cytometer (BD Biosciences Immunocytometry System, San Diego, CA). The following monoclonal antibodies were used: FITC-conjugated CD34 (Nippon Becton Dickinson [BD]) antibody, PE-conjugated CD235a and CD33 (DAKO) antibodies, FITC-conjugated CD15 (DAKO) antibody, and APC-conjugated CD45 (BD) antibody. FITC- and PE-conjugated mouse IgG1 antibodies (BD), APC-conjugated mouse IgG1 (BD), and FITC-conjugated IgM (DAKO) antibodies were used as isotype-matched controls. In the flow-cytometric analysis, dead cells were gated out first by propidium iodide staining and then with a forward versus side scatter window. For each analysis set, at least 10,000 events were collected.

Histological staining

Cultured cells (1×10^3 - 1×10^4/100 μL) were centrifuged onto slides with Cytospin (Shandon, Pittsburgh, PA) and stained with May-Grunwald-Giemsa (Merck, Darmstadt, Germany). Microscopic images were captured with an MP5Mc/OL digital camera (Olympus) and processed using Win Roof software, version 5.5.

Statistical analysis

Results are expressed as mean ± standard deviation (SD). A two-way paired ANOVA followed by *post hoc* Bonferroni's test was used for comparisons of multiple HbV-treated groups with the control (HbV; 0%) group. For analysis of the difference between two exposure times, unpaired two-tailed Student's *t* test was used. Values of $p < 0.05$ were considered significant.

RESULTS AND DISCUSSION

Clonogenic potential of CB hematopoietic cells

We first examined the effect of continuous exposure to HbVs (0.09%-3%) on the formation of BFU-E, CFU-GM, CFU-Mix, CFC, and HPP-CFC in the clonogenic assay. HbVs at 3% inhibited the formation of CFU-GM and tended to decrease the formation of CFC-C. Most notably, HbVs significantly inhibited the formation of HPP-CFC in a concentration-dependent manner (Fig. 1A). Although no change in the number of colonies of BFU-E was noted, the size of the colonies of BFU-E and CFU-GM tended to be smaller in the presence than in the absence of HbVs (Fig. 2). On the other hand, the empty liposomes (phospholipid vesicles devoid of Hb) had no inhibitory effect on the formation of mature-committed colonies or HPP-CFC (Fig. 1B).

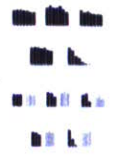

Figure 1. A: Effects of HbVs on the clonogenic activity of CB-derived hematopoietic cells. B: Effects of empty liposomes on the clonogenic activity of CB-derived hematopoietic cells. CB-derived mononuclear cells were seeded at 300 CD34$^+$ cells per dish in complete methylcellulose medium for human clonal-culture assays. Various concentrations of HbVs or empty liposomes (vol/vol %) were added to the medium containing the cells. BFU-E, CFU-GM, CFU-Mix, and CFU-C were scored after 14 days incubation. HPP-CFC was scored after 28 days incubation. Data represent the mean ± SD of three experiments performed on three separate CB donors in (A) and (B), respectively. A two-way paired ANOVA followed by Bonferroni's test was used for comparisons of multiple HbVs-treated groups with the control (HbVs; 0%) group. *$p < 0.05$, **$p < 0.01$ versus HbVs (0%).
[Normal View 84K | Magnified View 262K]

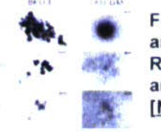

Figure 2. Effects of HbVs on the size of the colonies of BFU-E and CFU-GM formed in clonal cultures of CB-derived cells. Representative colonies of BFU-E and CFU-GM in the absence and presence of HbVs are shown. Scales represent 50 μm.
[Normal View 28K | Magnified View 103K]

As continuous exposure to HbVs had a marked inhibitory effect on the formation of HPP-CFC, we examined the effect of short-term exposure of CB hematopoietic cells to HbVs. Toward this end, the CB hematopoietic cells were exposed to HbVs for 20 h or for 3 days, washed to remove the HbVs, and then subjected to a clonogenic assay. Exposure to HbVs for 20 h had no inhibitory effect on the formation of either HPP-CFC or other mature-committed colonies (Fig. 3). Exposure to 3% HbVs for 3 days modestly inhibited the formation of HPP-CFC, however, a greater number

of HPP-CFC was formed when compared with that observed under continuous exposure to HbVs. No effect was observed on the formation of other mature-committed colonies (Fig. 3). From the clinical point of view, continuous exposure of hematopoietic stem/progenitor cells to HbVs in the marrow for 14 days or 28 days is unlikely. Rather, 1-3 days exposure is more relevant to the clinical setting as described below. In this sense, short-term exposure of hematopoietic progenitor cells to HbVs even at 3% had no adverse effect on the clonogenic activity of hematopoietic progenitor cells.

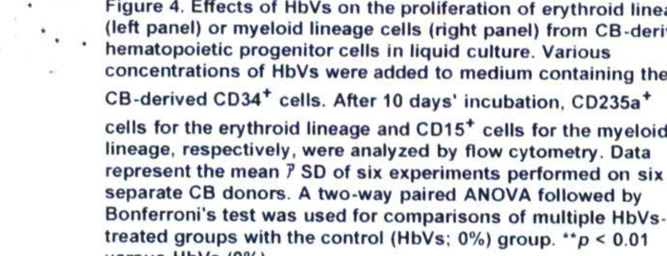

Figure 3. Effects of short-term exposure to HbVs on the clonogenic activity of CB-derived hematopoietic cells. CB-derived mononuclear cells were suspended in IMDM containing FCS, BSA, IL-3, SCF, G-CSF, and GM-CSF; then, different concentrations of HbVs were added to the cell suspension. The cells were incubated for 20 h (open column) or for 3 days (closed column). Subsequently, they were recovered, washed to remove the HbVs, and subjected to clonal assay. Data were expressed as the mean ± SD of the percentage of control. Three experiments were performed on three separate CB donors. **$p < 0.01$; 20 h versus 3-day exposure; unpaired Student's *t* test.
[Normal View 15K | Magnified View 33K]

Proliferation and differentiation of erythroid or myeloid lineage cells from CB hematopoietic progenitor cells in liquid culture

Because the numbers of HPP-CFC and CFU-GM were significantly reduced, and the size of the colonies of BFU-E and CFU-GM tended to be smaller in the presence than in the absence of HbVs, we next examined the effect of HbVs (0.75%, 1.5%, or 3%) on the proliferation of erythroid or myeloid lineage cells in a liquid culture of CB CD34$^+$ cells. As shown in Figure 4, the presence of HbVs throughout the culture period significantly inhibited the proliferation of CD235a$^+$ cells (erythroid lineage) and CD15$^+$ (myeloid lineage) cells in a dose-dependent manner. These results suggested that continuous exposure to HbVs had an inhibitory effect on the proliferation of hematopoietic progenitor cells. Thus, the reduced number of HPP-CFC and reduced colony size of BFU-E and CFU-GM in the clonogenic assay were surmised to be associated with reduced proliferation of the erythroid and myeloid lineage cells in the presence of HbVs throughout the culture period.

Figure 4. Effects of HbVs on the proliferation of erythroid lineage (left panel) or myeloid lineage cells (right panel) from CB-derived hematopoietic progenitor cells in liquid culture. Various concentrations of HbVs were added to medium containing the CB-derived CD34$^+$ cells. After 10 days' incubation, CD235a$^+$ cells for the erythroid lineage and CD15$^+$ cells for the myeloid lineage, respectively, were analyzed by flow cytometry. Data represent the mean ± SD of six experiments performed on six separate CB donors. A two-way paired ANOVA followed by Bonferroni's test was used for comparisons of multiple HbVs-treated groups with the control (HbVs; 0%) group. **$p < 0.01$ versus HbVs (0%).
[Normal View 7K | Magnified View 15K]

We further analyzed the subset of CD235a$^+$ cells and CD15$^+$ cells. The CD235a$^+$CD45$^-$ cells and CD15$^+$CD33$^-$ cells represented some of the more differentiated cells in the erythroid and myeloid lineage, respectively. Continuous exposure to HbVs significantly reduced the percentage of

CD233$^+$CD45$^-$ cells in the total cell population (Fig. 5A). Microscopic examination of a smear of cells cultured for 10 days revealed that while orthochromatic erythroblasts and erythrocytes (differentiated lineage) were present in the absence of HbVs, basophilic erythroblasts (less differentiated lineage) were more abundant in the presence of 3% HbVs (Fig. 5B).

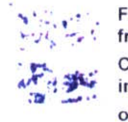

Figure 5. Effects of HbVs on the differentiation of erythroid cells from CB-derived hematopoietic progenitor cells in liquid culture. CB-derived CD34$^+$ cells were cultured in the medium for induction of erythroid lineage without or with HbVs (0.75%, 1.5%, or 3.0%). A: The percentage of CD235a$^+$ CD45$^-$ cells in the total cell population was analyzed by flow cytometry. Data represent the mean 7 SD of experiments performed on CB obtained from six separate donors. A two-way paired ANOVA followed by Bonferroni's test was used for comparisons of multiple HbVs-treated groups with the control (HbVs 0%) group. $^*p < 0.05$, $^{**}p < 0.01$ versus HbVs (0%). Representative results of flow cytometric analysis are shown at the bottom. B: Morphology of the cells generated in the liquid culture for erythroid lineage. Arrow head; basophilic erythroblasts, white arrow; orthochromatic erythroblasts, and black arrow; erythrocyte. Note that the differentiated erythroid cells are much fewer in number in the presence of HbVs when compared with that in the control (HbVs 0%). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]
[Normal View 58K | Magnified View 221K]

Similarly, continuous exposure to HbVs significantly decreased the percentage of CD15$^+$CD33$^-$ cells in the total cell population (Fig. 6A). Examination of a smear of the cells showed that while metamyelocytes (differentiated lineage) could be recognized in the absence of HbVs, myelocytes (less differentiated lineage) were more abundant in the presence of 3% HbVs (Fig. 6B). These results suggest that continuous exposure to HbVs also inhibited the differentiation of both erythroid and myeloid lineage cells.

