拍が多く見られた．Harrisonら[15]の報告によれば持続性心室頻拍が見られた患者では，右室流出路が動脈瘤化してPRとなっている傾向が見られた．Ghai[16]はファロー四徴症修復術後遠隔期死亡の患者12人を125人の生存例と比較して，遠隔期死亡した患者では持続性心室頻拍の既往，心電図でのQRS幅が180msec以上であること，中等度ないし重度のPR，中等度ないし重度の左室機能不全が見られたことを報告した．心電図でのQRS幅は右心室のサイズと密接な関係があることが知られている[17]．またKnauthら[18]は心臓MRIで測定した右心室のサイズが大きいことと修復時年齢が高いことの2つを，遠隔期死亡，重症不整脈，心不全の予見因子として挙げている．

評価法

PRはその評価法が困難である．心エコーや心臓カテーテル検査などの従来の評価法ではPRの定量的評価に技術的限界がある．これは主として右心室の形状が左心室に比較して立体的に複雑であることに起因している．一般的にはこれらの評価法を用いても中等度以上の臨床的に意味のあるPRか否かが判明できる程度である．従ってこれらの所見を基にして臨床症状でPRを評価しているのが現状である．しかし1990年代になってPRの定量的な評価が可能となりはじめ，右室メカニクスに与える影響を論じることができるようになった．心臓MRIは心室の容積，心室壁容積，機能，および逆流量を定量化することが可能であり，ファロー四徴症修復術後の患者における右室メカニクスと臨床経過の関係が明らかになってきた．まずPRの程度と右室拡張期径は密接な相関関係があることが分かった[19, 20]．また重度慢性大動脈弁閉鎖不全の際における左室機能の場合と同様に，慢性PRでも初期では右室収縮機能が温存される．この病期は数年間も持続し，多くの例では比較的無症状であることが多い．しかし，いったん代償機転が破綻すると，右室心筋容積ー心室容積比が減少して収縮末期容積が増加し，駆出率が低下する．Kurotobiら[21]は右室壁張力（後負荷）の増加，右室駆出率の低下，および症状の相関関係を示し，PRに対する手術時期に対してもより客観的な評価が可能になりつつある．

手術適応

手術適応となるPRの多くはtransannular patchを用いたファロー四徴症修復術が行われた遠隔期に生じるものである．多くの施設ではtransannular patchによるファロー四徴症修復術後数十年経過後に肺動脈弁挿入術の適応となった例は比較的まれである[22]．しかし1980年代以前に行われたものは，それ以降のものよりも術後同時期に発生するPRが多い．これは当時の修復術では右室切開が長く，右室心筋切除範囲が広く，心筋保護の精度が低く，経心房による心室中隔欠損閉鎖の頻度が少なかったことなどが影響しているものと考えられる．PRはその評価法が困難であることもあって，PRに対する手術すなわち肺動脈弁挿入術の適応をも含めた管理も議論が分かれている．

比較的一致した手術適応はPRで説明可能な心不全症状の出現である．この場合では，肺動脈弁挿入が心不全症状の改善をもたらすとの報告が多くされている[23~25]．このうちChandarら[23]はこの再手術によってNYHA心機能分類上少なくとも1度は改善することを示している．Ilbawiら[24]は2～20年後に行った49症例を報告している．この施設での適応基準は進行性の心拡大，または右心室の拡大やその機能低下の所見が得られた場合としている．そのほかにNew England Medical Center[26]やMayo Clinic[27]なども遠隔期の肺動脈弁挿入術の有効性を挙げている．

しかし患者が無症状の重度PRに関しては現

■表1　肺動脈弁閉鎖不全の手術適応

PR自体が中等度以上であり，かつ
①他に説明できない心不全症状を有するPR
②心室機能の低下（無症状であっても）
③進行性の右室拡大（無症状であっても）
④中等度以上の三尖弁閉鎖不全と合併する（無症状であっても）
⑤頻発する心房性または心室性頻拍型不整脈と合併する（無症状であっても）
など

時点では一致した手術適応がない．無症状であってもPRが長期に及ぶと心室機能[28]や運動能[29]に悪影響を及ぼすことが示されている．三尖弁閉鎖不全が合併した場合の多くはPRと相まってこれらに付加的な悪影響を及ぼすことが考えられる．また心室性または心房性頻拍型不整脈が無症状のPRに伴う場合，これらは心房や心室の容量負荷の続発症である可能性がある．一方で待機的PRの早期遠隔期成績を見ると，手術死亡率が1.1%，再手術後90%の患者がNYHA心機能分類上Ⅰ度で10年生存率が95%と報告されている[30]ように極めて良好である．従って，リスクと得られる利益のバランスから手術適応を考えた場合，無症状であることが手術適応から逸脱するものではないと考えることができる．

理論的には大動脈弁と同様に，右室系の進行性の拡大が見られた後で，かつ右室心筋の不可逆的な病理学的変化が生じる前の病期ということになるが，この最適な時期を見つけるための複合的な評価法はいまだ確立されていないのが現状である．

筆者らが考える現時点における手術適応を表1に示す．

すでに経皮的カテーテルによる弁挿入術[31]が開発されていることから，近い将来はこの方法が外科手術に取って代わる可能性がある．

3）肺動脈弁に対する術式

本疾患に対する外科的手術を歴史的に見ると，1948年Brock卿がclosed（閉鎖式）弁切開術を初めて試行して以来，人工心肺を用いて直視下に弁組織を切開する方法や，余剰弁尖組織の切除術が行われてきた．しかし，1990年代以降はバルーン肺動脈弁形成術が本病変に対する主な治療手段となっていて，孤立性PSは原則として外科手術による治療の対象ではなく，また孤立性PRは極めてまれである．従って肺動脈弁に対する外科手術の大部分は先天性心疾患に伴う一部分か，もしくはそれに対する再手術であり，その内訳は以下の3つの術式に大別することが可能である．

