Table 1 Correlations between clinicopathological characteristics and the micropapillary pattern ($N=383$)

Characteristics	*No. of cases*	*Micropapillary pattern*		*P-value*
		Negative (%)	*Positive (%)*	
Gender				
Male	214	105 (53)	109 (59)	
Female	169	94 (47)	75 (41)	0.202
Age (years)				
<60	127	60 (30)	67 (36)	
≥60	256	139 (70)	117 (64)	0.193
Smoking				
Nonsmoker	185	107 (54)	80 (44)	
Smoker	198	92 (46)	104 (56)	0.044
Maximum tumor diameter (mm)				
≤30	261	139 (70)	122 (66)	
>30	122	60 (30)	62 (34)	0.457
Lymph node metastasis				
Negative	267	164 (82)	103 (56)	
Positive	126	35 (18)	81 (44)	<0.001
Pleural invasion				
Negative	265	150 (75)	115 (63)	
Positive	118	49 (25)	69 (37)	0.006
Lymphatic invasion				
Negative	215	154 (77)	61 (33)	
Positive	168	45 (23)	123 (67)	<0.001
Venous invasion				
Negative	309	182 (92)	127 (69)	
Positive	74	17 (8)	57 (31)	<0.001
Dominant histological subtype				
Bronchioloalveolar	103	84 (42)	19 (11)	
Non-bronchioloalveolar	280	115 (58)	165 (89)	<0.001
Acinar	69	36 (18)	33 (18)	
Non-acinar	314	163 (82)	151 (82)	0.968
Papillary	182	65 (33)	117 (63)	
Non-papillary	201	134 (67)	67 (37)	<0.001
Solid with mucin	29	14 (7)	15 (8)	
Non-solid with mucin	354	185 (93)	169 (92)	0.680

times observed but their distribution was focal and random. The apical and basal sides of the cells were not clearly delineated (Figure 4a), and showed intercellular junctions in micropapillary tufts (Figure 4b). These findings were confirmed in all three cases. Some cells constituting micropapillary tufts appeared to accumulate without cell polarity.

Correlation of Micropapillary Pattern and Clinicopathological Factors

Clinicopathological characteristics of patients and their tumors were compared between micropapillary pattern-positive (with focal, moderate, and extensive extent as mentioned below) and micropapillary pattern-negative groups (Table 1). Smoking ($P=0.044$), lymph node metastasis ($P<0.001$), pleural invasion ($P=0.006$), lymphatic invasion ($P<0.001$), venous invasion ($P<0.001$), dominant non-bronchioloalveolar carcinoma subtype ($P<0.001$), and dominant papillary subtype ($P<0.001$) were significantly associated with the micropapillary pattern. Especially strong correlations between the micropapillary pattern and lymph node metastasis, lymphatic invasion, and dominant non-bronchioloalveolar carcinoma subtype were observed. There was no association with gender ($P=0.202$), age ($P=0.193$), or tumor size ($P=0.457$). In stageIA cases ($N=197$), smoking ($P=0.031$), lymphatic invasion ($P<0.001$), venous invasion ($P<0.001$), dominant non-bronchioloalveolar carcinoma subtype ($P<0.001$), and dominant papillary subtype ($P<0.001$) were significantly associated with the micropapillary pattern (Table 2).

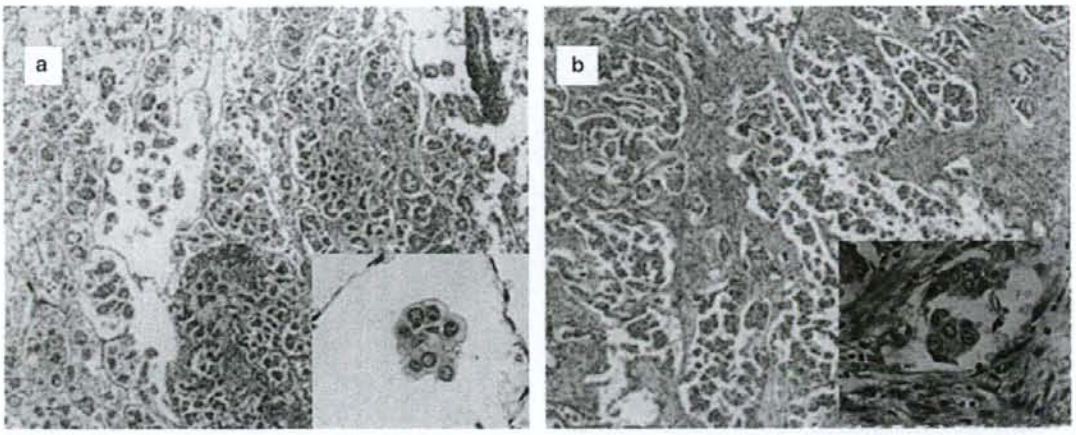

Figure 1 Representative histology of the micropapillary pattern. (**a** and **b**) The micropapillary pattern was characterized by small tufts with no fibrovascular core (**a**) lying in the alveolar space or (**b**) in spaces that in most cases represented retracted connective tissue. (**a** and **b**) Hematoxylin—eosin: ×40; inset: ×400.

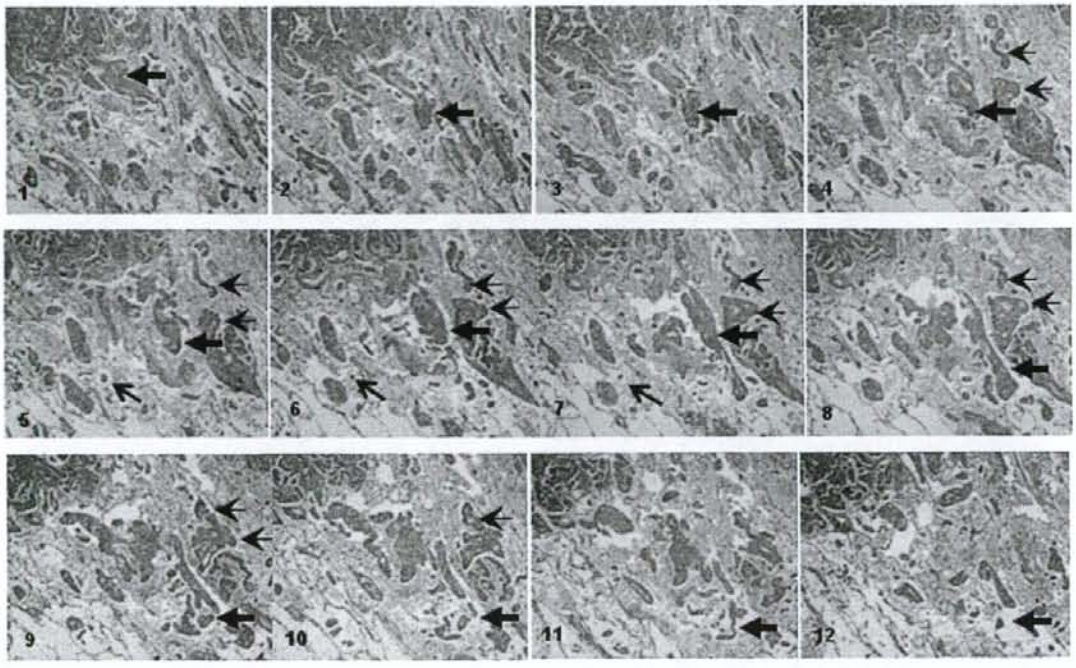

Figure 2 Serial sections of a micropapillary pattern-positive specimen. The tuft marked by (➡) in section 12 is consecutive with another tuft in section 8 and even with the main tumor in section 1. Two isolated tufts identified by (➔) in section 4 are shown to be consecutive in section 10. The tuft identified by (→) in sections 5, 6, and 7 could not be identified as continuous with any other tuft. Sections 1–16 hematoxylin–eosin at ×40.