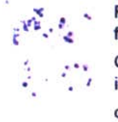

Figure 6. Effects of HbVs on the differentiation of myeloid cells from CB-derived hematopoietic progenitor cells in liquid culture. CB-derived CD34$^+$ cells were cultured in the medium for the induction of myeloid lineage without or with HbVs (0.75%, 1.5% or 3.0%). A: The percentage of CD15$^+$ CD33$^-$ cells in the total cell population was analyzed by flow cytometry. Data represent the mean 7 SD of experiments performed on six separate CB donors. A two-way paired ANOVA followed by Bonferroni's test was used for the comparisons of multiple HbVs-treated groups with the control (HbVs 0%) group. $^*p < 0.05$, $^{**}p < 0.01$ versus HbVs (0%). Representative results of flow cytometric analysis are shown at the bottom. B: Morphology of the cells generated in the liquid culture for erythroid lineage. Arrow head, macrophage; white arrow, myelocyte; and black arrow, metamyelocyte. Note that the differentiated myeloid cells are much fewer in number in the presence of HbVs when compared with that in the control (HbVs 0%). Scales represent 10 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]
[Normal View 43K | Magnified View 170K]

Next, the effects of the short exposure to HbVs, which is more relevant to the clinical setting, of

CD34$^+$ cells on the proliferative activity of both erythroid and myeloid lineage cells were examined. Exposure to HbVs even at 3% for 20 h or for 3 days did not affect the proliferative activity of either the CD235a$^+$ cells or the CD15$^+$ cells (Fig. 7). Furthermore, the percentages of CD235a$^+$CD45$^-$ cells and CD15$^+$CD33$^-$ cells in the total cell population were not affected by exposure to HbVs, either for 20 h or for 3 days (data not shown). Thus, HbVs exerted no inhibitory effects on the proliferation and differentiation of either erythroid or myeloid lineage cells following short durations of exposure.

Figure 7. Effects of HbVs on the proliferation of erythroid lineage (left panels) or myeloid lineage (right panels) cells from CB-derived hematopoietic progenitor cells in liquid culture. CB-derived CD34$^+$ cells were exposed to HbVs (0%, 0.75%, 1.5%, or 3%) for 20 h, 3 days, or 10 days. After culture for a total of 10 days, CD235a$^+$ cells for the erythroid lineage and CD15$^+$ cells for the myeloid lineage, respectively, were analyzed by flow cytometry. The number of CD235a$^+$ cells or CD15$^+$ cells at each concentration of HbVs is expressed as a percentage of the number in the control (HbVs 0%). Data represent the mean 7 SD of three experiments performed on three separate CB donors. A two-way paired ANOVA followed by Bonferroni's test was used for comparisons of multiple HbVs-treated groups with the control (HbVs 0%) group. $^*p < 0.05$, $^{**}p < 0.01$ versus HbVs (0%).
[Normal View 30K | Magnified View 78K]

Several hypotheses have been suggested to explain the inhibitory effects of continuous exposure to HbVs on hematopoietic progenitor activity including direct contact of the progenitor cells with HbVs, conversion of Hb in HbVs to met-Hb during culture, interaction of progenitor cells with several components from HbVs, which might degrade over time. The observation that the empty liposomes did not have any inhibitory effect on the clonogenic activity suggested that the progenitor activity was not inhibited by direct contact of the progenitor cells with the HbVs surface, but by the presence of Hb in the HbVs. In this case, higher dissolved oxygen concentrations in the culture medium were theoretically expected in the presence of HbVs than in the absence of HbVs, which may be involved in the inhibition of progenitor activity following to the prolonged exposure to HbVs. Furthermore, conversion of Hb to met-Hb within HbVs[24] cannot be excluded as the reason for the inhibition of progenitor activity caused by HbVs. In addition, there is a possibility that HbVs might degrade during long-term incubation, leading to the release of Hb. We determined the Hb level during the continuous presence of HbVs in liquid culture up to 10 days. At maximum, 6.7% of the Hb in the HbVs inputted at 3% (i.e., 0.02 g/dL) was released into the culture supernatant. This Hb concentration was calculated as ~3 μ*M*. According to the report by Fowler et al.,[25] 1 μ*M* of recombinant Hb did not affect the proliferation of erythroid or myeloid lineage cells from human bone marrow CD34$^+$ cells in liquid culture system. Therefore, we do not believe that the released Hb accounted for the inhibitory effect of long-term exposure to HbVs on the progenitor activity.

It is difficult to predict the events *in vivo* from the results of experiments *in vitro*, because the effects of HbVs on the immature hematopoietic stem/progenitor cells from the CB may not be the same as those on the hematopoietic stem/progenitor cells in the adult bone marrow. In addition, the concentration of HbVs used here is based on simple assumption and may not necessarily be relevant to the physiological conditions prevailing in humans. With regard to the exposure time to HbVs, continuous exposure of hematopoietic stem/progenitor cells to HbVs in the marrow for more than 10 days is unlikely in the clinical setting. Rather, 1-3 days exposure is

more relevant to the clinical setting, because a study in which an acute 40% exchange transfusion of HbVs was administered to rats showed that a significant amount of the HbVs was phagocytosed by the macrophages in the marrow by 1-3 days after the infusion. A significant decrease in the number of HbVs was observed at 7 days, with the vesicles becoming undetectable at 14 days. Under these conditions, hematopoietic activity, including the formation of erythroblastic islets was observed at 3 days in the marrow.[14] Moreover, the destination of HbVs in the bone marrow is macrophages, and the HbVs are degraded in the phagosomes. These findings imply that there is little possibility of direct contact between HbVs and the hematopoietic progenitor cells *in vivo*. The finding that short-term exposure to HbVs did not have any significant effect on the clonogenic activity or the proliferation and differentiation of erythroid and myeloid lineage cells in liquid culture is consistent with the results of animal experiments,[14][15] suggesting that the infusion of HbVs in humans may have no adverse effects on hematopoiesis.

In conclusion, our results suggest that HbVs, under conditions relevant to the clinical setting, have no adverse effect on human CB hematopoietic progenitor activity *in vitro*. The present results are of value for estimating the biocompatibility of HbVs and hematopoietic progenitor cells.

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Effects of endogenous ligands on the biological role of human serum albumin in S-nitrosylation ☆

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Abstract

Many proteins have been identified as targets for S-nitrosylation, including structural and signaling proteins, and ion channels. S-nitrosylation plays an important role in regulating their activity and function. We used human serum albumin (HSA), a major endogenous NO traffic protein, and studied the effect of mediators on S-nitrosylation processes which control NO bioactivity. By using NOC-7, S-nitrosoglutathione, and activated RAW264.7 cells as NO-donors we found that high-affinity binding of endogenous ligands (Cu^{2+}, bilirubin and fatty acid) can affect these processes. It is likely that the same effects take place in many clinical situations characterized by increased fatty acid concentrations in plasma such as type II diabetes and the metabolic syndrome. Thus, endogenous ligands, changing their plasma concentrations, could be a novel type of mediator of S-nitrosylation not only in the case of HSA but also for other target proteins.

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Keywords: Human serum albumin; Nitric oxide; Type II diabetes; Metabolic syndrome; Cysteine; S-Nitrosylation; Ligand binding; Fatty acids; Copper; Bilirubin

Post-translational modifications are essential in their functional regulation. Among these, changes of the redox state of cysteine residues are of great importance. The sulfhydryl moiety can interact with nitric oxide (NO) and thereby form S-nitrosothiols (RS-NO) [1–3]. RS-NOs may function as NO reservoirs and preserve the antioxidant and other activities of NO [4,5]. For example, it has been reported that S-nitroso human serum albumin (SNO-HSA) may serve in vivo as a circulating reservoir for NO produced by the endothelial cells [6]. The reservoir function was also reported to be operative when application of SNO-HSA to animals suffering from ischemia-reperfusion injury minimized the extent of tissue damage associated with reperfusion [7,8]. However, several pieces of evidence propose that RS-NOs are more than simply NO reservoirs [4]. Thus, the antibacterial and cytoprotective properties of SNO-HSAs [9] are most probably the results of S-transnitrosylation.

HSA is a single, non-glycosylated polypeptide that organizes to form a heart-shaped protein with approximately 67% α-helix but no β-sheet [10]. All but one (Cys-34) of the 35 cysteine residues are involved in the formation of

Abbreviations: HSA, human serum albumin; SNO-HSA, S-nitroso HSA; RS-NOs, S-nitrosothiols; GSH, glutathione; GS-NO, S-nitrosoglutathione; NOC-7, 1-hydroxy-2-oxo-3-(*N*-3-methyl-aminopropyl)-3-methyl-3′-triazene; OA, oleic acid; BR, bilirubin; DTT, 1,4-dithiothreitol; DTNB, 5,5′-dithiobis-2-nitrobenzoic acid; DTPA, diethylenetriamine-pentaacetic acid; EDTA, ethylenediaminetetraacetic acid; PBS, phosphate-buffered saline; NEM, *N*-ethylmaleimide.

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stabilizing disulfide bonds. In the circulation, normally about half of the Cys-34 residues are freely accessible, i.e., not oxidized or involved in ligand binding, and they represent the largest fraction of free thiols in blood.

HSA is the most abundant protein in blood plasma and serves, among other things, as a transport and depot protein for numerous endogenous and exogenous compounds [10]. We studied the effects of the strongly bound ligands oleate (OA), bilirubin (BR) and Cu^{2+} and the weakly bound ligands L-tryptophan, progesterone, ascorbate, Zn^{2+} and Fe^{2+} on S-nitrosylation of HSA by S-nitrosoglutathione (GS-NO), 1-hydroxy-2-oxo-3-(N-3-methyl-aminopropyl)-3-methyl-3′-triazene (NOC-7) and stimulated RAW264.7 cells.