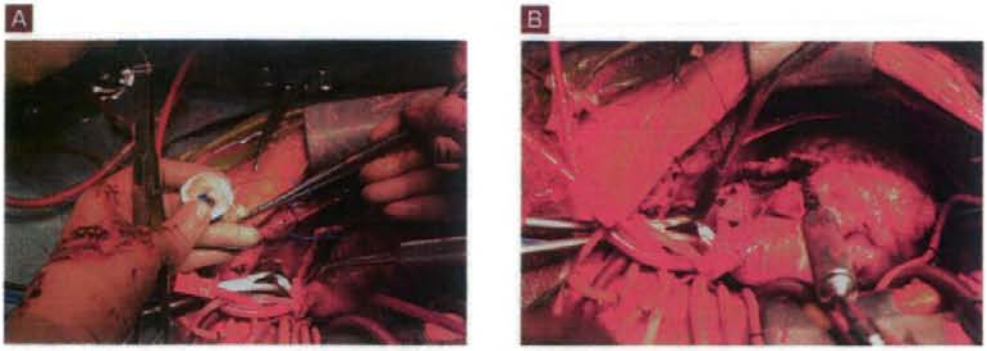
肺動脈弁置換（挿入）術

成人サイズに達した初回または再手術におけるPSまたはPRがその対象である．人工心肺を確立し，他の大動脈遮断下の心内操作の後，心拍動下に行うことが多い．

弁を挿入する位置については必ずしも本来の肺動脈弁輪にこだわる必要はなく，最も大きなサイズのものが挿入可能な位置を選択するが，peelの滑落による人工弁の閉塞を予防する意味では導管またはパッチの近位側が有利である．弁縫合輪への縫着方法に関しては特に制限はないが，筆者らは再弁置換となる可能性を考慮して人工弁を除去する際に血管内膜への損傷を最小限にするためにプレジェットなどの補強材料を使わず，3-0ポリプロピレン糸による連続縫合で行っている（図1）．

弁の種類の選択

生体弁（同種弁を含む）と機械弁の選択基準



■図1　成人ファロー四徴症の修復術における肺動脈弁挿入（左側が患者頭側）
A ステント生体弁縫合輪に3-0 ポリプロピレン糸を用いて連続縫合を始める．
B 挿入するレベルは必ずしも元の肺動脈弁輪に一致させる必要はない．この症例では肺動脈主幹部遠位の高さに挿入している．

には現在まで一定の意見はないが，生体弁の方が使用実績は圧倒的に多い．小児心臓外科医は伝統的に生体弁を用いた右室流出路再建術を行ってきたために患者が成人になった後の再手術においても同種類（生体弁）の弁を選択する傾向がある．

生体弁を肺動脈弁位に用いた術後10年までの中期遠隔期成績は生命や心事故発生に関して数多くの報告[32, 33]があり，現世代の異種生体弁の中期遠隔期の成績は同種弁のそれとほぼ同等にまで改善されてきている[34, 35]．従って遠隔期成績の差を理由として同種生体弁を積極的に選択する論理的背景は現代では消失している．

機械弁を肺動脈弁に使用する場合に考慮すべき点を以下に述べる．肺動脈弁は肺動脈圧が正常もしくは中等度までの高血圧であるほとんどの場合，開放閉鎖に導く圧較差が，大動脈弁や僧帽弁はもちろんのこと三尖弁に比較しても少なくなるので血栓弁になりやすい．従ってこの論点からは，他の位置の機械弁置換以上に厳重な（INR値が高値での）抗凝固療法が求められる．このように適切な抗凝固療法を行ったとしても生涯にわたって血栓塞栓症と同時に出血性合併症のリスクがある．肺動脈弁位に機械弁が用いられた後の血栓弁の発生も多く報告されている．ただしこれらのシリーズの中には，抗凝固療法が行われていなかったものや不適切であったものが含まれている．しかし一方では規模の小さい血栓塞栓症は直接肺動脈の末梢に向かうので，僧帽弁または大動脈弁位に比較すると耐性は高い．挙児希望の若年女性では特にワルファリンによる抗凝固療法を避けることは重要である．また生体弁の遠隔期における交換のための再手術または再々手術のリスクは低い．

出血性合併症のリスクも累積的である．成人に達している先天性心疾患の患者の大部分は若年者であるので，これらの患者年齢層を弁選択の際の考慮に入れるべきである．

生体弁の場合と同様に経過観察中の心エコーによる定期的なチェックは必要であり，その中で弁周囲のパンヌス形成や右室肺動脈導管が用いられている場合にはその内側のpeel形成を観察し，これらが閉塞を起こす前に再手術をする必要がある．

以上の論点を考慮に入れると，機械弁を選択する場合は，将来の再手術の際に再開胸操作によりlacerationが生じる危険が高い解剖的な心大血管の位置関係があり，かつ機械弁を用いることによって確実にもしくはほぼ確実に再手術が不要となるような場合，心房細動など他の理

由によりワルファリン療法を要する場合などの特殊なケースに限定される．

将来は現在研究が進みつつあるtissue-engineeringによって作成された弁を用いることが予想される．

弁性PS解除術

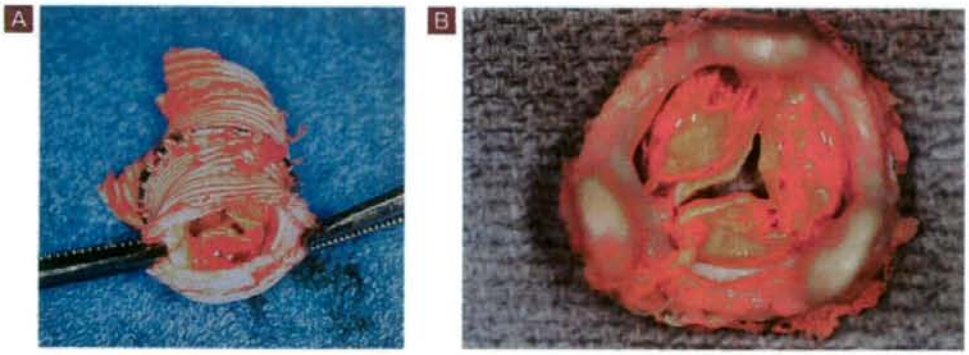
PS解除は，初回または再手術において他の先天性心疾患との合併症として存在するPSが手術の対象となる．弁置換術のときと同様に人工心肺を確立し，他の大動脈遮断下の心内操作の後，心拍動下に行うことが多い．

弁尖癒合によるPSの場合には弁の肥厚変性が中等度以下でかつ肺動脈弁交連の癒着が見られれば交連切開術を行う．大動脈弁と異なり，軽度のPRは許容されるので，弁輪（肺動脈壁）内に及ぶまで深く切開を入れる．

肺動脈弁輪狭窄例では肺動脈弁輪を切開し，弁付きパッチを用いて弁輪を拡大するtrans-annular patch術式となる．まず弁輪の遠位側または近位側に小切開を置いて肺動脈弁交連部の位置を確認する．二尖弁の形態を取ることがしばしば見られ，その場合交連部が前後である場合と左右である場合がある．可動性が豊富な弁尖がある場合には温存することが望ましい．この場合には温存した弁尖とcoaptationがマッチする位置にGore-tex®などの1弁を取り付ける．自己肺動脈弁が可動性に乏しい場合にはこれを切除し，1弁付きパッチはその反対側の肺動脈壁と十分なcoaptationを取るようなサイズと形状にデザインする．筆者らは弁を取り付ける位置の肺動脈切開線の2点間の肺動脈壁に沿う距離を糸で測定して，扇状に1弁を作成している．