Prognostic Significance of the Micropapillary Pattern

Survival curves showed that as the extent of the micropapillary pattern progressed, the prognosis tended to worsen, and that the disease-free and overall survival for the focal group were worse than in the none group ($P=0.027$ and $P=0.068$) (Figure 5a and b). In this study, cases in the none group (199, 52%) were therefore classified as micropapillary pattern-negative and the remainder of cases (184, 48%) were classified as micropapillary pattern-positive. Disease-free and overall survival for

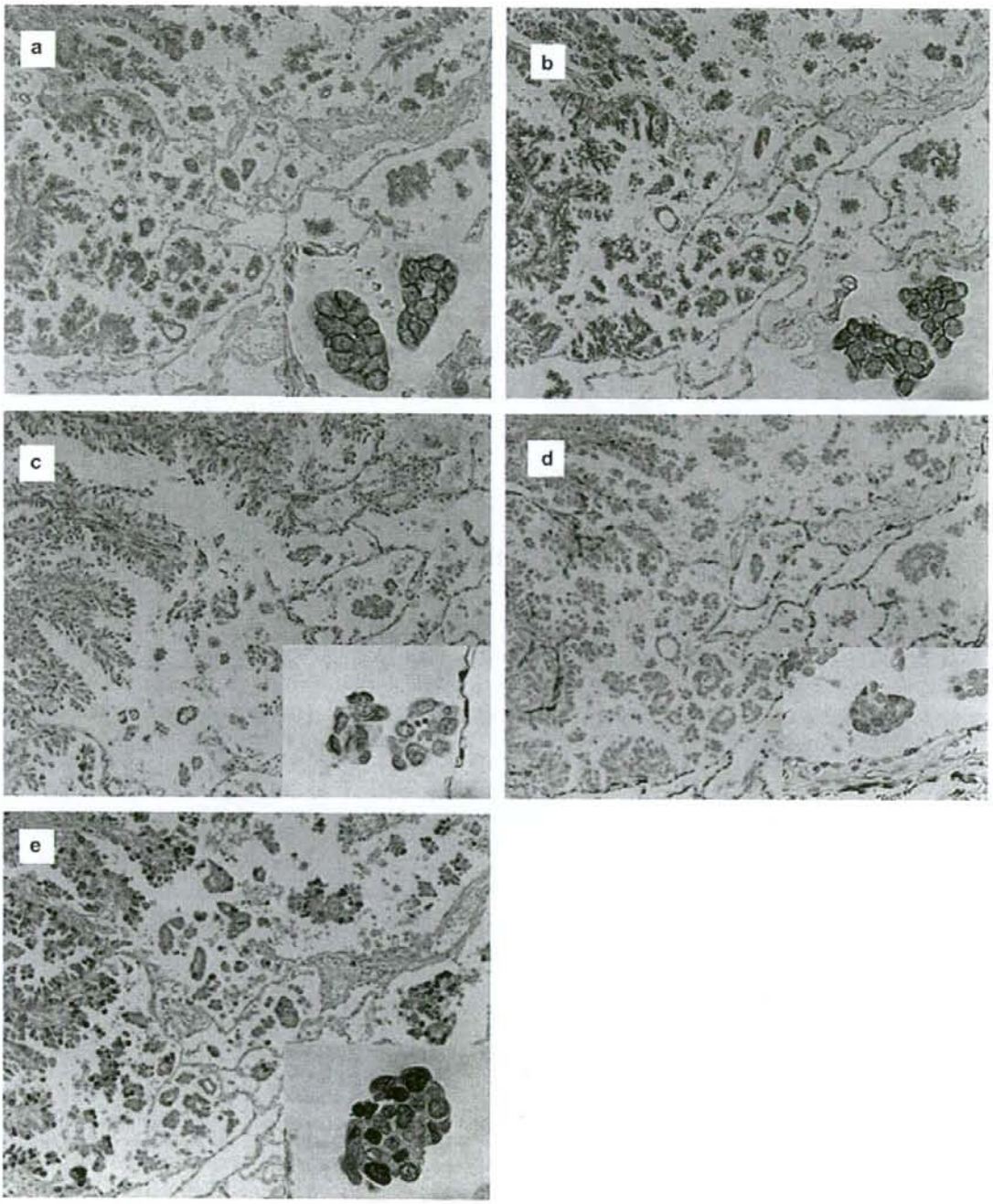

Figure 3 Immunohistochemical findings. (**a**) E-cadherin and (**b**) β-catenin were expressed at intercellular cell membranes of micropapillary tufts. (**c**) Laminin was found in the basement membrane of normal alveolar cells and in neoplastic cells in the main tumor, but was not found in cells constituting micropapillary tufts. (**d**) CD34-positive cells were enriched in the stroma of main tumor and the alveolar septum, whereas no CD34-positive cells were found in micropapillary tufts. (**e**) Cells constituting micropapillary tufts stained positive for Ki-67. (**a**) E-cadherin (**b**) β-catenin (**c**) Laminin (**d**) CD34 (**e**) Ki-67 × 40; inset: × 400.

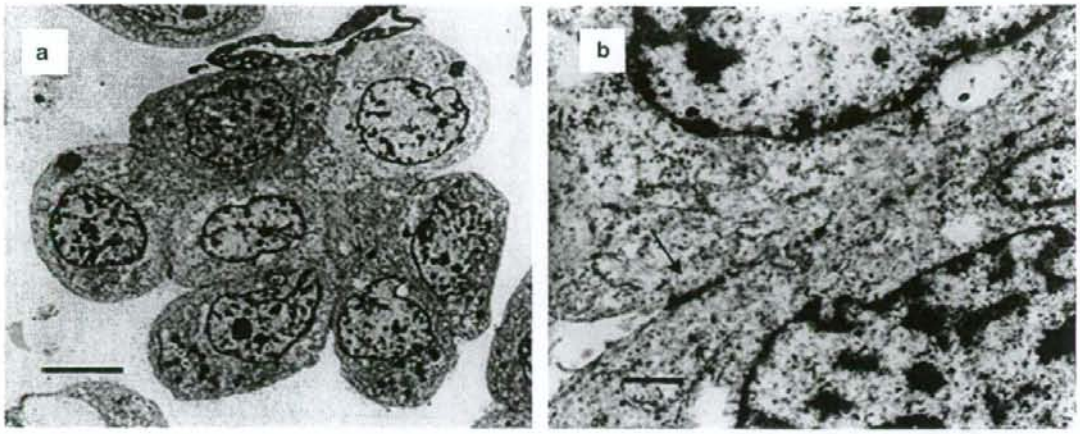

Figure 4 Electron microscopic findings. **(a)** No basement membrane or vascular structures were observed. Microvilli distribution was focal and random, and the apical and basal sides could not be clearly identified. **(b)** Intercellular junctions (arrow). **(a)** Scale bar = 5 μm; **(b)** scale bar = 500 nm.

Table 2 Correlation between clinicopathological characteristics and the micropapillary pattern in stageIA patients ($N = 197$)

Characteristics	*No. of cases*	*Micropapillary pattern*		*P-value*
		Negative (%)	*Positive (%)*	
Gender				
Male	108	60 (51)	48 (62)	
Female	89	59 (49)	30 (38)	0.125
Age (years)				
<60	68	36 (30)	32 (41)	
≥60	129	83 (70)	46 (59)	0.120
Smoking				
Nonsmoker	107	72 (60)	35 (45)	
Smoker	90	47 (40)	43 (55)	0.031
Lymphatic invasion				
Negative	157	111 (93)	46 (59)	
Positive	40	8 (7)	32 (41)	<0.001
Venous invasion				
Negative	180	116 (98)	64 (82)	
Positive	17	3 (2)	14 (18)	<0.001
Dominant histological subtype				
Bronchioloalveolar	79	64 (54)	15 (19)	
Non-bronchioloalveolar	118	55 (46)	63 (81)	<0.001
Acinar	28	17 (14)	11 (14)	
Non-acinar	169	102 (86)	67 (86)	0.971
Papillary	80	34 (27)	46 (59)	
Non-papillary	117	85 (73)	32 (41)	<0.001
Solid with mucin	10	4 (3)	6 (8)	
Non-solid with mucin	187	115 (97)	72 (92)	0.176

the micropapillary pattern-positive group were significantly worse than for the micropapillary pattern-negative group ($P < 0.001$ and $P = 0.027$).