Materials and methods

Materials. Non-defatted HSA (96% pure) was donated by the Chemo-Sera-Therapeutic Research Institute (Kumamoto, Japan), and it was defatted by treatment with charcoal as described by Chen [11]. Sephadex G-25 (φ1.6 × 2.5 cm), Blue Sepharose CL-6B (φ2.5 × 20 cm), and RESOURCE PHE columns (φ0.64 × 3 cm) were from Amersham Pharmacia Biotech (Tokyo, Japan). Enzymes for DNA assays were from Takara (Kyoto, Japan). The Pichia Expression kit was from Invitrogen (Carlsbad, CA). L-Tryptophan, ascorbic acid, $FeCl_2$, $(CH_3COO)_2Zn$, $CuSO_4{\cdot}5H_2O$, BR, progesterone, OA, 1,4-dithiothreitol (DTT), and glutathione (GSH) were purchased from Sigma–Aldrich (St. Louis, MO). Sulfanilamide, naphthylethylenediamine–hydrochloride, $HgCl_2$ and $NaNO_2$ were obtained from Nakalai Tesque (Kyoto, Japan). GS-NO, NOC-7, 5,5′-dithiobis-2-nitrobenzoic acid (DTNB), diethylenetriaminepentaacetic acid (DTPA), and ethylenediaminetetraacetic acid (EDTA) were obtained from Dojindo Laboratories (Kumamoto, Japan). Other chemicals were of the best grades commercially available, and all solutions were made in deionized and distilled water.

Synthesis and purification of recombinant HSA. Wild-type HSA and the H3A mutant were synthesized, using *P. pastoris* GS115 his4, and purified as previously described [9]. The mutagenic primers (sense and antisense) for making the mutant were:

5′-GCTCATCCGATGGCCACAAGAGTGAGG-3′, and 3′-CCTCA CTCTTGTGGCCATCGGATGAGC-5′.

The albumins were deionized and defatted via charcoal treatment, freeze-dried, and then stored at −20 °C until used. According to density analysis of Coomassie Brilliant Blue-stained protein bands on 12.5% SDS–PAGE, the purity of the protein samples were more than 97%.

Preparation of ligand-HSA solutions. First, HSA was treated with DTT as follows. HSA (300 μM) was incubated with DTT (molar ratio, protein:DTT = 1:10) for 5 min at 37 °C. After that, DTT was quickly removed by Sephadex G-25 gel filtration using 10 mM phosphate-buffered saline (pH 7.4) (PBS; Ca^{2+}, Mg^{2+} free). Stock solutions of 20 mM OA and 20 mM progesterone were made in methanol-H_2O (1:1, v/v) and ethanol-H_2O (1:1, v/v), respectively, whereas 20 mM BR was made in 0.1 N NaOH and protected against light. Later, these stock-solutions were diluted with PBS. Other ligands were directly dissolved in PBS. In all cases, the resulting solutions were mixed with PBS containing HSA. The ligand-protein solutions, except for those having $CuSO_4$, were incubated for 30 min at 37 °C in the dark. Freshly prepared $CuSO_4$-HSA solutions were also incubated for 30 min in the dark but at 4 °C, because the SH-group of HSA easily undergoes oxidation in the presence of Cu^{2+}. To remove free ligands, mixtures were applied to a Sephadex G-25 column, quickly eluted with PBS and concentrated by ultrafiltration. The protein content of all protein preparations used in this study was determined by the Bradford assay.

Accessibility of Cys-34. In reduced HSA this was estimated with Ellman's reagent, DTNB. Briefly, the accessibility was evaluated as A_5/A_{60}, where A_5 and A_{60} is the sample absorbance at 405 nm after 5 min and 60 min (maximal absorbancy), respectively, of incubation with DTNB [12].

S-Nitrosylation of HSA in cell-free reaction systems. SNO-HSA was prepared with protection against light and according to previous reports [13,14]. HSA (100 μM) with and without ligand was incubated with GS-NO or NOC-7 as NO donor (molar ratio, protein:NO donor = 1:5) in PBS for 10 min at 37 °C. To remove NO donors, S-nitrosylated products were applied to a Sephadex G-25 column, eluted with PBS containing 0.5 mM DTPA, and concentrated by ultrafiltration. These samples were stored at −80 °C until analyzed.

Determination of S-nitrosylation efficiency. The amounts of the S-nitroso moiety of SNO-HSA were quantified by HPLC coupled with a flow-reactor system, as previously reported [13,15]. The HPLC column was a gel filtration column for S-nitrosylated proteins (φ8 × 300 mm), Diol-120, YMC, Kyoto, Japan. Briefly, the eluate from the HPLC column was mixed with a $HgCl_2$ solution to decompose S-nitrosylated compounds to yield NO_2^- (via NO^+). The NO_2^- generated was then detected after reaction with Griess reagent in the flow-reactor system.

SNO-HSA production by cells in culture. RAW264.7 cells were cultured in 24-well plates (16-mm diameter; Falcon, Lincoln Park, NJ) with Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and nonessential amino acids (Life Technologies, Inc.). Cells at saturation density (1×10^6 cells/well) were stimulated with interferon-γ (Genzyme, Cambridge, MA) at 100 U/ml and lipopolysaccharide (*Escherichia coli* 026B; Difco) at 10 μg/ml for 12 h at 37 °C in a CO_2 incubator (5% CO_2, 95% air (v/v)). The culture medium was removed, and the cells were washed three times with PBS (pH 7.4). Cells were further incubated in the CO_2 incubator at 37 °C with 200 μl of PBS containing 0.5 mM L-arginine and 100 μM HSA alone or with bound ligand. After incubation for 10 min, the reaction medium was mixed with 1/10 volume of 5 mM DTPA dissolved in PBS (pH 7.4), followed by centrifugation at 10,000*g* for 10 min at 4 °C. The resultant supernatants were stored at −80 °C until applied to the HPLC-flow reactor system.

Statistical analysis. The statistical significance of collected data was evaluated using the ANOVA analysis followed by Newman–Keuls method for more than 2 means. Differences between groups were evaluated by the Student's *t* test. $P < 0.05$ was regarded as statistically significant.

Results and discussion

S-Nitrosylation of mercaptalbumin with bound ligands

HSA purified from serum has bound endogenous ligands, in particular fatty acids, and perhaps also exogenous ligands. Any effect of these ligands on the S-nitrosylation of HSA was examined by incubating non-defatted and charcoal-treated albumin with GS-NO. The S-nitroso moiety of the former preparation was 0.41 ± 0.02 ($n = 4$), whereas that of the latter was only 0.19 ± 0.01 ($P < 0.01$). Thus, the presence of ligands greatly enhanced the efficiency of S-nitrosylation. In order to identify ligands of importance for S-nitrosylation, individual ligands were added to HSA, which had been delipidated by charcoal and dialyzed extensively against deionized water. In these experiments, two kinds of S-nitrosylating agents were used, namely GS-NO which S-transnitrosylates via NO^+, and NOC-7 which S-nitrosylates mainly via NO and N_2O_3. The results obtained with equimolar amounts of protein and ligand are given in Figs. 1A and 2A. It can be seen that OA and BR enhances the efficiency of GS-NO, but not that of NOC-7, whereas Cu^{2+} increases the S-nitrosylation by NOC-7 but not that caused by GS-NO. In contrast, no significant effect was observed when adding L-tryptophan,

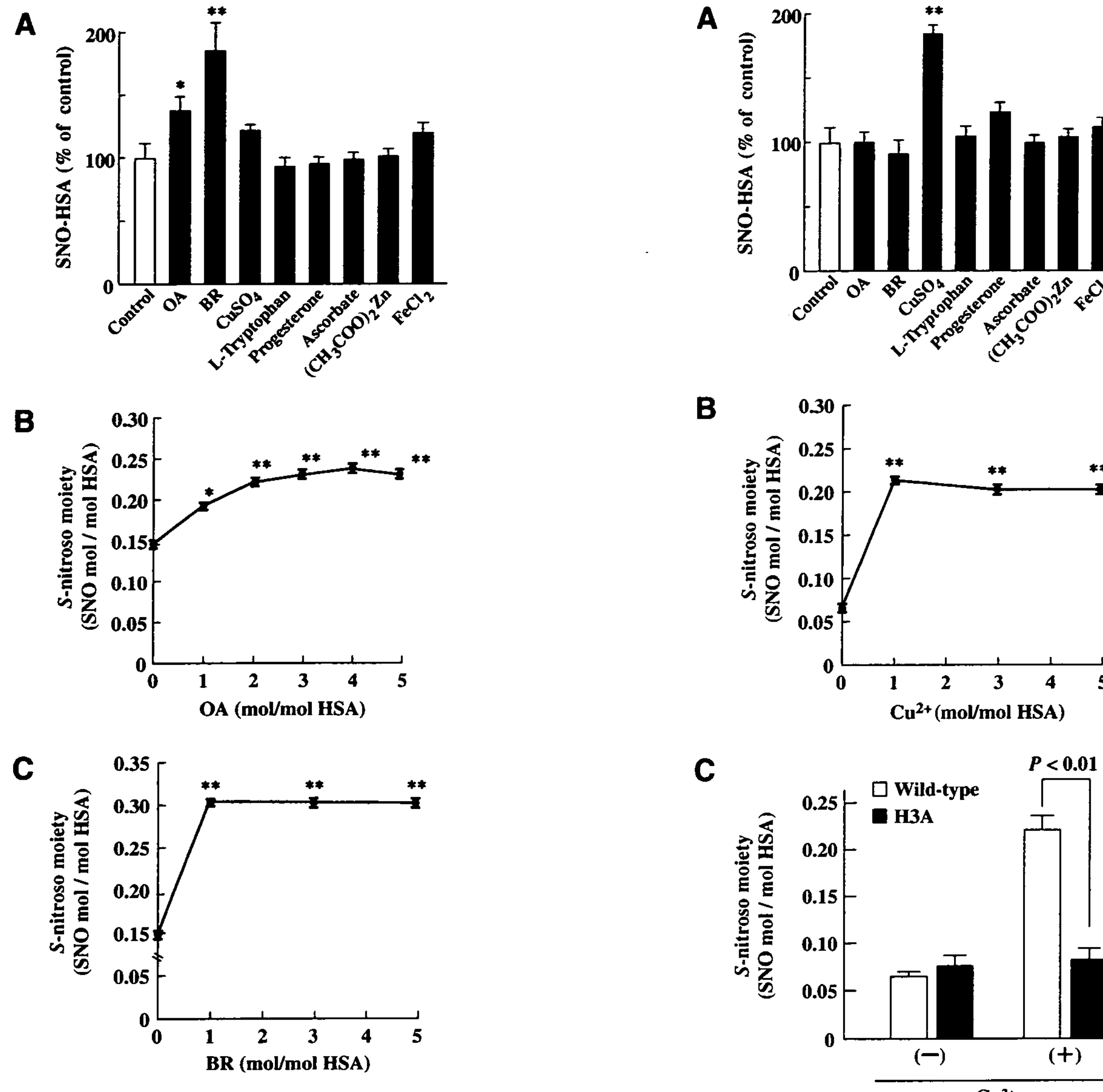

Fig. 1. Effect of ligand binding on S-nitrosylation of HSA by GS-NO. (A) 100 μM DTT-treated HSA was incubated with 100 μM of different ligands. (B) DTT-treated HSA was incubated with different molar ratios of OA. (C) DTT-treated HSA was incubated with different molar ratios of BR. In all cases, the GS-NO concentration was 500 μM. Data are expressed as means ± SEM ($n = 4$–6). $^{*}P < 0.05$, $^{**}P < 0.01$, as compared with control.

