Table 39.4 Some potential projects (investigators will be invited based on relevant expertise)

- Systems biologic approach to investigation of HBOC-mediated AEs (e.g., hypertension/vasoconstriction, enzyme abnormalities, cardiac abnormalities, etc.) utilizing molecular, genomic and proteomic analytical tools as HBOC interaction with cells/organs is dynamic multi-faceted process necessitating collaborative efforts of multi-disciplinary experts
- Robust global safety evaluation of HBOCs/OTs/MFRs via total body assessment based on organ proteomics and clinical assays
- Temporal and between group comparison of key physiological parameters before, during and after infusion of control and test HBOC/OT/MRF agents using in vivo analytical tools including single photon emission computed tomography (SPECT)
- Role of HBOCs in radical mediated toxicity and organ dysfunction in hemorrhagic shock/resuscitation
- Mechanism(s) of HBOC-mediated vasoconstriction/hypertension and it relationship to observed AEs and organ dysfunction
- Toxicities or harmful interactions between HBOCs and a patient's underlying disease
- Study of mechanisms of cell-free HBOC-mediated AEs/SAEs
- Role of vascular endothelial dysfunction (including NO and endothelin response and barrier function)/inflammation on physiological response to HBOCs
- Pathophysiologic relationship of post-trauma/hemorrhage immunosuppressive conditions and HBOC-mediated AEs
- Development of MFRs that include a crystalloid solution, oxygen carrier and procoagulant agents
- Others deemed necessary and appropriate

will be withdrawn from participation or conduct of certain studies. Qualifying investigators will submit a specific research proposal studying a selected HBOC/OT/MRF product(s) according to a format adopted by the consortium in consideration of potential funding sources (including a full budget proposal within a proposed direct cost cap). Most relevant high priority projects/investigators will be selected and included in the final consortium research proposal to be submitted to an appropriate funding agency. Ethics Committee, Data Safety Monitoring Board and strict adherence to local Institutional Review Board policies will be enforced as appropriate.

39.4.1 Proposed Activities of Consortium

- Coordination of collaboration in a concerted manner to bring about investigation with efficient use of resources.
- Identify and define highest priority issues/areas to resolve in HBOC/OT/MRF research/development for the consortium investigator to undertake.
- Evaluation of several distinct multi-product candidates (e.g., acellular and cellular HBOCs, OTs, MRFs, etc.).

- Data mining of literature (and possibly relevant FDA database if proper arrangement can be made) for in-depth analyses utilizing system's biology approach.
- Identify and develop avenues/means to undertake collaborative research including possible source of funding.
- Data/information exchange/workshop on focused topics.
- Identify and develop standardized methods/assays/tools/test HBOCs and specialty reagents and quality standards for preclinical and clinical tests.
- Repository for test HBOCs/OTs/MRFs, preclinical and clinical study data, relevant literature and regulatory information/advice.
- Others as deemed appropriate.

Specific terms of collaborative activities including nature of projects, execution of experiments, data management/dissemination, copyright/IP and other issues will be defined in a written Memorandum of Understanding (MOU).

39.4.2 Organizing Members

- Hae Won Kim, Ph.D., Brown University, Providence, RI, USA.
- Jonathan S. Jahr, MD, UCLA, Los Angeles, CA, USA.
- Andrea Mozzarelli, Ph.D., University of Parma, Parma, Italy.
- Hiromi Sakai, Ph.D., Department of Chemistry, Nara Medical University, Kashihara, Japan.

The core of the consortium will be U.S.-based. Dr. Hae Won Kim (Brown University, Providence, RI) and Dr. Jonathan Jahr (UCLA, Los Angeles, CA) will serve as co-Directors and share responsibilities in the overall management and coordination of consortium activities. Additional members with expertise in selected areas of interest will be included once priority areas/projects are determined (see Table 39.4, for potential projects). Specific roles and responsibilities of each consortium member will be defined in a written MOU agreement.