In all four dominant subtype groups, disease-free and overall survival rates for the micropapillary pattern-positive group tended to be worse than those for the micropapillary pattern-negative group (Figure 5c and d). The micropapillary pattern was therefore considered to affect on prognosis irrespective of dominant histological subtype.

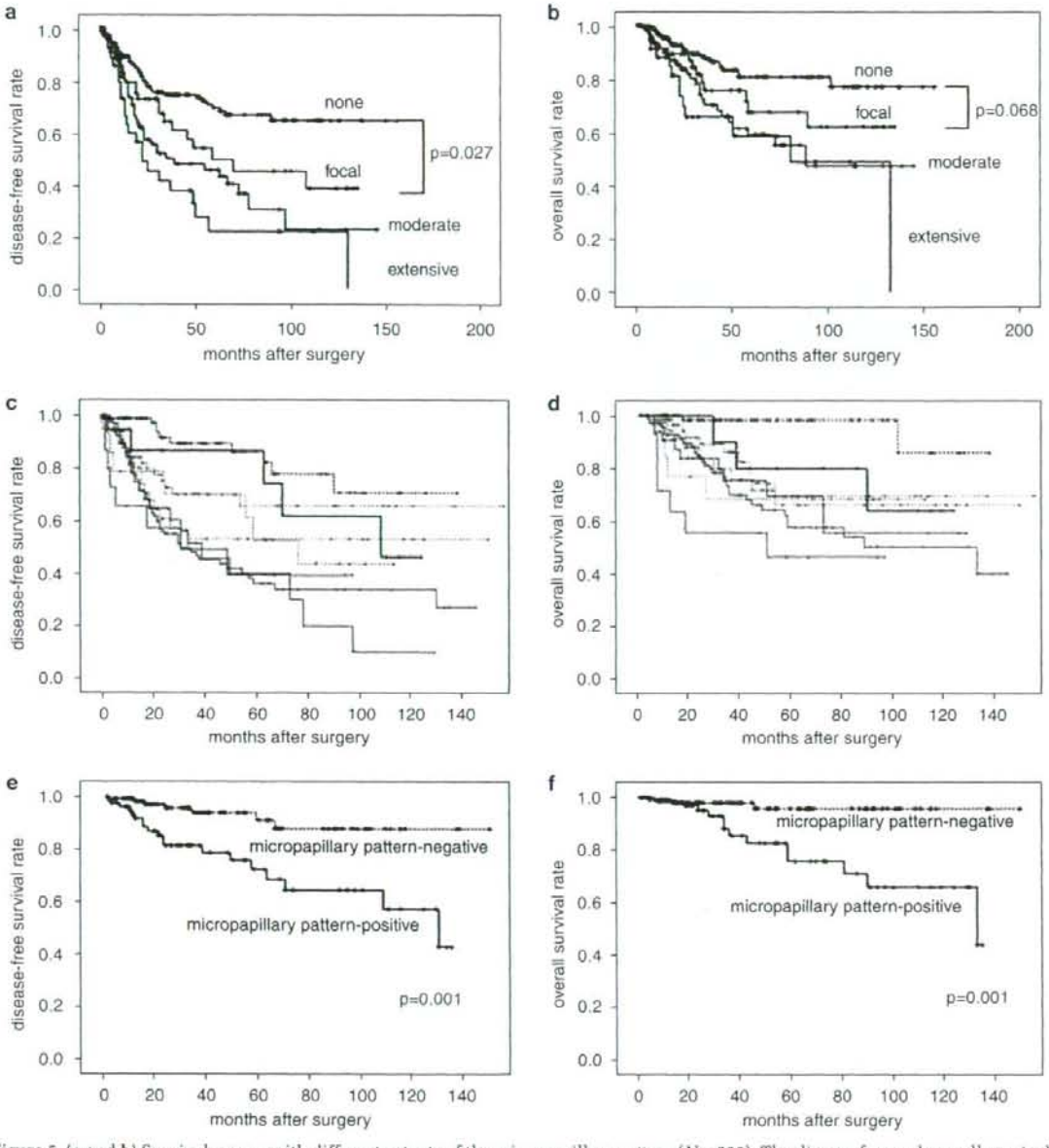

Figure 5 (**a** and **b**) Survival curves with different extents of the micropapillary pattern ($N=383$). The disease-free and overall survival of the focal group ($N=65$) were worse than those of the none group ($N=199$) ($P=0.027$ and $P=0.068$). (**c** and **d**) Survival curves grouped by the presence of the micropapillary pattern and dominant histological subtype. Black, blue, red, and green lines show dominant bronchioloalveolar carcinoma ($N=103$), acinar ($N=69$), papillary ($N=182$), and solid with mucin subtype ($N=29$), respectively. Solid and dotted lines show micropapillary pattern-positive and pattern-negative, respectively. No significant differences in survival between dominant acinar, papillary, and solid with mucin carcinomas were found. The difference between micropapillary pattern-positive and pattern-negative carcinomas was significant in the dominant papillary subtype (red) for disease-free survival ($P=0.008$) and in the dominant bronchioloalveolar carcinoma subtype (black) for overall survival ($P=0.046$). Differences were observed in other subtypes, but they were small and not significant. (**e** and **f**) The 5-year and 10-year overall survival rates of the micropapillary pattern-positive group ($N=78$) were 77.6 and 67.6%, respectively, which were significantly worse than those of the micropapillary pattern-negative group ($N=119$) (98.1 and 98.1%) in stageIA.

We then analyzed whether the micropapillary pattern had prognostic significance in the early stage when lymph node metastasis was absent at the time of surgery. At stageIA, disease-free and overall survival rates for the micropapillary pattern-positive group ($N=119$) were significantly worse than for the

micropapillary pattern-negative group ($N=78$) ($P=0.001$ and $P=0.001$) (Figure 5e and f). The 5-year and 10-year overall survival rates of the micropapillary pattern-positive group in stageIA were 77.6 and 67.6%, respectively, which were significantly worse than those of the micropapillary pattern-negative group (98.1 and 98.1%).

Discussion

On histology, micropapillary tufts were isolated from the main tumor and seemed to float in airspaces. Using serial sections, we identified an intricate structure of the micropapillary pattern in which the tufts appeared to extend into airspaces. Compared with other organs, the lung contains sufficient airspaces that cells constituting micropapillary tufts might extend easily and extensively beyond the main tumor. On histological diagnosis, it may therefore be difficult to discriminate the micropapillary pattern from intrapulmonary metastasis. However, some vascularities or stroma are normally found in intrapulmonary metastatic lesions, but not in micropapillary tufts. Both forms should be distinguished clearly. Because histopathological findings showed no vascularity in micropapillary tufts, the route of nourishment for their constitutive cells is uncertain. However, nutrients might be supplied from the surrounding epithelial lining fluids in the alveolar space.

Luna-More *et al*[13] suggested that neoplastic cells in invasive micropapillary carcinoma of the breast display reverse polarity. We explored the possibility that some of the cells constituting micropapillary tufts in lung adenocarcinomas also lost their cell polarity. Tufts composed of such cells are not micro 'papillary' but rather micro 'cluster.' Igaki *et al*[22] described that the loss of polarity in the presence of oncogenic Ras resulted in accelerated tumor invasion through the association of Ras and c-Jun N-terminal kinase pathway activation in *Drosophila*. The micropapillary pattern is believed to possess intense invasive potency considering its correlation with lymphatic, venous invasion. Loss of cell polarity might be related to this potency.