心外導管の交換術

小児期に行われた右室肺動脈心外導管における，患者成長に伴う相対的な導管のoutgrowing，導管内に挿入した生体弁の石灰化に伴う可動制限，導管内側面に付着するpeelの蓄積またはその滑落（図2），導管の感染などが交換の適応である．前回の手術で用いられた右室流出路再建術式でパッチや心外導管の胸骨胸壁への癒着度合いをCTなどの画像検査で評価して，開胸操作中に心大血管壁のlacerationが生じるリスクが高いケースでは送血部位を大腿動脈や腋窩動脈に，また脱血部位を大腿静脈からEdwards Lifesciences社製research medical FEM Ⅱカニューラなど多孔性静脈カニューラで確保して，



■図2　摘出された心外導管

生体弁内挿のダクロン®人工血管を用いた右室肺動脈心外導管修復術後の心外導管交換術の際に摘出された心外導管．生体弁の高度石灰化（A）と人工血管内面と外面に形成されたpeel（B）が認められる．

lacerationの際にはfull flowによる人工心肺運転が可能となるように準備してから，開胸操作を行う．

ほかに心内操作がない場合には大動脈遮断をせずに行うことが多い．術前の画像検査で心室レベルでの遺残短絡が認識されていない場合でも，高い右室圧によってマスクされている可能性を考慮すべきである．まず二酸化炭素ガスを術野に充満させておき，万一の際にガス塞栓症を生じるリスクを最小限にする．左心系への空気（ガス）混入に対してはTrendelenburg体位を取り，大動脈基部に挿入したカニューラを用いて持続的な空気抜きを行い，心室rapid pacingで心室の空気（ガス）の引き込みを最小限とする工夫を行う．

原則的には前回手術で挿入されている心外導管を全切除する．切除した後の後壁は通常導管の外側に厚いpeel自己線維組織で形成されている．このpeelからなる導管床をそのまま新しい後壁として利用し，人工血管から舟形に切り出したパッフルを前壁（屋根）にして縫着する[36]．この方法の成績は良好で，再導管交換術回避率は非常に高い[37]．前回使用されている心外導管の材質がダクロン®の場合は導管床の形成が顕著であり，この方法が容易に行える．材質がホモグラフトなどの生体材料やPTFE（polytetrafluoroethylene）の場合にはしばしば導管床の形成が乏しいので，この組織を用いて縫合することが困難なことが多く，新しい導管を導管の形状のままで遠位側，近位側それぞれに縫合する．

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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*. © 2008 Wiley Periodicals, Inc. J Biomed Mater Res 88A: 34–42, 2009

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; Stem-Cell 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

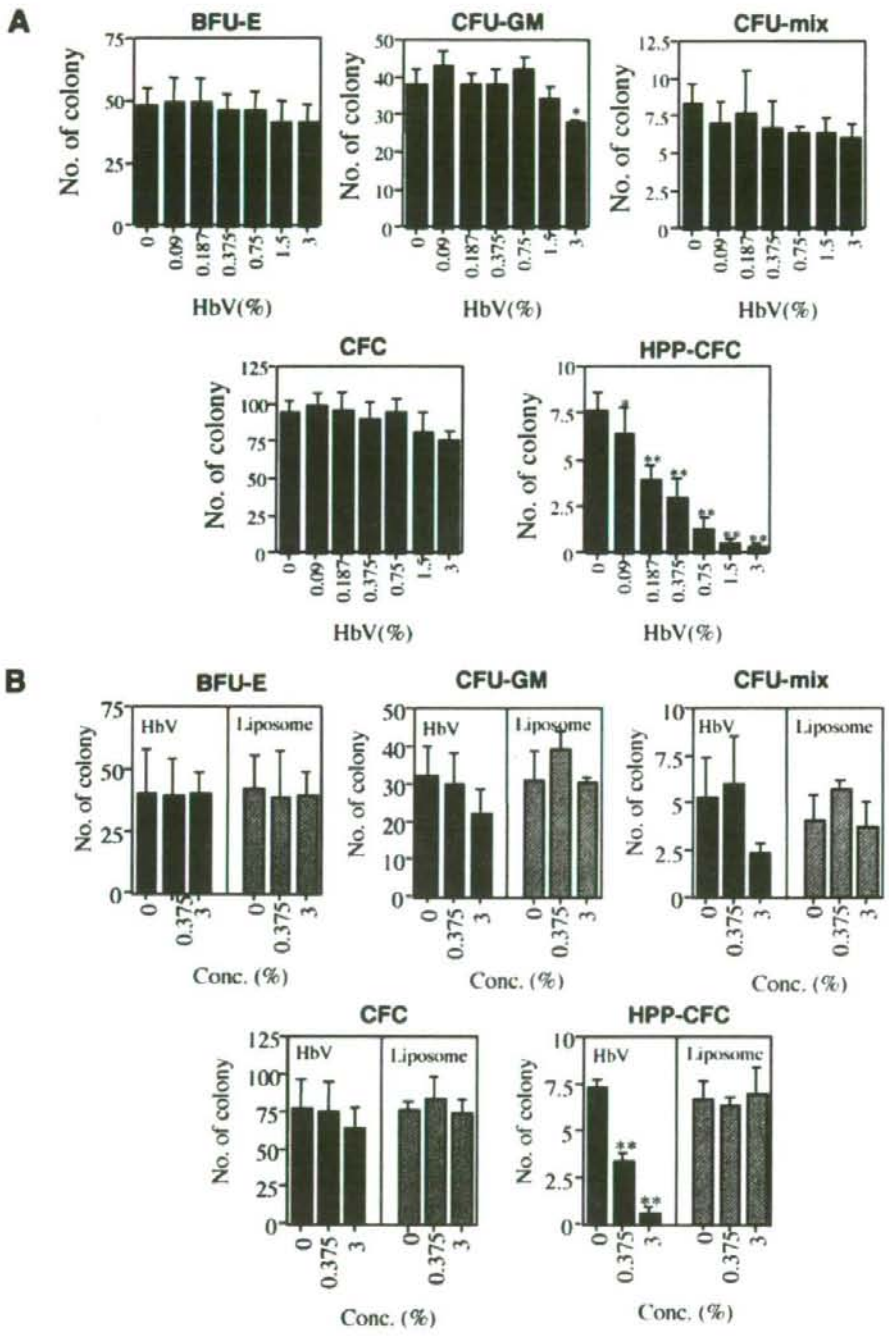

Figure 1. A: Effects of HbVs on the clonogenic activity of CB-derived hematopoietic cells. B: Effects of empty liposomes on the clonogenic activity of CB-derived hematopoietic cells. CB-derived mononuclear cells were seeded at 300 $CD34^+$ cells per dish in complete methylcellulose medium for human clonal-culture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

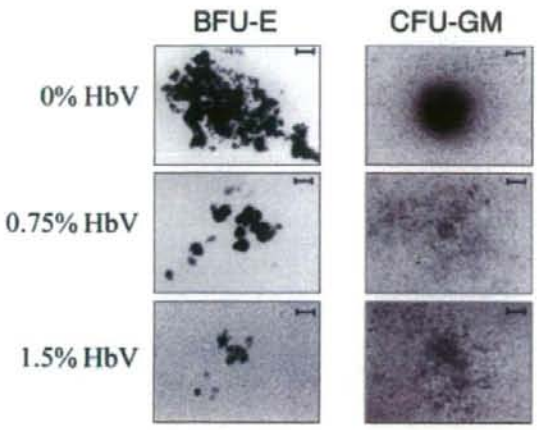

Figure 2. Effects of HbVs on the size of the colonies of BFU-E and CFU-GM formed in clonal cultures of CB-derived cells. Representative colonies of BFU-E and CFU-GM in the absence and presence of HbVs are shown. Scales represent 50 μm.