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平成24年度「独創性のある生命科学研究」プロジェクト型研究課題 抑制性マクロファージの機能発現に関わる分子基盤の解明

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旭川医科大学研究フォーラム 14　：85～87，2013

依頼稿（報告）

平成24年度「独創性のある生命科学研究」プロジェクト型研究課題 抑制性マクロファージの機能発現に関わる分子基盤の解明

東　　寛* 小林博也** 木村昭治***

背　景

マクロファージは活性化されると、大きく二つの性質を持つものに分化する。これをmacrophage polarization呼び、一方をM1 type、他方をM2あるいは、classically activated macrophageおよびalternatively activated macrophageという。M1 typeは、IFN-γ単独あるいはそれとTNF-αやGM-SCF等のサイトカイン、LPS等の細菌由来の刺激により誘導される。一方、M2 typeは、Th2サイトカインであるIL4, IL13等により誘導される。M1typeがantimicrobial/inflammatory responseを荷なっているのに対してM2 typeは、その対局としてanti-inflammatory作用とともに組織修復にも重要な役割を荷なっているとされている。

Tumor associated macrophage (TAM)はM2のフェノタイプを持ち、担がん状態の宿主で、特に腫瘍周辺に集積し、腫瘍免疫応答を抑制することが知られている。M2 type macrophageに関連するもう一つの細胞群として、immunosuppressive macrophage (Mis)がある。肺胞マクロファージの一部あるいはmycobacterial infection、protozoal infectionにおいて見いだされることが古くから報告されており、これも強力なT細胞増殖抑制効果をもっている[1)]。

Myeloid derived suppressor cell (MDSC)は、様々な病的状態（癌、感染等）において生体内に出現し、強力なT細胞機能抑制を誘導する、未熟な骨髄由来細胞の不均一な細胞集団と定義される。腫瘍組織周辺に存在するMDSCは、TAMと同じく腫瘍特異的T細胞の抑制に関与している。実際、MDSCの一部はin vivoでTAMに変化するとの報告もある[2)]。現状では、MDSCの機能を如何にコントロールするかは免疫学における重要な研究課題の一つとなっている[3)]。

MDSC, TAMそしてMisはいずれもM2 polarization側に位置するものと考えられており、実際これらの細胞群のT細胞増殖抑制の機序には幾つかの共通点がある。例えば、作用の発現にNOが関与していること、多くの場合、抑制効果の発現にはcell-to-cell contactが必要な事などである[1,4,5,6)]。しかしながら、cell-to-cell contactに関与する分子基盤に関しては、MDSC側およびT細胞側とも未だ明らかにされていない。

我々はラットにある種のリポソームを投与すると、脾臓内に強力なT細胞増殖抑制効果を持つマクロファージが誘導されること見いだした[7)]。その作用機序を解析した結果、抑制効果の発現にNOが関与していること、かつcell-to-cell contactが必要であることを見いだした。即ち、誘導されるMisは、その作用機序からは、既に報告されているMDSC（あるいはTAM）細胞群との共通点を有する。この系を用いれば、T細胞増殖抑制効果を持つMisそしてMDSC細胞群に特徴的に発現している遺伝子群およびcell-to-cell contactに関与する分子を見いだすことができると考えられる。

方　法

1．ラットへの操作

リポソームをラットに投与後、脾臓を摘出し、リポ

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ソーム貪食細胞を CD11b/c をマーカとし、磁気ビーズを用いて純化した。純化した脾細胞分画から RNA を抽出し、DNA マイクロアレイ解析の為の試料とした。コントロールは生理食塩水を静注したラットから採取した脾臓を用いて作成した。

2．DNA マイクロアレイによる遺伝子プロファイルの解析

上記のように抽出した RNA をから Low Input Quick Amp Labeling Kit (Agilent Technologies) を用い cDNA の合成と cRNA のラベルと増幅をおこなった。ラベルした cRNA をマイクロアレイ (whole rat, 44,000 gene, Agilent Technologies) にアプライし、ハイブリダイゼーションをおこなった。マイクロアレイの洗浄と乾燥後、Agilent Technologies Microarray Scanner を用いてスキャンした。得られた数値化データはグローバルノーマライゼイションによってアレイ間の補正をおこなった。生理食塩水 i.v. 後とリポソーム i.v 後の結果を比較し、前者と比較して 2.6 倍以上発現量の差がみとめられた場合を有意と判定した。

結果および考察

1．CD11b/c陽性細胞の純化結果

磁気ビーズを用いた純化操作により CD11b/c＋細胞の割合は数％から 50% 前後に高めることができたので、当該細胞集団の遺伝子発現プロファイルをよく反映することができるものと判断した。

2．遺伝子プロファイルの解析結果

遺伝子発現量をコントロールと比較した結果、2 回の実験で 2 回とも Fold increase が 2.6 以上であったものが 168 遺伝子あった。

その中から、数個の遺伝子についての結果を表にした（表 1）。

表 1

	Exp. 1	Exp. 2
Mmp14	57.2	51.3
Ccl9	37.4	46.43
ApoE	16.8	9.8
IL-18bp	11.39	13.1
IL-1α	5.6	9.9
CD276	5.27	7.21