Loss of vascularity and cell–matrix contact, as well as the preservation of intercellular junctions in micropapillary tufts were demonstrated in this study. The micropapillary pattern is believed to represent the morphological piling up of neoplastic cells. Normally, when displaced from the extracellular matrix, epithelial cells undergo apoptosis (anoikis).[23] In neoplastic cells, alterations in the expression of cell–matrix adhesion molecules, integrins, integrin-associated signaling molecules, or apoptosis regulators can lead to anoikis resistance.[23–24] In the present study, we further revealed that cells constituting micropapillary tufts had proliferation potency using Ki-67 staining, and also revealed a strong correlation between the micropapillary pattern and lymphatic invasion and metastasis based on clinicopathological analysis. These cells have most likely acquired anoikis resistance and facilitated anchorage-independent growth, which are advantageous for proliferation during lymphatic cancer metastasis. The micropapillary pattern is a distinct histobiological feature that may therefore help to elucidate the mechanism of lymphatic cancer metastasis.

Our data show that survival for the focal group was worse than for the none group, and also show that as the extent of the micropapillary pattern progressed, the prognosis tended to worsen. Makimoto *et al*[21] reported no significant difference in survival between the focal (extent: $<10\%$) group and the none group, but the criteria for the extent of the micropapillary pattern and the methods used to evaluate it have been inconsistent.[20]

We clearly showed that in stageIA when lymph node metastasis was absent at the time of surgery, the 5-year and 10-year overall survival rates of the micropapillary pattern-negative group were high (98.1 and 98.1%), whereas those of the micropapillary pattern-positive group were much lower (77.6 and 67.6%). Currently, therapeutic decisions are mainly based on the TNM staging of cancer. The micropapillary pattern could be a significant prognostic factor even in the early stage.

In conclusion, the micropapillary pattern in lung adenocarcinoma is a distinct histopathological variant with biological and prognostic significance. This entity should be recognized carefully on pathological diagnosis.

Acknowledgement

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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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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*.

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Key words: liposome-encapsulated hemoglobin; hemoglobin-vesicles; hematopoietic progenitor cells; colony assay; biocompatibility

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,

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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 ± 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 ($1 \times 10^3 - 1 \times 10^4$/100 μL) were centrifuged onto slides with Cytospin (Shandon, Pittsburgh, PA) and stained with May-Grünwald-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).

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

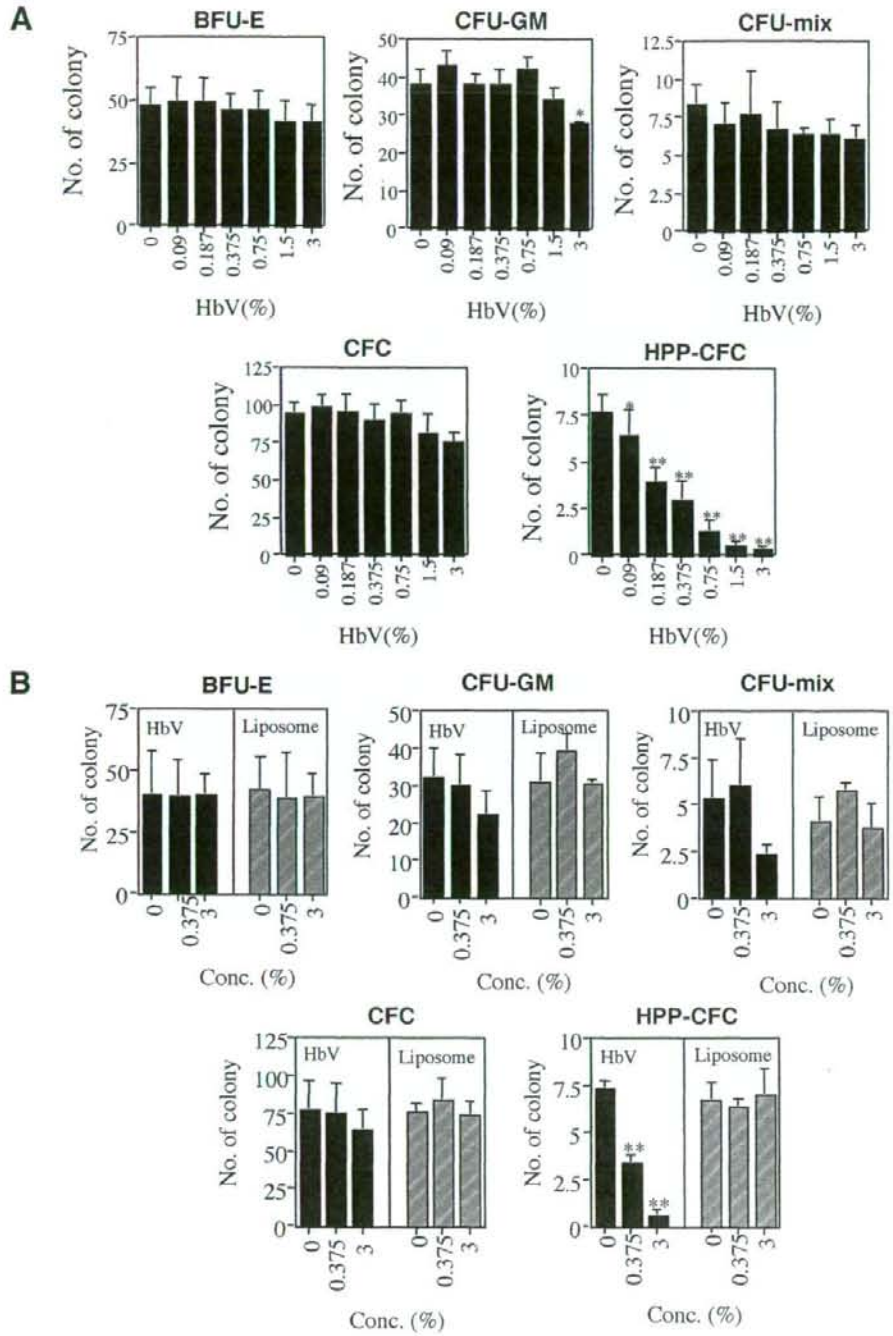

Figure 1. A: Effects of HbVs on the clonogenic activity of CB-derived hematopoietic cells. B: Effects of empty liposomes on the clonogenic activity of CB-derived hematopoietic cells. CB-derived mononuclear cells were seeded at 300 $CD34^+$ cells per dish in complete methylcellulose medium for human clonal-culture 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%).

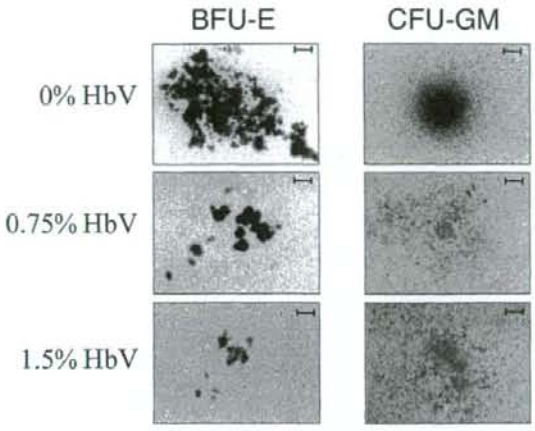

Figure 2. Effects of HbVs on the size of the colonies of BFU-E and CFU-GM formed in clonal cultures of CB-derived cells. Representative colonies of BFU-E and CFU-GM in the absence and presence of HbVs are shown. Scales represent 50 μm.

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.

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.