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.

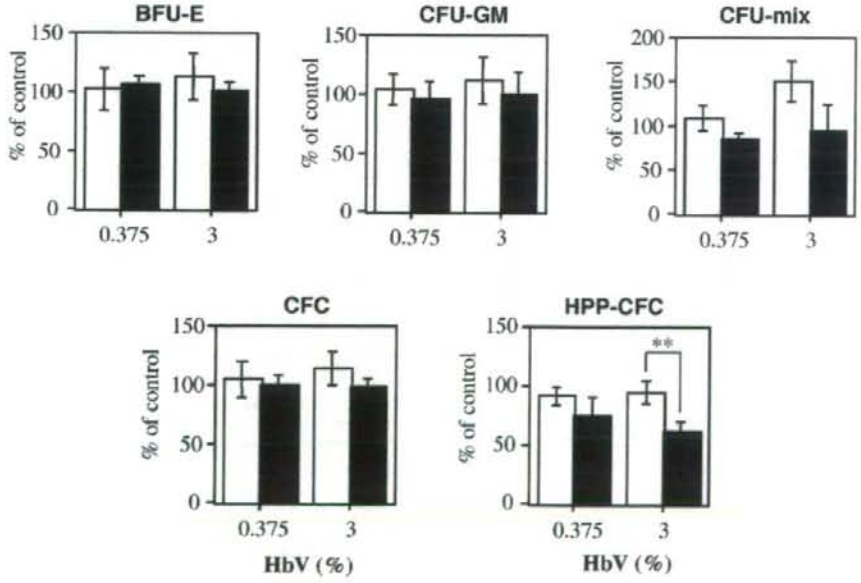

Figure 3. Effects of short-term exposure to HbVs on the clonogenic activity of CB-derived hematopoietic cells. CB-derived mononuclear cells were suspended in IMDM containing FCS, BSA, IL-3, SCF, G-CSF, and GM-CSF; then, different concentrations of HbVs were added to the cell suspension. The cells were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

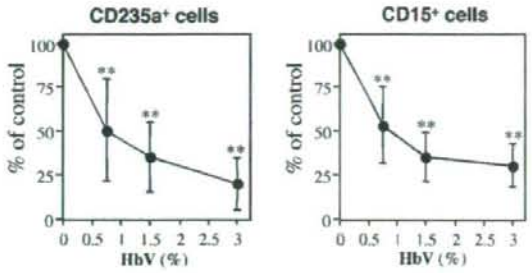

Figure 4. Effects of HbVs on the proliferation of erythroid lineage (left panel) or myeloid lineage cells (right panel) from CB-derived hematopoietic progenitor cells in liquid culture. Various concentrations of HbVs were added to medium containing the CB-derived $CD34^+$ cells. After 10 days' incubation,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

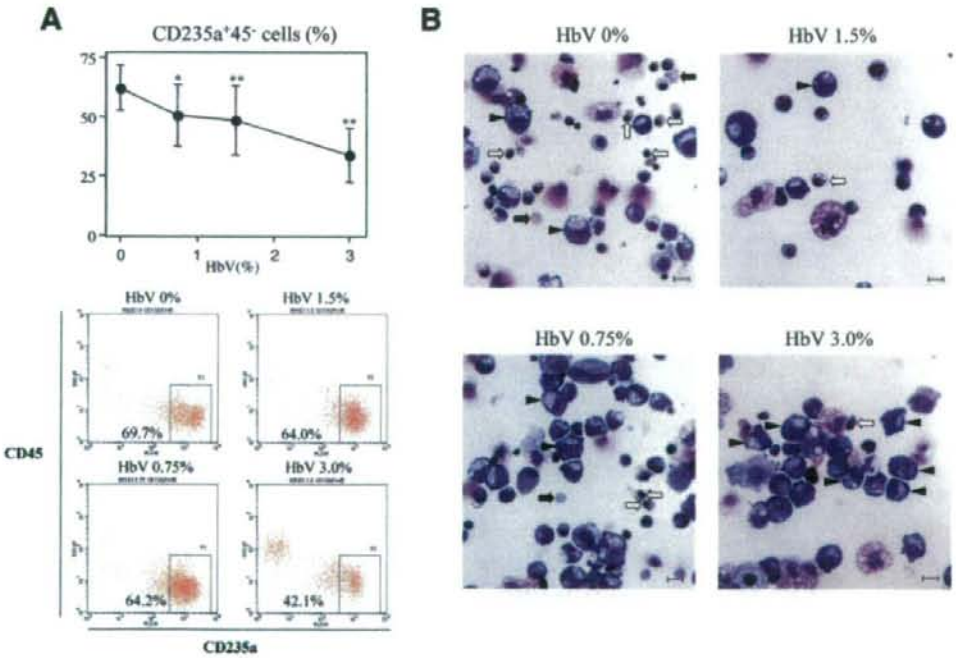

Figure 5. Effects of HbVs on the differentiation of erythroid cells from CB-derived hematopoietic progenitor cells in liquid culture. CB-derived $CD34^+$ cells were cultured in the medium for induction of erythroid lineage without or with HbVs (0.75%, 1.5%, or 3.0%). A: The percentage of $CD235a^+$ $CD45^-$ 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.]