Fig. 2. Effect of ligand binding on S-nitrosylation of HSA by NOC-7. (A) Hundred micromolars of DTT-treated HSA was incubated with 100 μM of different ligands. (B) DTT-treated HSA was incubated with different molar ratios of Cu^{2+}. (C) Wild-type HSA and the H3A mutant, without or with Cu^{2+}, were S-nitrosylated by NOC-7. In all cases, the NOC-7 concentration was 500 μM. Data are expressed as means ± SEM ($n = 4$-6). $^{**}P < 0.01$, as compared with control.

progesterone, ascorbate, $(CH_3COO)_2Zn$ or $FeCl_2$. In the following, we have studied in more detail the positive effects of OA, BR and Cu^{2+}, which bind to different high-affinity sites of HSA [10,16] (Fig. 3).

Fig. 1B shows an increasing effect of OA on S-nitrosylation of HSA by GS-NO. The increment is dose-dependent until a OA:HSA molar ratio of 3; increasing the molar ratio further to 4 or 5 did not result in additional S-nitrosylation. Because OA does not bind to Cys-34 (Fig. 3), the effect observed could be due to binding-induced conformational changes of HSA making Cys-34 more accessible to GS-NO [12,17]. Actually, the data given in Table 1 propose such a mechanism, because OA binding results in an almost linear increment in binding of the test-compound DTNB to Cys-34.

The effect of BR binding on S-nitrosylation by GS-NO was also studied at different molar ratios of ligand to protein (Fig. 1C). Without BR the amount of S-nitroso moieties was 0.15 ± 0.02 ($n = 3$), and with BR it was approximately 0.30 ($P < 0.01$). The latter value was obtained, whether the BR:HSA molar ratio was 1, 3 or 5. Thus, only high-affinity BR binding increases S-nitrosylation. Because this kind of binding takes place to another region of HSA than that housing Cys-34 (Fig. 3) the improving effect must be the result of conformational

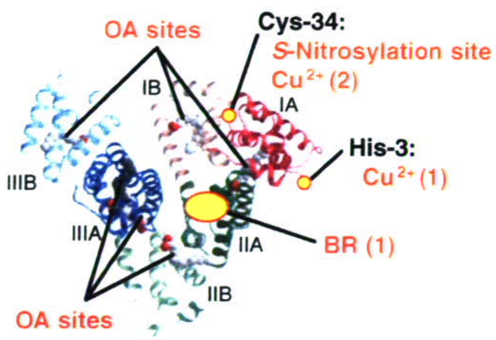

Fig. 3. Crystal structure of HSA showing locations of OA binding sites, high-affinity binding sites for BR (BR(1)) and Cu^{2+} (Cu^{2+}(1)) and Cys-34 which also is the site for secondary Cu^{2+} binding (Cu^{2+}(2)). The subdivision of HSA into domains (I-III) and subdomains (A and B) is indicated. The structure was simulated on the basis of X-ray crystallographic data for HSA-OA (PDB ID code 1gni) and modified with the use of Rasmol (downloaded from http://www.openrasmol.org).

Table 1
Effect of binding on the accessibility of Cys-34

Ligand/HSA	0	1	3	5
OA	0.17 ± 0.01	0.22 ± 0.02*	0.67 ± 0.03**	0.84 ± 0.04**
BR	0.17 ± 0.02	N.D.	N.D.	N.D.
Cu^{2+}	0.17 ± 0.02	0.18 ± 0.03	0.20 ± 0.04	0.19 ± 0.04

The accessibility was evaluated as A5/A60, where A5 and A60 is the sample absorbance at 405 nm after 5 min and 60 min (maximal absorbancy), respectively, of incubation with DTNB [12]. Results are given as means ± SEM ($n = 5$). N.D., not determined. $^{*}P < 0.05$, $^{**}P < 0.01$, as compared with Ligand/HSA = 0.

changes in the protein related to accommodation of the large BR molecule. That such conformational changes take place has previously been detected by techniques such as fluorescence spectroscopy [10]. By contrast to GS-NO, high-affinity binding of BR does not influence S-nitrosylation by NOC-7 (Fig. 2A). For testing whether this lack of effect could be caused by an interaction between NO and HSA-bound BR, we performed spectrophotometric experiments. These experiments showed that exposure of HSA-BR to NOC-7, but not to GS-NO, results in a fast decrease of the absorbancy at 470 nm (representing λ_{max} for HSA-BR) and a concomitant and pronounced increase at 650 nm (representing λ_{max} for HSA-biliverdin) (data not shown). Therefore, the following reaction seems to have taken place: (^{34}Cys-SH)-HSA-BR + ·NO → (^{34}Cys-SH)-HSA-BV + NO_2^-. Thus, the lack of effect of BR is due to a conversion to biliverdin (BV), and neither that ligand nor the NO_2^- formed can improve S-nitrosylation.

In contrast to the S-nitrosylating effect of GS-NO, the effect of NOC-7 was significantly increased by the presence of Cu^{2+} (Fig. 2A). The increasing effect was the same, whether the molar ratio of Cu^{2+} to protein was 1:1, 3:1 or 5:1 (Fig. 2B). Cu^{2+} binds with a very high affinity to a specific site in the N-terminal region of HSA, and His-3 is an essential element of that site [10]. In order to test whether high-affinity binding of Cu^{2+}, which takes place at a distance from Cys-34 (Fig. 3), is responsible for the improving effect of NOC-7, or whether the effect is caused by other means, e.g. secondary binding, we mutated His-3 for an alanine. The results of Fig. 2C show that the positive effect of Cu^{2+} disappears when mutating His-3. This finding strongly proposes high-affinity binding as the reason for the improving effect of Cu^{2+} on the S-nitrosylation by NOC-7. The positive effect of high-affinity Cu^{2+} binding is most probably caused by conformational changes induced in the HSA molecule, which render the SH-group of Cys-34 more reactive. Such a mechanism also seems to be supported by the results of Zhang and Wilcox [18]. These authors, using isothermal titration calorimetry and different spectroscopic techniques, found evidence for an interaction between the first Cu^{2+} binding site and Cys-34 in bovine serum albumin. However, these conformational changes are different from those caused by OA, because in contrast to OA binding of Cu^{2+} does not affect the accessibility of Cys-34 (Table 1). In contrast to the present findings Stubauer et al. [19] found no effect of high-affinity bound Cu^{2+} on RS-NO formation. RS-NO formation was only initiated, when that binding site was saturated, and the authors proposed S-nitrosylation of Cys-34 when also Cu^{2+} binds with a low affinity to the same residue. However, they used bovine serum albumin and NO gas in their studies.

S-Nitrosylation of mercaptalbumin-ligand complexes by NOC-7 and RAW264.7 cells

For studying S-nitrosylation of HSA in a biological system, we investigated the process caused by the murine macrophage cell line RAW264.7 (Fig. 4). The cell line had been activated by interferon-γ and lipopolysaccharide for expressing the inducible NO synthase. Binding of OA or BR does not affect S-nitrosylation of HSA by the cells. By contrast, Cu^{2+} binding facilitates S-nitrosylation (Fig. 4B). Fig. 4C shows that binding of Cu^{2+}, but not binding of OA or BR, decreases significantly the production of NO_2^-. These results propose that the formation of SNO-HSA by the cell line takes place via NO. This proposal was supported by findings showing that the effects of GS-NO in a similar experiment were different from those of NOC-7 and the RAW cells (data not shown).