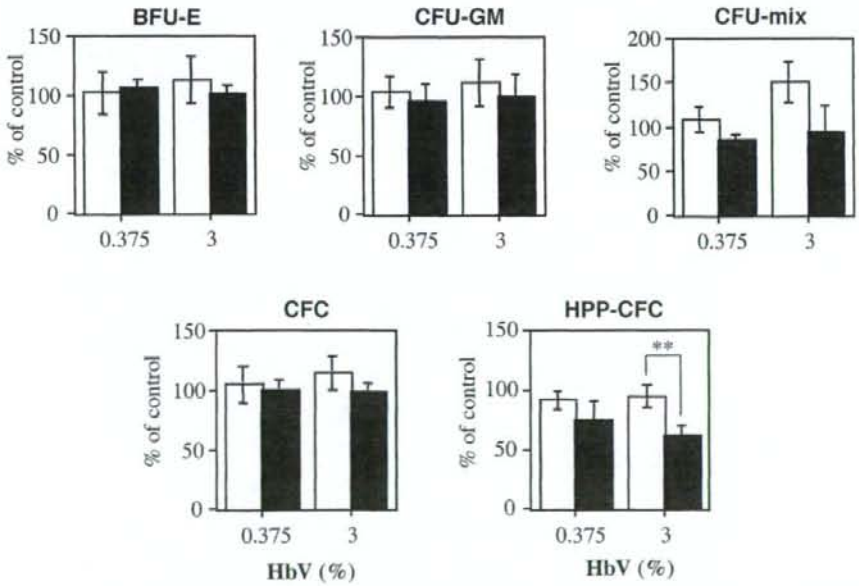

Figure 3. Effects of short-term exposure to HbVs on the clonogenic activity of CB-derived hematopoietic cells. CB-derived mononuclear cells were suspended in IMDM containing FCS, BSA, IL-3, SCF, G-CSF, and GM-CSF; then, different concentrations of HbVs were added to the cell suspension. The cells were 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.

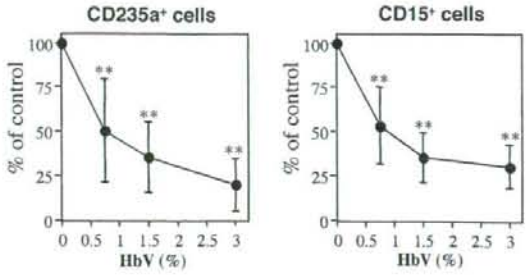

Figure 4. Effects of HbVs on the proliferation of erythroid lineage (left panel) or myeloid lineage cells (right panel) from CB-derived hematopoietic progenitor cells in liquid culture. Various concentrations of HbVs were added to medium containing the CB-derived $CD34^+$ cells. After 10 days' incubation, $CD235a^+$ 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%).

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).

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

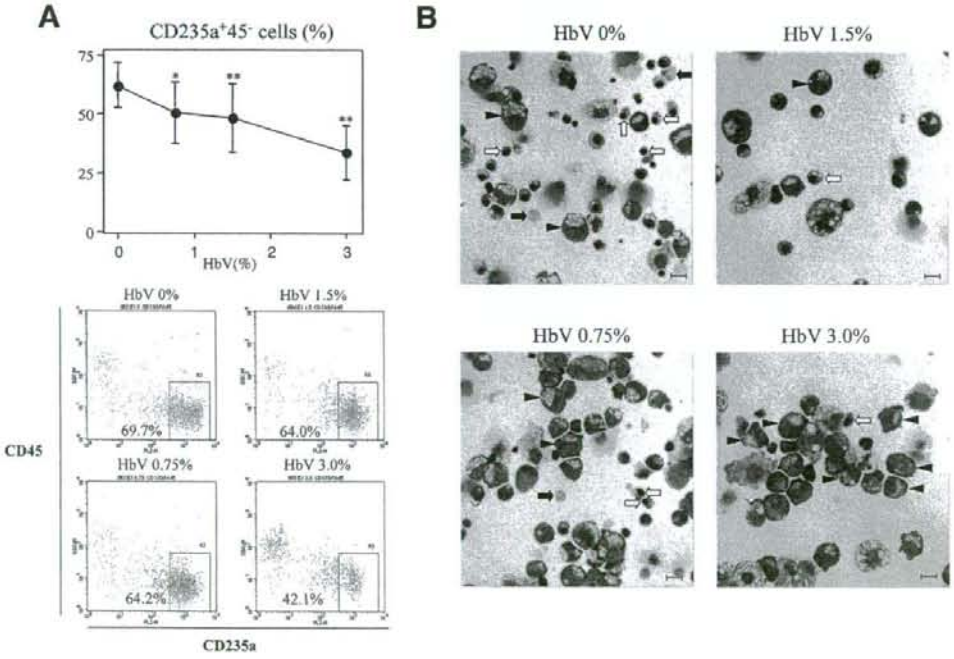

Figure 5. Effects of HbVs on the differentiation of erythroid cells from CB-derived hematopoietic progenitor cells in liquid culture. CB-derived $CD34^+$ cells were cultured in the medium for induction of erythroid lineage without or with HbVs (0.75%, 1.5%, or 3.0%). A: The percentage of $CD235a^+$ $CD45^-$ cells in the total cell population was analyzed by flow cytometry. Data represent the mean ± 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.]

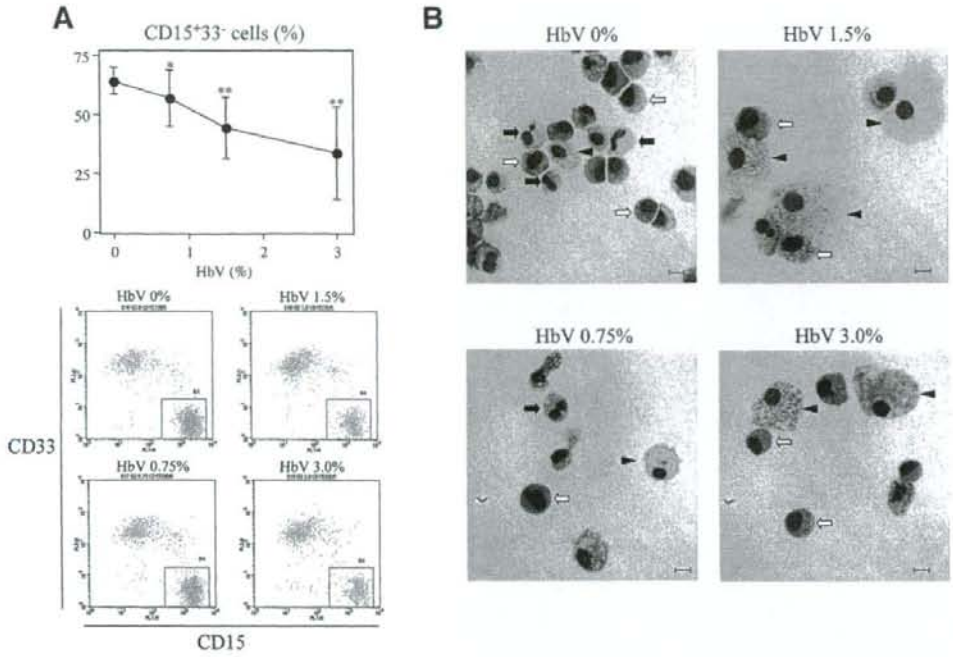

Figure 6. Effects of HbVs on the differentiation of myeloid cells from CB-derived hematopoietic progenitor cells in liquid culture. CB-derived $CD34^+$ cells were cultured in the medium for the induction of myeloid lineage without or with HbVs (0.75%, 1.5% or 3.0%). A: The percentage of $CD15^+$ $CD33^-$ cells in the total cell population was analyzed by flow cytometry. Data represent the mean ± 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.]

differentiation of both erythroid and myeloid lineage cells.

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.