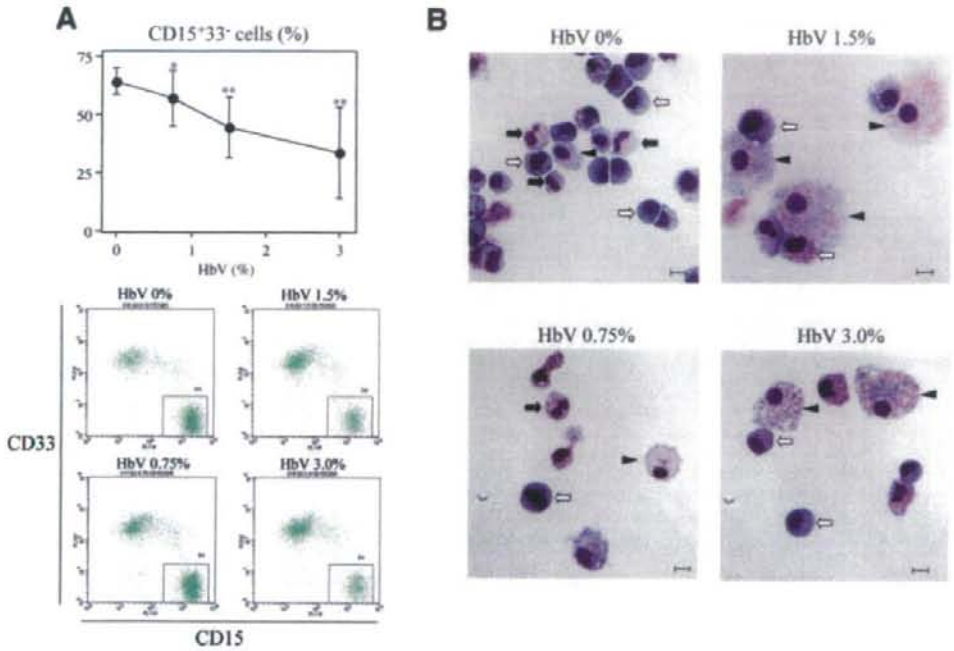

Figure 6. Effects of HbVs on the differentiation of myeloid cells from CB-derived hematopoietic progenitor cells in liquid culture. CB-derived $CD34^+$ cells were cultured in the medium for the induction of myeloid lineage without or with HbVs (0.75%, 1.5% or 3.0%). A: The percentage of $CD15^+$ $CD33^-$ 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

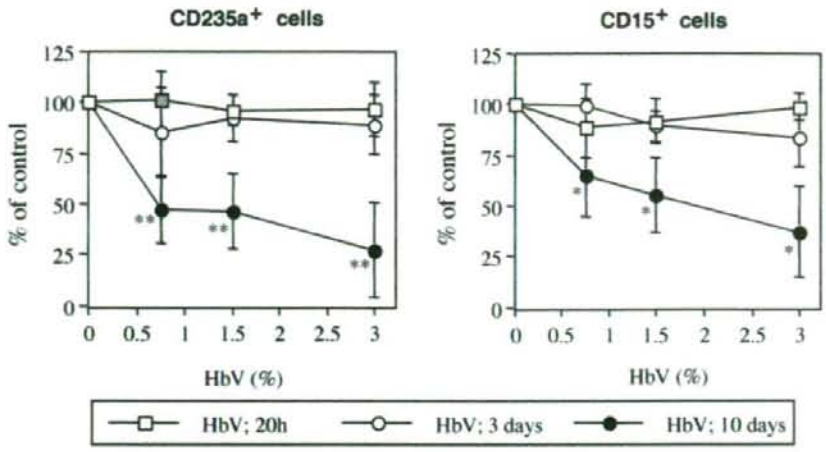

Figure 7. Effects of HbVs on the proliferation of erythroid lineage (left panels) or myeloid lineage (right panels) cells from CB-derived hematopoietic progenitor cells in liquid culture. CB-derived $CD34^+$ cells were exposed to HbVs (0%, 0.75%, 1.5%, or 3%) for 20 h, 3 days, or 10 days. After culture 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 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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Biocompatibility Study of Hemoglobin Vesicles, Cellular-Type Artificial Oxygen Carriers, with Human Umbilical Cord Hematopoietic Stem/Progenitor Cells Using an *In Vitro* Expansion System

Miki Yamaguchi,* Mitsuhiro Fujihara,* Shinobu Wakamoto,* Hiromi Sakai,† Shinji Takeoka,‡ Eishun Tsuchida,† Hirofumi Hamada,§ Hiroshi Azuma,* and Hisami Ikeda*

Hemoglobin vesicles (HbVs), liposomal oxygen carriers containing human hemoglobin, are candidates for development of a clinically useful transfusion alternative. Our previous *in vivo* animal studies of massive HbV dosages demonstrated the lack of any suppressive effect on hematopoiesis. Before starting clinical trials, we aimed to examine the details of the biocompatibility of HbVs with human hematopoietic stem/progenitor cells. We investigated the effect of HbVs at a concentration of up to 3 vol/vol (%) on expansion of human umbilical cord (CB) hematopoietic stem/progenitor cells, using a coculture system of human TERT-transfected bone marrow stromal cells and $CD34^+$ cells *in vitro*. The exposure of CB $CD34^+$ cells to HbVs up to 14 days suppressed the expansion of total cells and the $CD34^+$ cells themselves, whereas the empty liposomes, that did not contain Hb, had modestly inhibitory effects on the expansion of these cells. As a result, the number of colonies obtained from the expanded $CD34^+$ cells was inhibited by the exposure to HbVs. In contrast, exposure to HbVs for 3 days had no effect on the expansion of $CD34^+$ cells and only slightly decreased the number of total cells. Our *in vitro* experimental condition does not fully recreate the physiological condition, and the effect of the direct contact of HbV would be magnified because of the absence of shielding by the vasculature and the lack of the reticuloendothelial system and blood stream. However, the present data raise some concern regarding hematopoiesis, and one has to pay attention to this in future human clinical trials. *ASAIO Journal* 2009; 55:000–000.

Hemoglobin vesicles (HbVs) or liposome-encapsulated Hbs comprise human hemoglobin encapsulated with a phospholipid bilayer membrane and have been developed as an artificial oxygen carrier.[1–3] Hemoglobin vesicles are promising candidates for clinically useful transfusion alternatives, because several lines of evidence have demonstrated that HbVs transport oxygen as efficiently as red blood cells.[4–7] The distribution study using infusion of technetium-99m-labeled HbVs in animals demonstrated that the HbVs were mainly distributed in the liver, spleen, and bone marrow.[8] Because bone marrow is the major site of hematopoiesis, there has been concern whether the HbVs which have accumulated in the bone marrow might adversely affect hematopoiesis.