Concluding remarks

Normally, the molar ratio of endogenous fatty acids to HSA is about 1.5 or lower [10], and those of BR and Cu^{2+} are below unity. Strenuous exercise or other adrenergic stimulation can rise the molar ratio for fatty acids to about 4 [10]. The molar ratios of all three ligands can be elevated in pathological conditions, e.g., metabolic syndrome, Type II (non-insulin-dependent) diabetes (fatty acids), increased catabolism of hemoglobin or hepatic

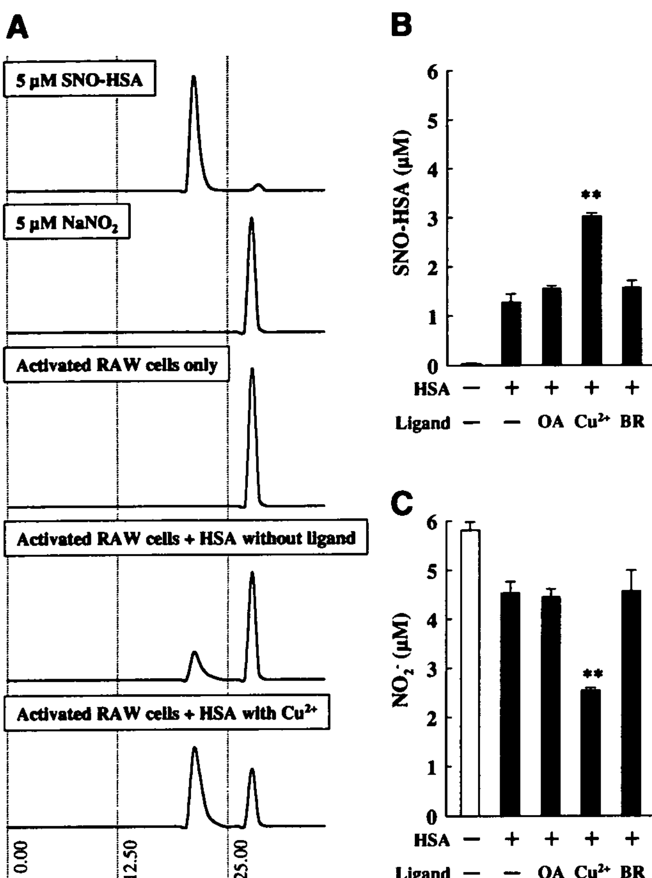

Fig. 4. Effect of high-affinity binding of OA, Cu^{2+} and BR on S-nitrosylation of HSA by activated RAW264.7 cells. (A) Elution profiles for the standard solutions (5 μM SNO-HSA and 5 μM $NaNO_2$) and for different cell media made by the HPLC-flow reactor system. The retention time for SNO-HSA and NO_2^- is about 21 and 27 min, respectively. (B) Production of SNO-HSA from HSA with and without bound ligand. (C) Production of NO_2^- in the presence of HSA with and without bound ligand. Data are expressed as means ± SEM ($n = 4$–6). $^{**}P < 0.01$, as compared with HSA without bound ligand.

disorders (BR) and Wilson's disease (Cu^{2+}). Thus, several physiological, clinical and pathological situations can affect the concentration of the quantitatively important SNO-HSA via modified high-affinity binding of OA, BR or Cu^{2+}.

In addition to HSA, other proteins possessing a free cysteine residue can be targets for S-nitrosylation, e.g. α_1-protease inhibitor [20]. Many of these proteins also bind ligand(s) the amounts of which can change in different biological or pathological conditions. Therefore, the present observations made with HSA could be general effects also applying to other proteins binding ligands and NO.

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Changes of net charge and α-helical content affect the pharmacokinetic properties of human serum albumin

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Abstract

The pharmacokinetics of 17 genetic variants of human serum albumin with single-residue mutations and their corresponding normal albumin were studied in mice. In all cases, the plasma half-life was affected, but only variants with +2 changes in charge prolonged it, whereas changes in hydrophobicity decreased it. Good positive and negative correlations were found between changes in α-helical content taking place in domains I+III and domain II, respectively, and changes in half-lives. No correlation was found to type of mutation or to changes in heat stability as represented by ΔH_v. Liver and kidney uptake clearances were also modified: α-helical changes of domains I+III showed good negative correlations to both types of clearances, whereas changes in domain II only had a good positive correlation to kidney uptake clearance. No correlation between the other molecular changes and organ uptakes was observed. The relatively few correlations between changes in molecular characteristics and the organ uptakes of the variants are most probably due to different handling by plasma enzyme(s) and the various types of cell endocytosis. Of the latter, most lead to destruction of albumin, but at least one results in recycling of the protein. The present information should be useful when designing recombinant, therapeutical albumins or albumin products with a modified plasma half-life.
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Keywords: Human serum albumin; Genetic variant; Pharmacokinetics; Half-life; Hepatic uptake; Renal disposition

1. Introduction

Human serum albumin (HSA) is produced in the parenchymal cells of the liver and after removal of its pre–pro sequences, it is secreted into the circulation, where it is the most abundant plasma protein. It is an important circulating carrier of endogenous and exogenous ligands in the blood, and contributes to the maintenance of osmotic pressure, plasma pH and to the Donnan-effect in the capillaries [1,2]. The protein is formed by a single polypeptide chain of 585 amino acids and has a molecular mass of approximately 67,000 [2]. According to X-ray crystallographic analyses of HSA and of its recombinant version, albumin has about 67% α-helix but no β-sheet. The analyses also showed that the polypeptide chain forms a heart-shaped protein with three homologous domains (I–III), each comprised of two subdomains (A and B) with distinct helical folding patterns that are connected by flexible loops [3,4]. HSA has 35 cysteine residues, and all of these but one, Cys 34, are involved in the formation of stabilizing disulfide bonds.

Clinically, HSA is used to treat severe hypoalbuminemia or traumatic shock [2], and the usual dosages of HSA are in excess of 10 g/dose. To date, albumin has been produced by fractionation of whole blood. However, there is the potential risk of HSA contamination with blood-derived pathogens. In addition, human plasma is in limited supply in countries like Japan. Therefore, the development of an alternative method of industrial production of HSA is desired as this would greatly assist in the general movement of, for example, Japan toward self-sufficiency in blood and blood products. Because of these

Abbreviations: HSA, human serum albumin; Alb, albumin; Alb A, normal (wild-type) albumin; ΔH_v, van't Hoff enthalpy

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problems, recombinant HSA, that is highly expressed by *Pichia pastoris*, most probably will be commercially available in the near future. Protein engineering will also enable the creation of recombinant HSAs with modified properties such as extended half-life in the circulation. A modified half-life will also be beneficial, when HSA is used as a fusion protein to extend the half-life of small proteins like soluble CD4 and hirudin [5,6]. Despite these possibilities, not much has been done to clarify the effects of mutations on the pharmacokinetics of HSA.

Previous studies with chemically modified albumin have demonstrated that changes in hydrophobicity and net charge on the protein surface affect its in vivo clearance [7,8]. In a previous study in which we produced six recombinant HSAs, our findings suggested that even single-residue mutations of surface or more internally placed amino acids can affect both the structural properties and the in vivo elimination of HSA [9]. Even though these studies indicated that the elimination of HSA is dependent on its structural properties more detailed studies of the relationships between structural and pharmacokinetic properties are needed to design useful recombinant HSAs with modified half-lives in the blood.

In our search for such candidates, we paid our attention to HSA genetic variants. Until now, more than 60 inherited variants of HSA have been identified and structurally characterized [10]. Usually, these genetic variants are expressed in heterozygous form and without any known association to disease [2]. Therefore, unlike lethal mutations, such as may occur for hemoglobin and coagulation factors, studying the pharmacokinetic properties of HSA variants is a good way of gaining information which can be used when designing recombinant HSAs, because we can consider the effects of molecular variation without worrying about complications such as antigenic effects.

Previously, we have shown that inherited single-residue substitutions affected the structure (α-helical content) and thermal stability (van't Hoff enthalpy (ΔH_v)) of HSA [11]. In addition, good linear correlations between mutation-induced changes of α-helical content and ΔH_v were found. In the present study, we examined the pharmacokinetics of 17 structurally different genetic variants with single-residue mutations isolated from heterozygote carriers, namely Alb Malmö-95 (D63N), Alb Tregasio (V122E), Alb Hawkes Bay (C177F), Alb Herborn (K240E), Alb Niigata (D269G), Alb Caserta (K276N), Alb Canterbury (K313N), Alb Brest (D314V), Alb Roma (E321K), Alb Sondrio (E333K), Alb Parklands (D365H), Alb Milano Slow (D375H), Alb Kashmir (E501K), Alb Maku (K541E), Alb Church Bay (K560E), Alb Verona (E570K) and Alb Milano Fast (K573E). We labeled the variants and their corresponding, endogenous normal albumin with ^{111}In, and examined the pharmacokinetic properties of these albumins in mice. Then, we determined the effects of the natural mutations on plasma half-life and organ uptakes. Finally, we related these results to the previously reported changes in α-helical content and ΔH_v.

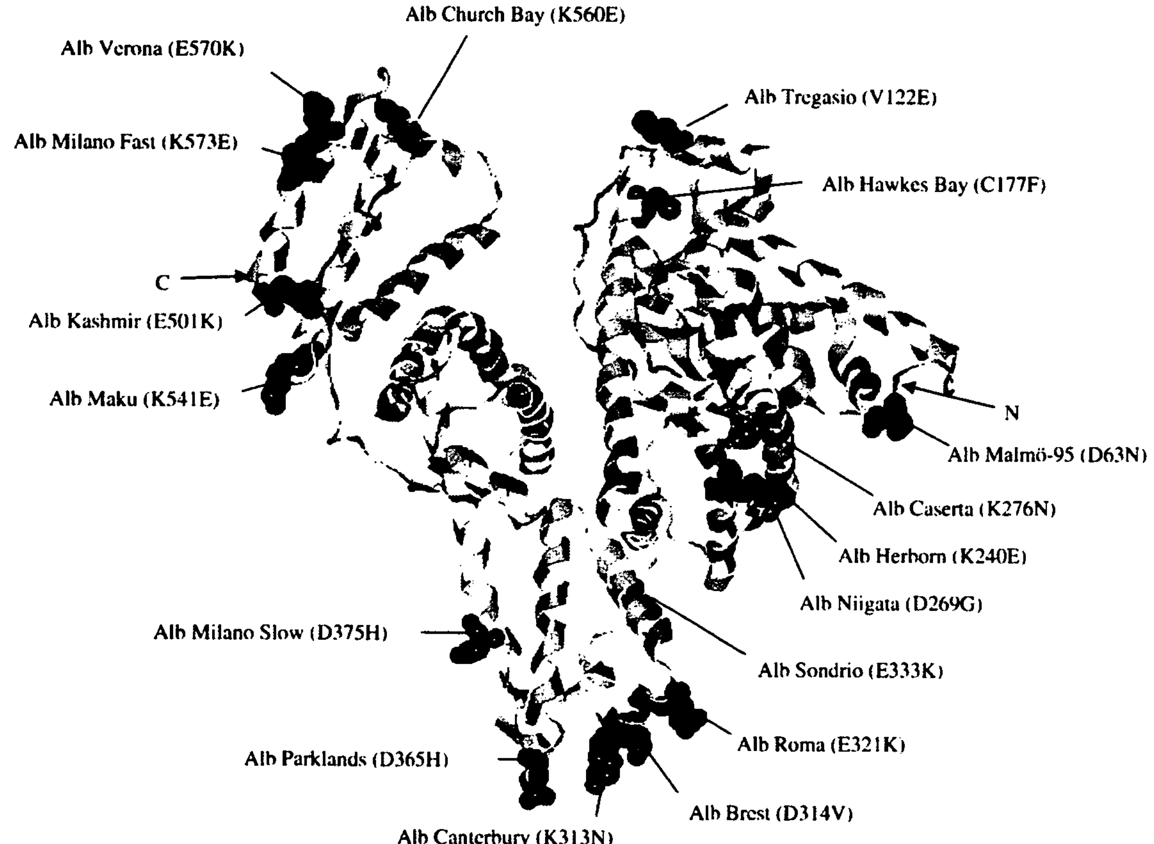

Fig. 1. The crystal structure of HSA showing the locations of the single-residue mutations of the 17 genetic variants used in this study. N and C stand for the N-terminal and the C-terminal ends, respectively.