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

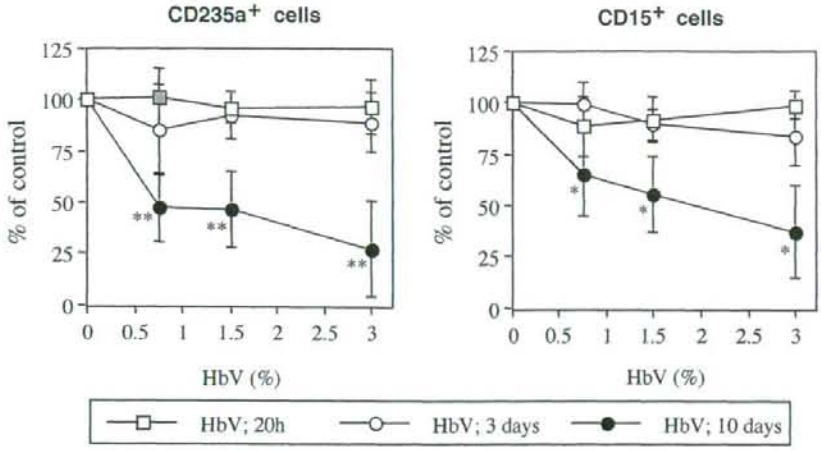

Figure 7. Effects of HbVs on the proliferation of erythroid lineage (left panels) or myeloid lineage (right panels) cells from CB-derived hematopoietic progenitor cells in liquid culture. CB-derived $CD34^+$ cells were exposed to HbVs (0%, 0.75%, 1.5%, or 3%) for 20 h, 3 days, or 10 days. After culture 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 ± 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%).

g/dL) was released into the culture supernatant. This Hb concentration was calculated as ~3 μM. According to the report by Fowler et al.,[25] 1 μM 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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総　説

ヘモグロビン小胞体のin vitroにおけるヒト血液細胞および血漿タンパクへの適合性

Biocompatibility of Hemoglobin Vesicles, a Cellular-type Artificial Oxygen Carrier, on Human Blood Cells and Plasma Proteins in Vitro

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Mitsuhiro Fujihara, Hiroshi Azuma, Hisami Ikeda

和文抄録

ヘモグロビン小胞体（hemoglobin vesicles; HbV）は，脂質二重膜からなるリポソームの中にヘモグロビンを内包させたセル型の人工酸素運搬体である．HbVはいわゆる「人工赤血球」として血管内に輸注されるため，その臨床応用に向けた安全性の評価には，生体適合性，とくに血液構成成分に対する適合性をみることが重要である．そこで我々はヒト血液細胞およびヒト血漿タンパク系を用いたin vitroの評価に焦点をあてた検討をおこなってきた．止血や炎症反応に関与する血小板に対し，HbV自体には活性化作用はみられず，またアゴニストによる活性化への影響もみられなかった．自然免疫に重要な働きをする好中球の機能（走化能，脱顆粒，活性酸素産生）の活性化に対しても，HbVは影響を与えなかった．造血機能に対しては，HbVはin vitroでの共存が3日間までの短期間であれば造血前駆細胞活性を損なうことはないことが示された．またリポソームの特性から補体の活性化が懸念されたが，現行のHbVでは活性化はみられず，さらに凝固系，カリクレイン・キニン系にも影響はみられなかった．以上のことから，HbVはヒト血液細胞ならびに血漿タンパクに対し高い適合性を持つことが示唆される．

Abstract

Hemoglobin vesicles (HbV), a cellular-type artificial oxygen carrier, are composed of human hemoglobin encapsulated within a phospholipid bilayer membrane. As HbV are injected intravenously, biocompatibility of the HbV with blood components is very important to ensure safety of this material for clinical use. We have evaluated this biocompatibility by focusing on the influence of HbV on human blood cells as well as plasma proteins in vitro. 1) As to the influences to platelets which are involved not only in the hemostasis but also in inflammation, HbV themselves did not activate platelets, and had no aberrant effect on agonist-induced platelet activation. 2) HbV did not affect on agonist-induced activation of neutrophil functions (chemotaxis, degranulation, and production of superoxide) which play important roles on innate immunity. 3) HbV had no effect on the hematopoietic progenitor activity, if the exposure period is brief. 4) The present HbV (containing DHSG) did not activate complement system, although the old-type HbV (containing DPPG, no PEG modification) did markedly. 5) The coagulation as well as kallikrein-kinin cascades were not affected by the present HbV. Thus, our in vitro studies show that HbV are highly biocompatible with human blood cells and human plasma proteins.

Keywords

artificial oxygen carrier, biocompatibility, platelets, neutrophils, hematopoietic progenitor cells, complement, coagulation, kallikrein-kinin

はじめに

赤血球製剤の人工代替物として人工酸素運搬体の開発が進められてきている．人工酸素運搬体の臨床応用における利点は，輸血時の血液型判定の必要がないこと，製剤の長期保存が可能となること，同種抗原感作や細菌感染およびウイルス感染の伝播を回避できること等があげられる[1]．これらの利点から，災

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害時の緊急事態において大量の血液製剤が必要とされる時，また血液型検査および適合した血液製剤の供給を待機する間，人工酸素運搬体を応急措置として用いることが想定される．現行の同種血輸血や自己血輸血との組み合わせにより，人工酸素運搬体は合理的な輸血システムを構築することに寄与することが期待される．

ヘモグロビン小胞体（hemoglobin vesicles; HbV）は，粒子径約250nmのリポソーム包埋型ヘモグロビンで[1]，細胞膜を模擬し人工的に作成したリン脂質／コレステロール／陰性荷電脂質／PEG脂質からなる脂質二重膜のリポソームの中にヘモグロビンを内包させたセル型の人工酸素運搬体である．HbVの人工赤血球としての酸素運搬能に関する有効性，組織分布，代謝等についてはこれまで詳細に検討されている[1]．一方，HbVの臨床応用を進めていく上では，その生体適合性を評価することが重要である．生体適合性を評価すべき項目は多岐にわたる[2]．その中で我々は，HbVが輸注された際に相互作用を起しうる血液構成成分，即ち，血液細胞および血漿タンパクに対しHbVがどのような影響をあたえるかについて焦点をあてた検討をおこなってきた．本稿においてはヒト血液細胞として血小板，好中球，造血前駆細胞を，ヒト血漿タンパクとして補体系，血液凝固系，カリクレイン-キニン系をとりあげ，HbVのin vitroにおけるそれぞれへの影響について我々の検討結果を概説し，HbVの生体適合性について言及したい．

1．ヘモグロビン小胞体の血小板活性化に対する影響

げっ歯類の動物モデルにおいては，リポソーム包埋型ヘモグロビン投与による副作用が知られ，その症状の一つとして一過性の急性血小板減少が報告されている[3-5]．同様な一過性の急性血小板減少は，陰性荷電のリポソームの投与においてもみられている[6,7]．この急性血小板減少の発生原因を解明するため，in vitroにおいてリポソームとげっ歯類の血小板との結合に関して研究がなされた結果[8]，リポソームの構成成分が負電荷の脂質である場合，ラット多血小板血漿とのインキュベーションによりリポソームは血漿中の補体成分C3bに覆われ，C3bを介したリポソーム - C3b - 血小板上CR1 receptorの結合による凝集塊が形成されることが示された．そしてin vivoにおいては，この凝集塊が速やかに網内系によって処理されることが急性血小板減少の原因と考えられている[8]．これに対しヒト血小板上にはCR1 receptorが存在しないため，リポソームと血小板との凝集塊は観察されない[8]．従ってヒトにおいてはリポソームの構成成分が負電荷の脂質であったとしても，動物モデルで観察されるリポソーム輸注後の急性血小板減少は発症しないと推測される．

一方，血小板はその顆粒中に種々の炎症性生理活性物質を含有していることや，膜表面の接着分子を介して白血球と結合することから，止血血栓の形成のみならず炎症反応にも関与していると考えられている[9,10]．HbVの投与により急性の血小板減少症が起こらないとしても，HbVが生体内で血小板を活性化したり，あるいはアゴニストによる反応を助長させたりするような影響を及ぼし，血小板由来生理活性物質の放出が起こるという事態は，生体にとって好ましいことではない．