Hematopoietic stem cells (HSCs) are generally defined as cells having the self-renewing potential and the capacity to give rise to differentiated cells of all hematopoietic lineages.[9] Colony-forming assays, as *in vitro* models of hematopoiesis, have been widely used to investigate the proliferation and differentiation of both pluripotent HSCs and of the different progenitor cells of blood cell lineages, and were utilized for screening for the safety of the compound in preclinical testing.[10,11] In addition, liquid culture system in a certain combination of hematopoietic cytokines allows hematopoietic progenitor cells to proliferate to single lineage cells such as erythroid and myeloid cells.[12,13] Using these systems, we have recently investigated the influence of HbVs on *in vitro* hematopoiesis of human hematopoietic progenitor cells obtained from umbilical cord blood (CB), and have demonstrated that longer exposure of CB progenitor cells to HbVs negatively affected progenitor activity, whereas such an effect was minimal under shorter exposure to HbVs.[14]

However, the expansion of HSCs in culture while maintaining their functions has been extensively carried out.[15–17] Because contact between HSCs and stromal cells in bone marrow is important for maintaining the function of HSCs, several methods that support expansion of HSCs and progenitor cells derived from human bone marrow or CB using cocultures with primary stromal cells have been reported.[18–20] Our group also achieved the expansion of hematopoietic stem/progenitor cells from CB using a coculture system with human telomerase catalytic subunit (hTERT)-transfected human bone marrow stromal cells.[21] In the study, we also demonstrated that the expanded $CD34^+$ cells contained transplantable stem cells through a transplantation experiment using nonobese/severe combined immunodeficiency mice.[21] Thus, in the present study, we sought to evaluate whether HbVs had any adverse

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effect on the expansion of human CB stem/progenitor cells using this coculture system.

Materials and Methods

HbVs

Hemoglobin vesicles were prepared under sterile conditions in a similar manner as previously described.[22,23] In brief, 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 mM pyridoxal 5'-phosphate (PLP) at a molar ratio of [PLP]/[Hb] = 2.5 as an allosteric effector. 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 Co. Ltd, Osaka, Japan), and 1,2-distearoyl-sn-glycero-3-phosphatidylethanol-amine-N-[poly(ethylene glycol) (5,000)] (NOF Corp., Tokyo, Japan), at a molar ratio of 5:5:1:0.033. The physicochemical parameters were P_{50}, 27 Torr; 262 ± 77 nm particle diameter; and <3% MetHb content. 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 reticuloendothelial system, including the spleen, liver, and bone marrow. The circulation half-life of HbVs in the elimination phase in humans has been estimated to be approximately 66 h by the study of circulation kinetics using rats and rabbits,[8] and the percentage of the 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.[8] 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 could 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).[24] From these values, the amount of HbVs in the human bone marrow can be calculated to be about 2 to 3 vol/vol%.

Preparation of Human CB

Experiments using human umbilical CB were approved by the Committee of Hokkaido CB Bank. Cord blood was obtained during normal full-term deliveries. Cord blood $CD34^+$ cells were prepared as described previously.[25] Briefly, after the sedimentation of red blood cells by incubating CB samples with the same volume of 6% (w/v) hydroxyethyl starch dissolved in Ringer's solution (Veen-D Inj., Nikken Chemical, Tokyo, Japan) at room temperature for 30 min, low-density (<1.077 g/ml) mononuclear cells were collected with Ficoll-Paque (Pharmacia biotech, Uppsala, Sweden). The cells were then enriched further with $CD34^+$ cells using a MACS CD34 Progenitor Isolation Kit (Milenyi Biotec, bergish-Gladbach, Germany) according to the manufacturer's instructions. The purity of $CD34^+$ cells was >85%.

Coculture of $CD34^+$ Cells and hTERT-Transfected Stromal Cells

The coculture of $CD34^+$ cells and hTERT-transformed cells was carried out as previously described.[25] Briefly, hTERT-transfected human stromal cells were cultured in 25 cm^2 flasks at 37°C in 5% CO_2 in a humidified atmosphere using a culture medium comprising Iscove's modified Dulbecco's medium (IMDM, Invitrogen, Carlsbad, Ca), 12.5% FCS (Equitech Bio Inc., Igram, TX), 12.5% horse serum [Invitrogen], 1 μM hydrocortisone (BSA; Sigma Chemical, St Louis, MO), and 50 μM β-mercaptoethanol. The cells were cultured until confluent. On the day of the coculture, the stromal cells were washed several times with phosphate buffered saline before the addition of CB $CD34^+$ cells. Some 5,000 CB $CD34^+$ cells were seeded on a monolayer of hTERT-stromal cells that had been preestablished in 4 ml of serum-free medium, X-VIVO 10 (BioWhittaker, Walkersville, MD), supplemented with 50 ng/ml human thrombopoietin (TPO: a gift from Kirin Brewery Co LTD, Tokyo, Japan), 10 ng/ml stem cell factor (SCF), and 100 ng/ml human Flt-ligand (FL: a gift from Immunex Corporation, Seattle, WA), in the presence of HbVs (final concentration, up to 3%). After 1 week of coculture, 4 ml of fresh complete medium containing these same concentrations of cytokines without HbVs were added, and the coculture was continued for 1 week. At this point, each concentration of HbVs was halved. At the end of the second week, both expanded hematopoietic cells that had not attached to the stromal cells and those that had attached weakly to them were collected by gentle pipetting. Cells attached strongly to stromal cells were also recovered by treatment with ethylenediamine-tetra-acetic acid and trypsin after the removal of nonadherent cells. Stromal cells were removed by culturing in a tissue culture flask for 1 h. A mixture of the hematopoietic cells was washed to remove HbVs and the population of $CD34^+$ cells was determined with a flow cytometer. Subsequently, expanded cells (300 $CD34^+$cells/dish) were subjected to a clonal assay.

In the experiment for examining the effect of the exposure period to HbVs, HbVs (0%, 1.5%, or 3%) were added to the coculture for either 3 days or 7 days, at the end of which both hematopoietic cells that had not attached to the stromal cells and those that had attached weakly to them were collected by gentle pipetting, washed by repeated centrifugation to remove HbVs, and resuspended in complete culture medium without HbVs. The monolayers of h-TERT stromal cells were washed with phosphate-buffered saline twice to remove the residual HbVs, and were cultured with the resuspended hematopoietic cells as described above up to a total of 14 days.

Clonal Cell Culture

The methylcellulose clonal culture was carried out as described previously, except for absence of HbVs. The population of $CD34^+$ cells among the mononuclear cells was determined by flow cytometry, and CB-derived mononuclear cells were seeded at 300 $CD34^+$ cells/dish. Burst-forming units of erythrocyte (BFU-E), colony-forming units of granulocytes/macrophages (CFU-GM), CFU-mix, and colony forming units in culture (CFC) were scored with an inverted microscope after 14 days of incubation. Densely packed colonies that reached >1 mm in size were scored as high-proliferative potential colonies (HPP-CFC) after 28 days of incubation.

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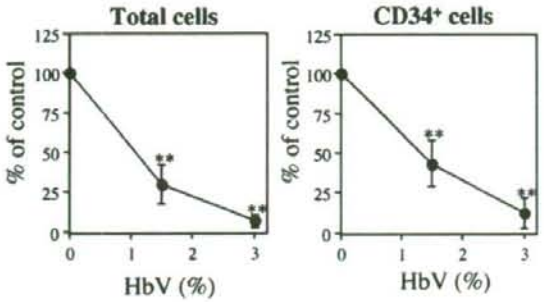

Figure 1. Effects of hemoglobin vesicles (HbVs) on expansion of stem/progenitor cells *in vitro*. Five thousand CD34$^+$ cells derived from (cord blood) CB were cultured on a monolayer of h-TERT-transfected human stromal cells in the presence of thrombopoietin (TPO), stem cell factor (SCF), and Flt-ligand (FL). Various concentrations of HbVs were added to the coculture system. Two weeks later, the total number of expanded cells, and the number of expanded CD34$^+$ cells were determined. Data represent the mean ± SD of three experiments performed on three separate CB donors. *$p < 0.01$ versus HbVs (0%).