2. Materials and methods

2.1. Protein samples

The 17 HSA genetic variants used in this study have been named after the place from where the first detected carrier originates. The variants have single-residue substitutions, and the location of the mutations is shown in Fig. 1. All variants, except one, have net charges which differ from that of normal (wild-type) HSA (endogenous Alb A) at physiological pH: Alb Roma (E321K) [12], Sondrio (E333K) [13], Kashmir (E501K) [14] and Verona (E570K) [15] are all +2 variants (i.e., they have two positive charges more than Alb A); Alb Malmö-95 (D63N) [16], Niigata (D269G) [17], Brest (D314V) [18], Parklands (D365H) [19] as well as Milano Slow (D375H) [20] are +1 variants; Alb Tregasio (V122E) [21], Caserta (K276N) [22] and Canterbury (K313N) [23] are −1 variants; and, finally, Alb Herborn (K240E) [24], Maku (K541E) [25], Church Bay (K560E) [26] and Milano Fast (K573E) [27] are −2 variants. The only variant with no change in net charge is Alb Hawkes Bay (C177F) [28]. Most of the substitutions are placed in domain II (residues 200–391), namely 9. Domains I (residues 1–199) and III (residues 392–585) are represented by three and five examples, respectively.

After isolation from heterozygous sera by ion-exchange chromatography, the individual albumins were checked for homogeneity by native electrophoresis, and no denaturation or significant (no more than 5%) cross-contamination between variant and Alb A was detected. The proteins were donated to us by Drs. M. Galliano and L. Minchiotti, University of Pavia, Pavia, Italy; Dr. S.O. Brennan, Canterbury Health Laboratories, Christchurch, New Zealand; Dr. A.L. Tárnoky, University of Reading, Reading, UK; and Dr. O. Sugita, Niigata University School of Medicine, Niigata, Japan. Before use, the albumins were delipidated by treatment with hydroxyalkoxypropyldextran at pH 3.0, as described elsewhere [29]. After defatting, the albumins were dialysed extensively against deionized water, lyophilized and stored at −20 °C until used. Thus, the albumins from a donor have been exposed to exactly the same conditions from the time the blood samples were taken until the present experiments were performed.

Fraction V HSA (96% pure), assumed to be Alb A, was donated by the Chemo-Sera-Therapeutic Research Institute (Kumamoto, Japan) and defatted using the charcoal procedure described by Chen [30], deionized, freeze-dried and then stored at −20 °C until used.

2.2. Chemicals and animals

$^{111}InCl_3$ (74 Mbq/mL in 0.02 N HCl) was donated by Nihon Medi-Physics (Takarazuka, Japan). All chemicals were of the highest grade commercially available, and all solutions were prepared using deionized, distilled water.

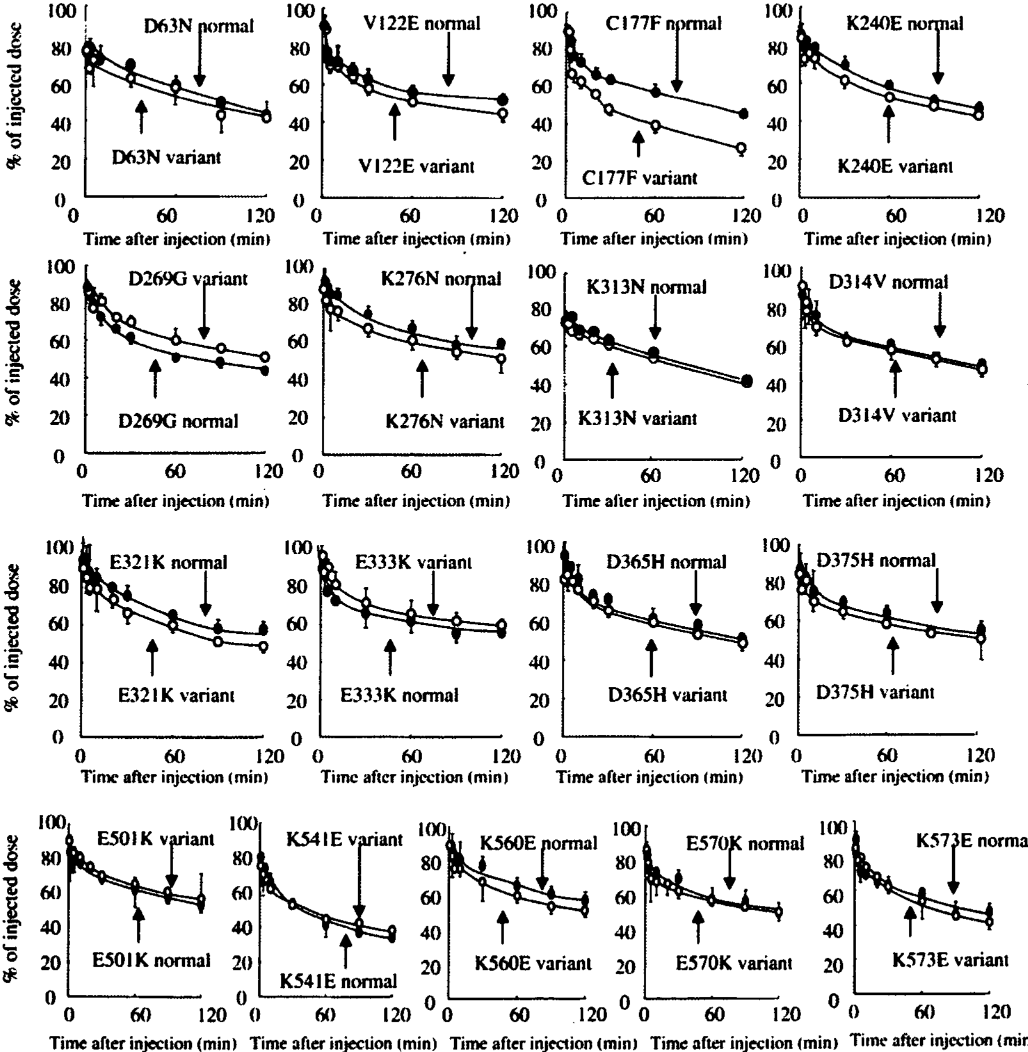

Fig. 2. Relative plasma amounts of ^{111}In-labeled HSA variants and their corresponding Alb A after intravenous administration in mice. ^{111}In-albumin was injected as a bolus dose into the tail vein. Relative amounts are plotted against time after injection. Each point represents an average value obtained for 3–6 mice (±S.D.). The open and closed circles represent variant and normal albumin, respectively.

Male ddY mice (26–32 g) were purchased from the Shizuoka Agricultural Cooperative Association for Laboratory Animals (Shizuoka, Japan), and were maintained under conventional housing conditions. All animal experiments were conducted in accordance with the principles and procedures outlined in the National Institute of Health Guide for the Care and Use of Laboratory Animals.

2.3. In vivo experiments

All proteins were radiolabeled with ^{111}In using the bifunctional chelating reagent DTPA anhydride according to the method of Hnatowich et al. [31], which has been described in detail in a previous paper [32]. In previous works, we found no significant differences in pharmacokinetic properties among these albumins, when ^{111}In-labeled mouse, rat, bovine or human serum albumin was administered to mice (unpublished), suggesting that immunogenic behavior does not occur in mice. Therefore, we chose the mouse as a reasonable model for the study of the pharmacokinetics of the HSAs. Mice received tail vein injections of ^{111}In-labelled proteins in saline, at a dose of 0.1 mg/kg and were housed in metabolic cages to allow the collection of urine samples. Urine samples were collected throughout the 120 min of the experimental period. In the early period after injection, the efflux of ^{111}In radioactivity from organs is assumed to be negligible, because the degradation products of ^{111}In-labeled proteins using DTPA anhydride cannot easily pass through biological membranes [33]. This assumption was supported by the fact that no ^{111}In was detectable in the urine after 120 min. At 1, 3, 5, 10, 30, 60, 90 or 120 min after injection, blood was collected from the vena cava under ether anesthesia and plasma was obtained by centrifugation. After blood collection, the animals were sacrificed, organs were excised, rinsed with saline and weighed. The radioactivity of each blood and tissue sample was measured in a well-type NaI scintillation counter (ARC-500, Aloka, Tokyo).

Pharmacokinetic analyses were performed as follows. The plasma ^{111}In radioactivity concentrations (C_p) were normalized with respect to the percentage of injected dose and analyzed using the nonlinear least-square program MULTI [34]. The two-compartment model was fitted according to the Akaike information criterion by Eq. (1).

$$C_p = Ae^{-\alpha t} + Be^{-\beta t} \tag{1}$$

The half-lives of the HSAs were determined as β-phase elimination within a 120-min period. The tissue distribution patterns were evaluated using tissue uptake clearances (CL_{uptake}) according to the integration plot analysis. CL_{uptake} was calculated using Eq. (2).

$$CL_{uptake} = \frac{X_t/C_t}{AUC_{0-t}/C_t} \tag{2}$$

where X_t is the tissue accumulation at time t, AUC_{0-t} is the area under the plasma concentration time-curve from time 0 to t, and C_t is the plasma concentration at time t. CL_{uptake} was obtained from the slope of the plot of X_t/C_t versus AUC_{0-t}/C_t. We estimated the organ uptake clearances within a 30-min period.