このような背景のもと，われわれはリポソームの構成成分としてDPPG（1,2-dipalmitoyl-*sn*-glycero-3-phosphatidyl glycerol）を含有するHbVの血小板活性化への影響について，RANTESの放出反応を指標として検討した[11]．RANTESは血小板のα顆粒に存在する炎症性ケモカインで，好塩基球，好酸球，単球およびリンパ球に対する強力な走化作用を有する[9,12]．そこでヒト多血小板血漿と最大20％（vol/vol）までの濃度のHbVを予めインキュベーション後，アゴニストであるコラーゲンで刺激した反応上清中のRANTESの濃度の増加を測定した．その結果，コラーゲンの濃度に依存したRANTESの放出がみとめられたが，20％ HbVと予めインキュベーションさせた場合においては，RANTESの放出への影響はみられなかった（Table 1）．またアゴニスト刺激なしの場合においても，RANTESの放出に関して血小板とHbVを予めインキュベーションした影響はなんらみとめられないことから，HbVが血小板を活性化する作用もないと考えられた．

次に，リポソームの構成成分としてDPPGをカルボン酸脂質DHSG（1,5-*O*-dihexadecyl-*N*-succinyl-L-glutamate）に置き換えた現行のHbVの血小板への影響について，HbV濃度最大40％まで検討した[13]．血小板活性化の評価は，RANTESに加え同じくα顆粒に存在する炎症性ケモカインβ-thromboglobulin（β-TG），濃染顆粒に存在するセロトニンの放出反応，ポジティブフィードバックによって血小板活性化を増幅するThromboxane A_2（TXA_2）の産生，さらにα顆粒由来のCD62Pの膜表面への発現，および血小板膜糖タンパクαIIbβ3の構造変化を認識する抗体PAC-1の結合を指標とした．なお，TXA_2は半減期が短いためその代謝産物のThromboxane B_2（TXB_2）を測定した（Table 1）．

Table 1. Effect of HbV on human platelets .

Index	Stimulant	Type of HbV	Conc. of HbV	Effect
RANTES release	Collagen (+)	DPPG-HbV	≦ 20%	No effect
	(-)	DPPG-HbV	≦ 20%	No effect
RANTES release	Collagen (+)	DHSG-HbV	≦ 40%	No effect
	(-)	DHSG-HbV	≦ 40%	Marginal reduction
β-TG release	Collagen (+)	DHSG-HbV	≦ 40%	No effect
	(-)	DHSG-HbV	≦ 40%	No effect
Serotonin release	Collagen (+)	DHSG-HbV	≦ 40%	No effect
	(-)	DHSG-HbV	≦ 40%	No effect
TXB_2 production	Collagen (+)	DHSG-HbV	≦ 40%	No effect
	(-)	DHSG-HbV	≦ 40%	No effect
CD62 expression	ADP (+)	DHSG-HbV	≦ 40%	No effect
	(-)	DHSG-HbV	≦ 40%	No effect
PAC-1 binding	ADP (+)	DHSG-HbV	≦ 40%	Slight potentiation
	(-)	DHSG-HbV	≦ 40%	No effect

セロトニンは血小板の濃染顆粒に存在する生理活性アミンで，血管収縮作用をはじめ繊維芽細胞増殖作用，マクロファージの活性酸素産生促進作用，NK細胞活性化促進作用を有する[9]．CD62Pはセレクチンファミリーに属する接着分子で[9]，血小板

活性化に伴ってα顆粒から膜表面に分布するため，血小板活性化マーカーとして汎用されている[14]．PAC-1は血小板上のαIIbβ3（GPIIb-IIIa）が血小板活性化にともなって構造変化を起こした時に発現するエピトープを認識するモノクローナル抗体である[14,15]．低濃度のADP刺激によっても発現の増加がみられることから，高感度でかつ特異的な血小板活性化マーカーとして有用であるとされている．αIIbβ3はインテグリンファミリーに属する接着分子であり，血小板においてフィブリノーゲンやvon Willbrand因子のレセプターとして機能し，血小板凝集や粘着の重要な分子である[16]．

ヒト多血小板血漿に20%および40%の濃度のHbVを1時間予めインキュベーション後，アゴニスト刺激の無い場合は，血小板反応上清中のRANTES，セロトニン，β-TG，TXB_2および血小板表面CD62Pのレベルの上昇はみられないことから，HbVに血小板活性化作用は無いと考えられた．この際，RANTESレベルはむしろ減少したが，この程度の変化での臨床的意義は少ないと推察された．アゴニスト刺激により血小板の脱顆粒を惹起させると，いずれの濃度のHbV処理においてもRANTES，セロトニン，β-TGの放出，TXB_2の産生，およびCD62P発現が引き起こされ，そのレベルはHbV未処理のものと有意差はみられなかった（Table 1）．一方，PAC-1の発現はHbV未処理では影響がないものの，HbV処理により低濃度のADP刺激血小板において僅かな亢進がみられた．PAC-1の発現の亢進は空の小胞体処理においても観察されたため，ヘモグロビンの有無にはよらず，小胞体それ自体または小胞体の構成成分に起因する可能性が考えられた．以上のことから，HbVは低濃度のアゴニスト存在下でαIIbβ3の構造変化を僅かに促進するものの，CD62Pの膜への発現，RANTES，セロトニン，β-TGの放出，TXB_2の産生にはアゴニスト刺激の有無にかかわらず影響を与えないことから，ヒト血小板に対し高い生体適合性があると結論された（Table 1）．

2．ヘモグロビン小胞体の好中球活性化に対する影響

HbVがヒトに投与される状況として大量出血が想定される．その際に，生体防御のファーストラインを担う好中球の機能に対し，HbVが抑制的に働くならば，感染症などを合併する危険性が懸念される．循環血中の好中球は活性化を受け，炎症部位に遊走し，生体を脅かす細菌を効率的に殺菌する必要がある．好中球は，ザイモザンや細菌由来のペプチド（*N*-formyl-methionyl-leucyl-phenylalanine（fMLP））によって活性化を受け，多岐にわたる機能を発揮することが知られているが，PEG-distearoylphosphatydylethanolamine（PEG-DSPE）修飾を受けたある種のリポソームは，これらの刺激に対する好中球の反応を阻害することが報告されている[17]．逆に，フォスファチジルコリンやフォスファチジルセリンからなるリポソームが，マウスのアレルギーモデルにおいて，好中球の肺への集積を昂進させたという報告がなされている[18]．そこで，HbV自体がPEG-DSPE修飾をうけていることもあり，fMLP刺激によって好中球が発揮する機能に対し，どのような影響を及ぼすかについて検討を加えた[19]．

Table 2. Effect of DPPG-HbV on fMLP-induced neutrophil functions.

Function	Conc. of HbV	Effect
Chemotaxis	≤ 0.6%	No effect
Upregulation of CD11b expression	≤ 0.6%	No effect
Degranulation of Gelatinase B	≤ 6%	No effect
Superoxide production	≤ 6%	No effect

この検討においては，リポソームの構成成分としてDPPGを含有するHbVを用いた．fMLP刺激による好中球の機能評価として，(1) 好中球の走化能，(2) β_2インテグリンであるCD11bの膜発現，(3) 脱顆粒にともなうgelatinase B（matrix metalloproteinase-9）の放出，(4) 活性酸素の産生をとりあげた．Table 2にこれらの結果を示した．