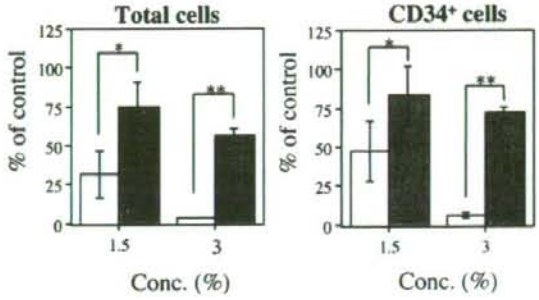

Figure 2. Effects of empty liposomes on the expansion of stem/progenitor cells. Experimental conditions were the same as those of Figure 1 except that either 3% (vol/vol) empty liposomes (closed column) or hemoglobin vesicles (HbVs) (open column) were added to the coculture for 14 d. *$p < 0.05$, **$p < 0.01$, empty liposomes vs HbVs; paired Student's t test.

Statistical Analysis

Results are expressed as the mean ± standard deviation (SD). A two-way nonrepeated analysis of variance followed by Bonferroni's test was used for multiple comparisons between the control (HbVs; 0%) group and a treatment group. For analysis of the difference between HbVs and empty liposomes, paired t test was used. Values of $p < 0.05$ were considered statistically significant.

Results

Effects of HbVs on Expansion of Hematopoietic Stem/Progenitor Cells

The coculture of hematopoietic stem/progenitor cells with bone marrow-derived stromal cells mimics the physiological hematopoiesis in bone marrow more closely than does a liquid culture. CD34$^+$ cells were cocultured for 2 weeks with h-TERT stromal cells in the presence of different concentrations of HbVs. The total cell numbers obtained without HbVs were $1.28 \times 10^6 \pm 2.7 \times 10^5$, showing an approximately 260-fold increase of cells. The continuous presence of HbVs at 1.5% for 1 week and then at 0.75% for an additional week, caused a significant reduction in the proliferation of cells. Furthermore, the presence of HbVs at 3% for 1 week and then at 1.5% for an additional week, caused a marked reduction (**Figure 1**). The CD34$^+$ cells obtained without HbVs were $9.3 \times 10^4 \pm 3.9 \times 10^4$, showing an approximately 20-fold expansion. Again, the presence of HbVs for 14 days dose-dependently inhibited the expansion of CD34$^+$ cells (**Figure 1**).

A colony assay of the CD34$^+$ cells expanded with or without HbVs was carried out (**Table 1**). The number of CD34$^+$ cells expanded in the presence of 3% HbVs was 17.7% of those without HbVs. Similarly, the numbers of BFU-E, CFU-GM, CFU-mix, CFC, and primitive HPP-CFC obtained from the CD34$^+$ cells expanded in the presence of HbVs were 49.9%, 15.8%, 18.7%, 18.2%, and 12.3%, respectively, of those without HbVs (**Table 1**).

Effects of Empty Liposomes on the Expansion of Hematopoietic Stem/Progenitor Cells

We then tested the question whether the inhibitory effect of HbVs on the expansion of total cells as well as CD34$^+$ cells themselves was due to either the liposomes themselves or Hb in HbVs. To do this, CD34$^+$ cells were cocultured with h-TERT stromal cells in the presence of either HbVs or empty liposomes for 14 days. As shown in **Figure 2**, the 14-day exposure of CD34$^+$ cells to empty liposomes reduced expansion of the

Table 1. Clonogenic Assay of Expanded Cells

HbV	CD34$^+$ Cells	BFU-E	CFU-GM	CFU-Mix	CFC	HPP-CFC
0%	$9.6 \times 10^4 \pm 6.4 \times 10^3$	1370 ± 1150	15500 ± 2570	1270 ± 1360	18240 ± 3440	2530 ± 1260
3%	$1.7 \times 10^4 \pm 9.5 \times 10^3$ (17.7%)	683 ± 76 (49.9%)	2450 ± 700 (15.8%)	237 ± 70 (18.7%)	3320 ± 715 (18.2%)	310 ± 90 (12.3%)

Five thousand CD34$^+$ cells derived from CB were cultured without or with HbV as shown in the legend of Figure 1. After 14 d of coculture, the total hematopoietic cells were counted, and the population of CD34$^+$ cells was determined with flow cytometry. Subsequently, expanded hematopoietic mononuclear cells were seeded at 300 CD34$^+$ cells/dish. The numbers of colonies shown in the Table were calculated by the following equation: (the numbers of colony formed) × (total CD34$^+$ cells)/300.

Parentheses show the percentage of the control (HbV 0%).

Data represent the mean ± SD of 3 experiments performed on 3 separate CB donors.

HbV, Hemoglobin vesicles; BFU-E, burst-forming units of erythrocyte; CFU-GM, colony-forming units of granulocytes/macrophages; CFC, colony forming units in culture; HPP-CFC, high-proliferative potential colonies.

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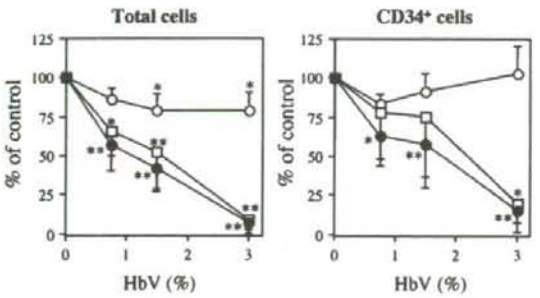

Figure 3. Effects of short exposure to hemoglobin vesicles (HbVs) on the expansion of stem/progenitor cells derived from cord blood (CB). Cord blood-derived $CD34^+$ cells were exposed to HbVs (0%, 1.5%, or 3%) for 3 d (open circle), 7 d (open square), or 14 d (closed circle). After culture for a total of 14 d, the total number of expanded cells and the number of expanded $CD34^+$ cells were determined, respectively. Data represent the mean ± SD of three experiments performed on three separate CB donors. A two-way paired analysis of variance (ANOVA) followed by Bonferroni's test was used for comparisons of multiple HbV-treated groups with the control (HbVs 0%) group. $^*p < 0.05$, $^{**}p < 0.01$ versus HbVs (0%).

total cells. However, the liposomal inhibitory effects were much less than those of HbVs. Similarly, the 14-day exposure of $CD34^+$ cells to empty liposomes only modestly inhibited the expansion of $CD34^+$ cells themselves as compared with those by HbVs (**Figure 2**).