Table 1
Half-lives and organ uptake clearances of ^{111}In-labeled variants and corresponding Alb A in mice

Variant name (mutation)		Half-life[a] (min)	Clearance (μL/h)[a]	
			Liver	Kidney
Alb Malmö-95 (63 Asp→Asn)	Variant	259.4±7.26	53.14±12.09*	65.09±18.83*
	Alb A	264.2±6.14	79.72±11.74	111.64±18.18
Alb Tregasio (122 Val→Glu)	Variant	250.3±6.65**	73.22±18.70*	71.42±12.74
	Alb A	270.4±1.23	129.13±11.87	104.87±24.94
Alb Hawkes Bay (177 Cys→Phe)	Variant	176.3±8.68**	494.40±38.82**	374.08±43.19**
	Alb A	250.8±5.58	23.99±2.68	69.11±11.67
Alb Herborn (240 Lys→Glu)	Variant	265.8±6.97	132.45±22.19**	137.86±25.61
	Alb A	269.3±4.51	41.10±5.37	110.69±12.94
Alb Niigata (269 Asp→Gly)	Variant	269.7±1.68	14.12±2.39	83.69±19.97
	Alb A	275.2±4.68	18.00±3.27	97.37±23.26
Alb Caserta (276 Lys→Asn)	Variant	254.8±4.89*	45.86±6.29**	203.48±35.37**
	Alb A	269.3±7.64	21.77±5.03	81.00±13.51
Alb Canterbury (313 Lys→Asn)	Variant	221.6±4.65	119.37±12.12	331.98±60.07**
	Alb A	231.6±4.64	128.80±29.97	64.36±20.01
Alb Brest (314 Asp→Val)	Variant	260.8±7.64*	35.48±11.53	102.87±13.27
	Alb A	276.4±5.46	25.31±6.10	99.75±16.32
Alb Roma (321 Glu→Lys)	Variant	269.4±4.51	56.18±5.89**	117.48±11.06*
	Alb A	266.1±3.65	13.34±2.91	83.49±14.23
Alb Sondrio (333 Glu→Lys)	Variant	251.1±4.98	44.72±10.58	172.70±40.37
	Alb A	247.6±7.89	28.92±8.71	169.51±16.82
Alb Parklands (365 Asp→His)	Variant	267.3±6.51	30.99±4.11**	119.35±14.93*
	Alb A	273.1±4.49	63.58±6.54	75.63±20.15
Alb Milano Slow (375 Asp→His)	Variant	268.7±7.61	43.88±10.33*	47.34±9.81
	Alb A	272.9±1.43	17.57±8.33	59.65±22.73
Alb Kashmir (501 Glu→Lys)	Variant	273.2±4.62	44.21±6.34*	76.48±16.91*
	Alb A	270.6±5.48	64.36±9.46	138.53±18.94
Alb Maku (541 Lys→Glu)	Variant	245.9±7.32	144.86±15.01**	285.50±44.74**
	Alb A	251.3±6.51	276.01±47.95	90.25±20.42
Alb Church Bay (560 Lys→Glu)	Variant	270.3±3.56*	13.37±4.39	77.06±16.01*
	Alb A	276.3±1.03	17.61±8.08	136.67±30.62
Alb Verona (570 Glu→Lys)	Variant	274.1±3.98*	99.12±18.83	128.12±20.68
	Alb A	262.4±4.14	63.85±11.72	121.06±16.44
Alb Milano Fast (573 Lys→Glu)	Variant	263.9±2.46	119.41±18.90	218.05±33.41**
	Alb A	263.5±6.45	85.88±11.35	89.64±10.02

[a] The data are average values of 3–6 experiments (±S.D.). *$P<0.05$, **$P<0.01$ as compared with endogenous Alb A.

2.4. Analysis of experimental data

The effects of the single-residue mutations were evaluated by using the following relationship:

$$\text{Percent change} = \frac{(\text{Result for variant}) - (\text{Result for Alb A})}{(\text{Result for Alb A})} \times 100\% \quad (3)$$

In Eq. (3), the result can be a value determined for plasma half-life, organ uptake clearance, α-helical content or for ΔH_v.

2.5. Statistical analysis

Statistical analyses were performed by using the Student t-test. A probability value of $P<0.05$ was considered to indicate statistical significance.

3. Results

3.1. Pharmacokinetic properties of HSA variants

Fig. 2 shows the time courses for radioactivity in mouse plasma after intravenous administration of ^{111}In-labeled preparations of the variants and their corresponding Alb A. For all 17 variants, the mutation affected the elimination of HSA. In particular, Alb Hawkes Bay (C177F) showed rapid elimination as compared with Alb A. However, in this example the variant has undergone a major conformational change, see below, because it has lost a disulfide bond [28]. Table 1 gives the plasma half-lives, calculated by β-phase using the nonlinear least-square program MULTI and Eq. (1), and liver and kidney uptake clearances, determined by an integration plot analysis (Eq. (2)). As a control we have compared the pharmacokinetic results obtained for endogenous Alb A (Table 1) with those obtained with commercial HSA (not illustrated), because both types of preparations are assumed to represent the normal protein. The average half-life for Alb A is 264.2 min. This value is comparable to that determined for commercial HSA, i.e., 268.2±7.2 min (n=6). The liver and kidney uptake clearances for Alb A are on an average 64.64 μL/h and 100.2 μL/h, respectively, whereas those for commercial HSA are 54.31± 8.23 μL/h and 83.12±5.43 μL/h, respectively. The slightly lower values found for commercial HSA are most probably due to differences in isolation and/or defatting procedures.

Table 2 shows the effects of the mutations on plasma half-lives and organ uptake clearances as determined by Eq. (3). As seen, 12 of the mutations diminished plasma half-life, i.e., negative percent changes, whereas one mutation had almost no effect (Alb Milano Fast). By contrast, the remaining variants, all of which are +2 variants, had a prolonged half-life. These findings are in accordance with those of Iwao et al. [9], who, by using oxidized and recombinant mutants, observed that increasing the net negative charge of HSA increased its elimination from the circulation. The results of Table 2 also propose that a change in hydrophobicity diminish plasma half-life. Thus, Alb Malmö-95, Niigata, Caserta, Canterbury, Brest, Parklands and Milano Slow all are more hydrophobic than Alb A, and all of them have negative values for the effect of mutation. Principally the same was found to be the case for Alb Tregasio which is less hydrophobic than its normal protein.

All genetic variants also have a modified liver and kidney uptake clearance (Table 2). About half of the mutants have diminished liver uptake clearance, and about half of them have a reduced kidney uptake. In general, the increases in organ uptake clearance are more pronounced than the decreases. Thus, all percent changes of organ uptake clearances of 100% or more are associated with increases in uptake. For liver uptake it is Alb Hawkes Bay (1961%), Roma (321%), Herborn (222%), Milano Slow (150%) and Caserta (111%), and for kidney uptake it is Alb Hawkes Bay (441%), Canterbury (416%), Maku (216%), Caserta (151%) and Milano Fast (143%). However, no clear correlation could be found between changes of protein net charge and hydrophobicity, respectively, and liver and kidney uptake clearance, respectively.

3.2. Relationships between structure, stability and pharmacokinetic properties of HSA variants

Previously, we have studied the effects of the single-residue substitutions on the α-helical content and thermal stability of

Table 2
Percent changes of half-life and organ uptake clearances of HSA variants in mice

Variant name (mutation)	Half-life (%)	Liver clearance (%)	Kidney clearance (%)	α-helical content[a] (%)	ΔH_v[a] (%)
Alb Malmö-95 (63 Asp→Asn)	−1.82	−33.34	−41.69	−9.68	−46.71
Alb Tregasio (122 Val→Glu)	−7.43	−43.30	−31.89	2.02	9.76
Alb Hawkes Bay (177 Cys→Phe)	−29.70	1960.58	441.31	−10.00	−8.53
Alb Herborn (240 Lys→Glu)	−1.30	222.26	24.54	−4.12	−21.59
Alb Niigata (269 Asp→Gly)	−2.00	−21.56	−14.05	−3.92	−18.83
Alb Caserta (276 Lys→Asn)	−5.38	110.68	151.21	0.68	5.74
Alb Canterbury (313 Lys→Asn)	−4.32	−7.32	415.82	1.47	3.28
Alb Brest (314 Asp→Val)	−5.64	40.17	3.13	1.57	10.53
Alb Roma (321 Glu→Lys)	1.24	321.26	40.70	−1.77	11.31
Alb Sondrio (333 Glu→Lys)	1.41	54.66	1.88	−1.57	−12.14
Alb Parklands (365 Asp→His)	−2.12	−51.26	57.81	−3.13	29.19
Alb Milano Slow (375 Asp→His)	−1.54	149.77	−20.63	−5.30	−32.23
Alb Kashmir (501 Glu→Lys)	0.96	−31.30	−44.79	0.37	−0.60
Alb Maku (541 Lys→Glu)	−2.15	−47.52	216.36	0.40	−20.76
Alb Church Bay (560 Lys→Glu)	−2.17	−24.12	−43.62	1.18	5.28
Alb Verona (570 Glu→Lys)	4.46	55.24	5.83	2.55	36.24
Alb Milano Fast (573 Lys→Glu)	0.17	39.03	143.24	−2.95	−16.64

[a] The values for α-helical content and ΔH_v are taken from Kragh-Hansen et al. [11].

albumin [11], and the results of that study are included in Table 2. The α-helical contents were estimated from far-UV intrinsic circular dichroism spectra by using the ellipticity values at 222 nm. The effect of the mutations on the reversible thermal denaturation (25–85 °C) was also monitored by circular dichroism at 222 nm. These additional data allow for a more detailed comparison between molecular characteristics and pharmacokinetic properties. Fig. 3 gives the correlations between mutation-induced changes in α-helical content and pharmacokinetic parameters. For the mutations in domain I and III, i.e., positions 63–177 and 501–573, respectively (Fig. 1), the same tendencies between the data were observed; therefore they have been gathered in Fig. 3A–C. For these mutations, an increase in α-helical content implies increased plasma half-lives, but decreased liver and kidney uptake clearances. Surprisingly, mutations in domain II, i.e., positions 240–375 (Fig. 1), have the opposite effects. In these cases, an increase in α-helical content results in decreased plasma half-lives (Fig. 3D) but in an increment of kidney uptake clearances (Fig. 3F). By contrast, there is no correlation between changes in α-helical content and liver uptake clearances (Fig. 3E).