走化性（ケモタキシス）は，細胞が化学物質の濃度勾配に従い，一定の方向に運動性を示すことであり，ヒト単離好中球を異なる濃度（最大0.6%）のHbVと37℃にて30分間予めインキュベーション（プレインキュベーション）後，ケモタキシス・チャンバー法によって評価をおこなった．fMLP（1 μM）刺激によって好中球の走化能が活性化されたが，これに対するHbVの影響はみとめられなかった．CD11bはCD18と二量体を形成して好中球の遊走能における接着因子Mac-1として機能する分子であるが，HbVとプレインキュベーションさせた好中球において，fMLP刺激によるCD11bの発現亢進は，HbVを作用させなかった好中球の場合と同等であった．gelatinase Bは，好中球の組織浸潤に関与する酵素であるが，fMLP刺激による好中球からの放出の経時変化をHbVとのプレインキュベーションの有無で比較したが，両者には違いはみとめられなかった．また好中球の殺菌能に関与する活性酸素産生についても，HbVとプレインキュベーションした好中球からのfMLPによる産生量は，HbVを作用させなかった好中球の場合と同等であった．以上の結果より，fMLP刺激によって発揮される好中球の少なくとも4つの機能において，用いたHbVの濃度ではなんら影響はみられず，HbVの好中球への高い適合性が示唆された．

PEG-DSPE修飾したリポソームがfMLP刺激による好中球の走化能活性化を阻害するという報告[17]を上述したが，リン脂質濃度にして大過剰の濃度を用いたにもかかわらず，HbVでは阻害がみられないことの理由は不明である．リポソームの構成成分であるリン脂質の種類，脂肪酸の飽和度の程度，脂肪酸の側鎖の数が，fMLPやザイモザンによる好中球の走化能に影響することが報告されている[20]．前述のPEG-DSPE修飾したリポソームとHbVとの間では，これらのパラメーターを始め，PEG-DSPEとリポソームのモル比が異なっており，これらの違いが異なる結果を得た理由となっている可能性が考えられる．

以上，HbVの好中球への高い適合性が示されたが，より高濃度即ち臨床で用いられる20%および40%での検討をみる必要性は残されている．

3．ヘモグロビン小胞体の造血前駆細胞に対する影響

血管内投与したリポソーム製剤は，漸次，肝臓のクッパー細胞，脾臓および骨髄のマクロファージなどの細網内皮系において捕捉される[21]．HbVにおいても，アイソトープラベルを用いた追跡によって組織分布が詳細に検討され，肝臓，脾臓，骨髄に主として分布していることが示されている[22]．他の組織学的検討においても，HbVは細網内皮系において捕捉されることが報告されている[23]．骨髄にHbVが移行することから，骨髄を主たる場とする造血に対し少なからず影響するのではないかと考えられた．40%脱血しHbVを輸血したラットさらにビーグル犬の場合，脱血による赤血球造血の亢進によってヘマトクリットは1週間以内には回復し，特に造血系への影響は認められていない[24,25]．さらに，20%のHbVをtoploadさせたラットの場合においても，末梢血の血球数は投与後1週間観察しても一定に保たれていた[26]．とはいえヒトへの投与を考えた場合，その造血前駆細胞の増殖・分化への影響について，in vitroの系において検討する必要があると考えられ検討をおこなった[27]．

造血のin vitroのアッセイ系としては，コロニー形成細胞の数を評価するコロニーアッセイと造血前駆細胞の増殖の度合いを評価する液体培養系を用いた．そして臍帯血から分離したCD34陽性細胞を含む単核球を，造血を支持する各種サイトカインを添加したメチルセルロースからなる半固形培地に播種し，一定期間培養後に形成したコロニーの数をカウントした．赤芽球バーストとよばれるBFU-E（Burst forming unit of erythrocyte），顆粒球・マクロファージ系コロニーとよばれるCFU-GM（Colony forming unit of granulocyte and macrophage），両者が混在したCFU-mix，これらのコロニーの総数であるColony-forming cells（CFC）は培養2週間後に，増殖能が高くコロニーのサイズが1 mm以上になったものをHPP-CFC（High proliferative potential-colony forming cell）と定義し，培養4週間後にそれぞれカウントした．用いたHbVはPEG修飾されたDHSGを構成成分とするHbVで，その濃度は最大3%と設定した[27]．

コロニーアッセイの培地にHbVを添加し，14日間共存させると，それぞれのコロニーの数およびサイズがHbVの濃度に依存して減少する傾向がみられた．さらに14日間HbVと共存後にコロニー数を測定するHPP-CFCの数は著しく減少した（Fig. 1A）．一方，HbVにCD34陽性細胞を含む単核球を20時間だけ曝してコロニーアッセイした場合は，HPP-CFCの数に影響はなく，3日間曝した場合でも，HPP-CFCの数の減少の程度は少なかった（Fig. 1B）．次に液体培地に血清と造血支持サイトカインを添加して，赤芽球系または顆粒球系の細胞へと増殖させる系にHbVを添加させ影響を評価した．10日間の培養期間，HbVが継続して存在する場合には赤芽球系または顆粒球系への細胞の増殖が抑制された．これに対し，コロニーアッセイの場合と同様に，HbVに20時間または3日間曝しただけの場合は，赤芽球系または顆粒球系への細胞の増殖は影響されなかった（Table 3）．

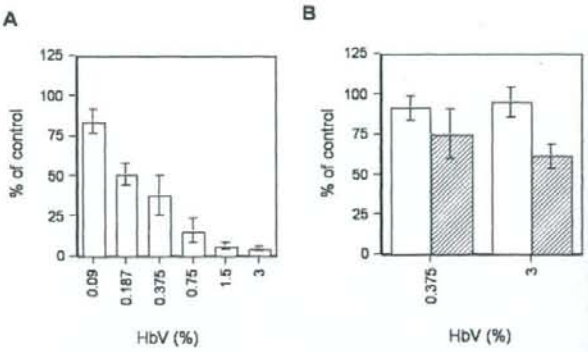

Fig. 1. Effects of HbVs on the formation of high proliferative potential colony forming cells (HPP-CFC) in the clonogenic assay of cord blood-derived hematopoietic cells. Cord blood-derived mononuclear cells were seeded at 300 at CD34$^+$ cells per dish in complete methylcellulose medium for human clonal culture assays. HPP-CFC was scored after 28 days incubation. (A) HbVs (0 to 3%) were added to the cells until the end of culture. (B) Cord blood-derived mononuclear cells were incubated for 20 h (open column) or for 3 days (slashed column) with different concentrations of HbVs in IMDM containing FCS, BSA, and cytokines. Subsequently, they were recovered, washed to remove the HbVs, and subjected to the clonal assay. Data are expressed as the mean ± SD of the percentage of control (HbV 0%) performed on three separate cord blood donors in (A) and (B).

以上HbVは，in vitroの閉鎖系において，長期間にわたり共存することにより造血前駆細胞の活性を阻害するという結果が，2つの評価系から明らかとなった．しかしながら，動物モデルでHbVを投与した場合，骨髄において1-3日にHbVの蓄積が認められ，以後1週間では激減することが報告されている[24]．

Table 3. Effect of DHSG-HbV on the proliferation of erythroid and myeloid lineage cells in liquid culture.

Exposure period to HbV	CD235a$^+$ cells			CD15$^+$ cells		
	HbV conc.(%)			HbV conc. (%)		
	0.75	1.5	3.0	0.75	1.5	3.0
20h	93.7 ± 10.0	94.9 ± 1.2	92.2 ± 8.8	100.8 ±14.3	96.3 ±7.9	96.6 ±13.3
3 days	85.2 ± 22.3	92.6 ± 11.5	89.0 ± 14.5	92.9 ± 6.1	95.8 ± 5.4	91.7 ± 4.7
10 days	47.5 ± 16.6**	46.6 ± 18.2**	27.3 ± 23.4**	65.2 ± 20.3*	55.2 ± 18.6*	37.2 ± 22.8*

Various concentrations of HbVs were added to the medium containing the cord blood-derived CD34$^+$ cells. After 10 days' incubation, CD235a$^+$ cells for erythroid lineage and CD15$^+$ cells for myeloid lineage, respectively, were analyzed by flow cytometry. The number of CD235a$^+$ cells or CD15$^+$ cells at each concentration of DHSG-HbV is expressed as a percentage of the number in the control (HbV 0%). Data are represented as the mean ± SD from three experiments performed on three separate cord blood donors. *$p < 0.05$, **$p < 0.01$ versus HbV (0%).