Effects of Short Exposure to HbVs on the Expansion of Hematopoietic Stem/Progenitor Cells

Next, the effects of the short exposure of $CD34^+$ cells to HbVs on the expansion of both total cells and $CD34^+$ cells were examined. Exposure to HbVs even at 3% for 3 days only slightly inhibited the increase of the number of the total cells and did not affect expansion of $CD34^+$ cells (**Figure 3**). In the case of 7-day exposure, the expansion of total cells and $CD34^+$ cells was reduced by a similar extent as was observed in the case of continuous exposure.

Discussion

We previously examined the biocompatibility of HbVs with CB hematopoietic progenitor cells by testing whether HbVs might affect the clonogenic activity of progenitor cells in a semisolid culture, and the proliferation and differentiation of erythroid and myeloid lineages from progenitor cells in a liquid culture. In the study, we showed that a short exposure (1–3 days) to HbVs had no adverse effects on progenitor activity, although a longer exposure (14 days) suppressed progenitor activity.[14] Under these two culture conditions, hematopoietic stem/progenitor activity could not be tested.

In the present study, therefore, we used the coculture system with hTERT-transfected human stromal cells and $CD34^+$ cells, which mimics the physiological conditions of hematopoiesis in the bone marrow in terms of the interaction between hematopoietic stem/progenitor cells and stromal cells, and importantly supports *in vitro* expansion of hematopoietic stem/progenitor cells.[21] Under this system, we found that 14-day exposure to HbVs significantly suppressed expansion of hematopoietic stem/progenitor cells, although the effect of 3-day exposure to HbVs was minimal. This observation is in agreement with our previous result.[14]

It has been shown that hemin (the ferric chloride salt of heme) exerts a proliferative effect on erythroid progenitor cells. The administration of hemin to mice increases the number of BFU-E within the bone marrow *in vivo*.[26] Hemin was also shown to stimulate a two-fold increase in a murine erythroid colony compared with those stimulated by Epo alone in the *in vitro* culture.[27] Along with hemin's effect on erythroid progenitor cells, purified and cross-linked hemoglobin has also been shown to have erythropoietic activity.[28] Purified and other chemically modified hemoglobin, as a blood substitute, has been shown to support expansion of erythroid progenitor cells from CB-derived $CD34^+/CD38^-$ and $CD34^+/CD33^-$ stem/progenitor cells in a liquid culture.[29] In addition, under a human $CD34^+$ bone marrow cell liquid culture system, a cross-linked human recombinant hemoglobin alone did not affect the proliferation of erythroid and myeloid lineage cells, but protected erythroid lineage cells from 3′-azido-3′-deoxythymidine (AZT)-mediated inhibition.[30] The protection of this recombinant hemoglobin from AZT toxicity was also achieved in animal models.[31] The authors speculated that free heme could be released from hemoglobin before its uptake by erythroid progenitor cells or, alternatively, intact hemoglobin could be taken up intracellularly by the cells before the release of heme. As a result, heme stimulates proliferation and differentiation of erythroid progenitor cells. These erythropoietic activities observed in an acellular type of hemoglobin substitute were not observed in HbVs, cellular-type artificial oxygen carriers, in our culture conditions. HbVs might degrade during long-term incubation, leading to the release of Hb. However, our previous study showed that the released Hb level in the liquid culture in the presence of 3% HbVs was low enough not to affect the proliferation of erythroid and myeloid lineage cells.[14] Another possible explanation is that the uptake of HbVs may not occur in erythroid progenitor cells as efficiently as that of modified hemoglobin.

The suppression of expansion by 14-day exposure to HbVs appeared to be mostly attributed to Hb itself in the HbVs, because the effect of empty liposomes was much less than that of HbVs. The modest inhibitory effect observed in the liposomes might be caused by physical suppression of the interaction of $CD34^+$ cells and stromal cells.

There are several explanations for the observed suppression by 14-day exposure to HbVs. Higher dissolved oxygenations in the culture medium were theoretically more expected in the presence of HbVs than in the absence of HbVs, which may be involved in the inhibition of expansion of hematopoietic stem/progenitor cells after the prolonged exposure to HbVs. In addition, Hb in HbVs might autoxidize during 14 days in culture, producing reactive oxygen species, which in turn cause lipid peroxidation of liposomes, resulting in the generation of lipid radicals. The production of lipid peroxides and their by-products ultimately causes the loss of membrane function and integrity of cells including hematopoietic stem/progenitor cells.[32,33] In fact, conversion of Hb to Met-Hb within HbVs occurs.[34] In addition, concentrated Hb within HbVs interacted with exogenously added H_2O_2, and subsequently Met-Hb and ferrylHb were formed inside the HbVs.[35] However, there was no peroxidation of lipids comprising the vesi-

cles,[35] due to the saturated lipid membrane of HbVs. Furthermore, when vesicles composed of egg yolk lecithin as unsaturated lipids were added to the mixture of HbVs and H_2O_2, no lipid peroxidation was observed in vesicles composed of egg yolk lecithin.[35] This is due to the stable encapsulation within HbVs of the products of Met-HbV and ferrylHb. Thus, the possibility that lipid peroxidation may be involved in a suppressive effect associated with longer exposure is less likely.

We found exposure time-dependent suppression of stem/progenitor activity by HbVs in this *in vitro* study. In contrast, our animal studies demonstrated a lack of any suppressive effects of HbVs on hematopoiesis. For instance, rats and beagle dogs after receiving an acute 40% exchange-transfusion with HbVs, showed elevated erythropoietic activity and completely recovered from the decreased hematocrit within 7 days.[36,37] In addition, the number of red blood cells, leukocytes, and platelets were maintained constantly for 1 week after the infusion of HbVs into rats at 20% of the whole blood volume.[38] In the animal model, HbVs accumulated transiently in the bone marrow by 1–3 days and significantly decreased at 7 days, and then became undetectable at 14 days after infusion of HbVs.[36] The HbVs distributed in the bone marrow were accumulating in bone marrow macrophages, and the HbVs were degraded in the phagosomes in the reticuloendothelial system.[39] However, an *in vitro* coculture system lacks any reticuloendothelial system in addition to any shielding by the vasculature and blood stream, and these closed systems might magnify the direct contact between HbVs and $CD34^+$ cells, resulting in the different outcome (*i.e.*, the exposure time-dependent suppression of stem/progenitor activity).

In conclusion, although the absence of adverse effects on hematopoiesis by HbVs in animal models suggests that HbVs, when transfused into humans, may have minimal side effects on hematopoiesis, the present data raise some concern regarding hematopoiesis, and one should therefore pay attention to the effect of HbVs on various hematological aspects in future clinical setting.

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