The effect of the mutations on albumin stability was examined in terms of heat stability, which was quantified by determining van't Hoff enthalpies (ΔH_v-values) (Table 2). Fig. 4 shows that no correlation was found between the percent changes in ΔH_v and any of the pharmacokinetic parameters. Principally the same observations were made when pooling all the results for domains I–III; in that case the P-values were in the range 0.79–0.97 (not shown).

4. Discussion

From a clinical point of view it would be beneficial, if protein engineering could result in the production of HSA with a prolonged half-life in the circulation. In addition, because of its half-life of 19 days in humans, its ease of synthesis and its known structure albumin is an attractive candidate for use in recombinant fusion proteins. These proteins combine a therapeutic protein (that would otherwise be rapidly cleared) and a plasma protein with a slow clearance in a single polypeptide chain. Furthermore, HSA has been proposed as a carrier in drug delivery systems. Also in the two latter types of examples it

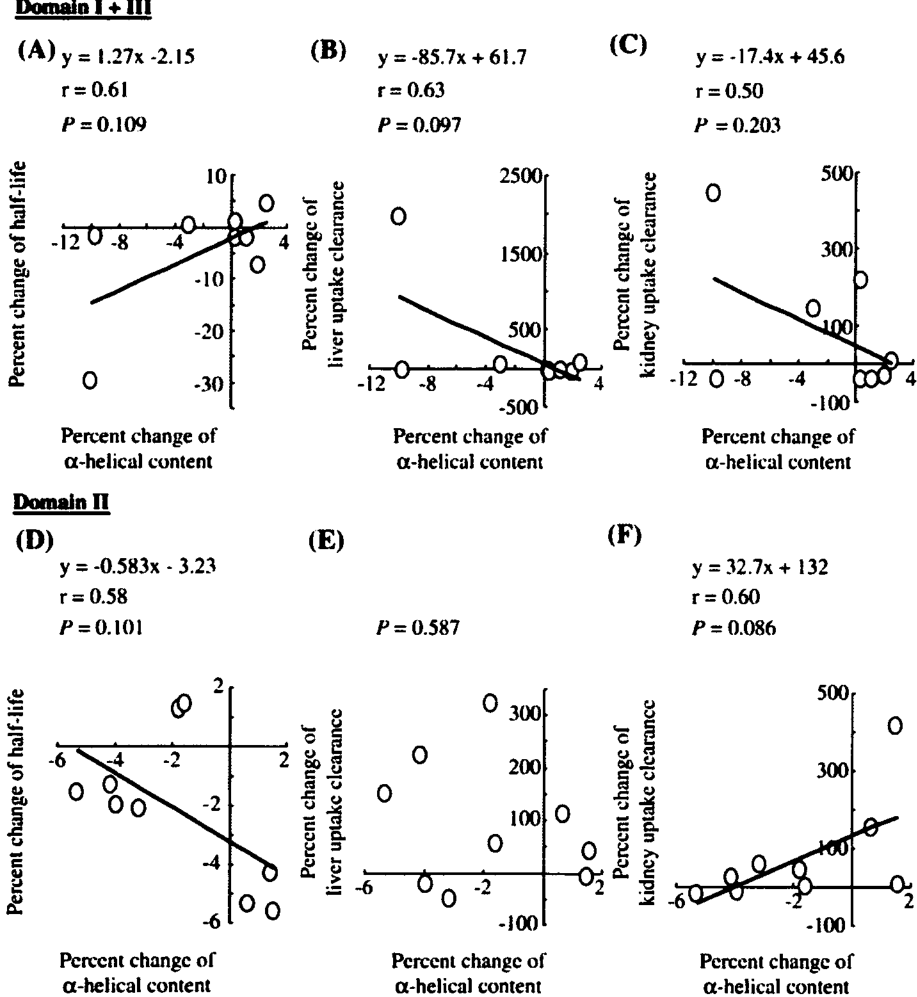

Fig. 3. Relationships between percent change of mutation-induced alterations of α-helical content and percent changes of half-life (A and D), liver uptake clearance (B and E) and kidney uptake clearance (C and F).

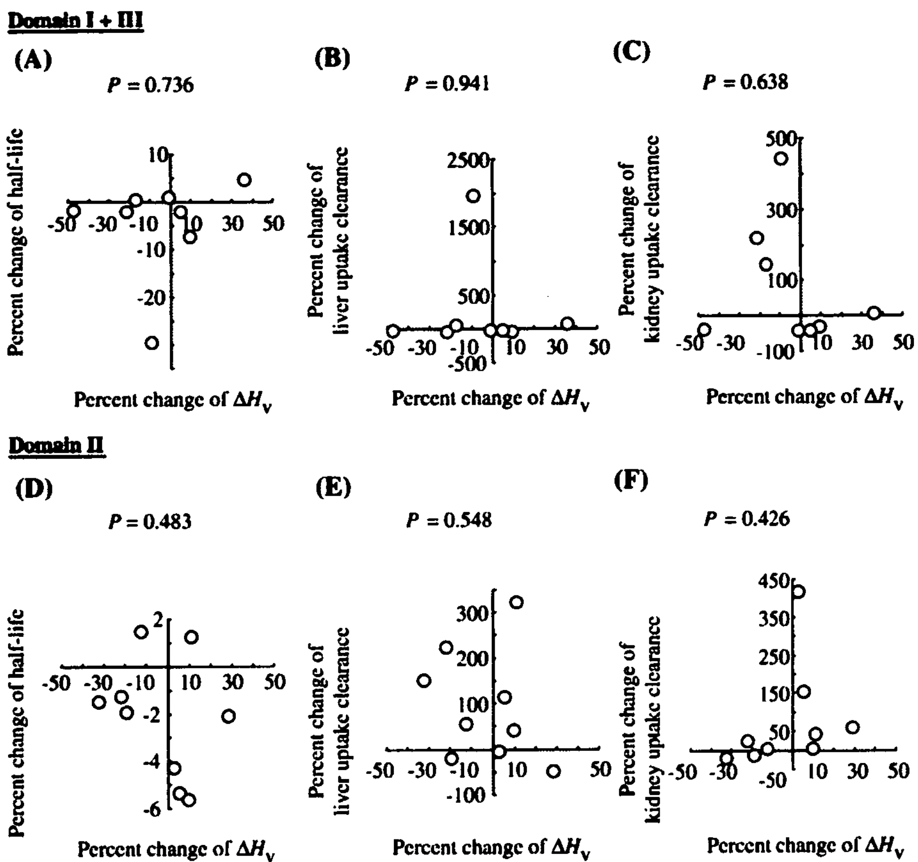

Fig. 4. Relationships between percent change of mutation-induced alterations of ΔH_v and percent changes of half-life (A and D), liver uptake clearance (B and E) and kidney uptake clearance (C and F).

would be advantageous to be able to modify the plasma half-life of the protein product. Therefore, in the present study, we investigated the half-life and organ uptakes of a series of genetic HSA variants. Since the isoforms have known single-residue mutations, we tried to identify molecular characteristics responsible for any modified pharmacokinetics.

The present variants have been detected by electrophoresis performed under native conditions. Therefore, their changes in charge are located on the surface of the protein, where they are exposed to the solvent. The only exception is Alb Hawkes Bay which rather is detected due to major conformational changes caused by the loss of the disulfide bond between Cys 168 and Cys 177 [28]. Thus, the amino acid substitutions affect the hydrophilic/hydrophobic characteristics of accessible regions of the protein. It should be mentioned that we did not quantify these effects experimentally. Instead, we used the knowledge of the amino acid substitutions, and when doing that we found that pronounced decreases in net charge (i.e., +2 variants) resulted in increased plasma half-lives. By contrast, increases in hydrophobicity implied shorter half-lives (Tables 1 and 2). These observations are in full accordance with those of Iwao et al. [9], who studied the effects of oxidation and recombinant mutations. Furthermore, Sheffield et al. [35], who used rabbit serum albumin, found that the recombinant mutant D494N, but not the charge-neutral mutant V14T, had a shorter half-life than the wild-type protein.

In addition to change in net charge and surface hydrophobicity we considered whether the modified plasma half-lives could be correlated to other molecular parameters. Therefore, in Fig. 3A and D the half-lives have been related to changes in α-helical content, which in this case were caused by single-residue substitutions; the results showed a good correlation between the two parameters ($P=0.1$). However, surprisingly, the effect depended on the location of the molecular change, because changes in α-helical content taking place in domains I and III showed a positive correlation to changes in half-life, whereas changes situated in domain II had the opposite effect. It can also be seen that most changes in α-helical content resulted in faster elimination from the circulation. The changes in half-life were also compared to mutation-induced changes in heat stability; quantified by using ΔH_v. However, in this case there was no correlation between the two parameters (Fig. 4A and D).

Iwao et al. [9] found that the diminished half-lives of their HSA preparations were accompanied by increased liver uptake clearances. Nakajou et al. [36] reported that a recombinant mutant of HSA in which the lysine residues in positions 199, 439 and 525 all had been substituted for alanine disappeared faster than the wild-type protein from the circulation. The authors suggested a faster than normal loss of the mutant to extravascular spaces. However, the triple-residue mutant also exhibited increased liver and kidney clearances. For being able to explain, at least in part, the modified plasma half-lives of the present