ApoE メッセージの増加は、貪食したリポソームの構成成分である脂質を代謝する為には、必須の反応であると考えられる。ApoE メッセージの増強が観察されたことから、回収した細胞群が、目的の細胞を十分に含んでいたことを示しているものと考えられる。

Mmp14 と Ccl9 はいずれの実験でも非常に強い発現の増強を認めたが、その意義については不明である。

CCL9 は主にマクロファージから産生され、その受容体が CCR1 である。実験結果から、リポソーム貪食細胞が、CCR9 を産生して、CCR1 を発現している細胞を集積する作用を発揮する事が推測できる。

IL-1 α は主としてマクロファージから産生されるものであり、リポソーム投与後の CD11b/c＋細胞群で遺伝子発現の増強を認めていることが示されたが、IL-1 β のメッセージの増強は認めなかった。

IL18bp もマクロファージから産生され、IL18 と結合することにより、IL-18 の IFN-γ の産生増強効果を減弱する作用を有する。この事が、T 細胞増殖抑制にどのように関与しているのかは、不明である。

いずれにしても、CCL9, IL-1α , IL-18bp はいずれもマクロファージの産生する物質であり、我々の実験系における immuno suppressive macrophage を特徴的づける遺伝子発現パターンを示しているものと思われる。

CD276 は、B7-H3 分子と同じものであり、免疫応答の制御に関わる分子である。その機能に関しては、T 細胞機能を促進するという報告と抑制するという相反する 2 つの報告がある[8]。我々の系においては、B7-H3 が T 細胞の増殖を抑制する事に関与している可能性があるので、今後 B7-H3 の役割について、検討を進めて行きたいと考えている。

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人工赤血球の開発とこれからの展望

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はじめに

血液の構成成分とその代用物を一覧にまとめてみた(Table 1)。殆どの血漿成分は何らかの代替物が既に存在していると言っても良いが、血球成分についてはまだ開発段階にある。本稿では赤血球の代替物に関する研究の現状と展望について述べたい。

Table 1. 血液の成分とその代替物

分画		成分〔役割〕	代替物
血漿成分 (55 vol%)	血漿蛋白	アルブミン 〔循環血液量の維持〕	代用血漿剤 (ヒドロキシエチルスターチ、デキストラン、ゼラチン、遺伝子組換えアルブミン)
		グロブリン〔免疫抗体〕	抗生物質 人工免疫グロブリン
		フィブリノゲン 凝固因子	フィブリン接着剤 各種遺伝子組換え凝固因子
	電解質 低分子	Na^+、K^+、Ca^{2+}、Mg^{2+}、Cl^-、HCO_3^-、HPO_4^{2-}	電解質輸液
		ビタミン類、アミノ酸、糖類、脂質など	栄養輸液 (アミノ酸、トリグリセリド、糖類)
血球成分 (45 vol%)		血小板	人工血小板(研究開発中)
		白血球	(抗生物質で対処?)
		赤血球	**人工赤血球(研究開発中)**

一番下の赤血球の代替物として研究されているのが人工赤血球である。

人工赤血球とは

血液に含まれる蛋白質のうち、最も沢山あるのがヘモグロビン(Hb)である。Hbは酸素を可逆的に結合-解離する蛋白質であり、血液の酸素輸送機能が生命維持にとって最も重要な要素であることを意味しているのかもしれない。しかし、高等動

物ではHbは赤血球(長径約8μm)の袋の中に極めて高濃度に(35g/dL)封じ込まれており、最も沢山あるにも関わらず、溶血によって一旦赤血球の外に遊離すると、様々な副作用(毒性)を生じる。Hbを使った人工酸素運搬体の研究は随分と長い歴史がある。Hbの毒性は早くから知られていたので、欧米のグループが、二量体への解離を抑制するために分子内架橋(crosslink)をしたり、分子量を大きくするために重合(polymerize)したり、あるいは水溶性高分子を結合させて(polymer conjugation)大きくするなど、実に数多のCell-free Hb based oxygen carriers(HBOCs)が試されたが、それでも副作用が残った[1]。血管内皮弛緩因子である一酸化窒素(NO)をHbが捕捉することにより、血管収縮により血圧が亢進し、末梢血管抵抗が増大する状況に陥るのである(ウシ由来のHbを架橋剤グルタルアルデヒドで重合したPolyHbは、南アフリカとロシアのごく限られた地域でのみ認可されていると聞くが、副作用が心配されている)。この結果から、私たちは、やはりHbは本来、赤血球あるいは赤血球構造に類似するカプセルの中にあるべき、という考えが正しいことを認識し、"細胞型(cellular)"の人工赤血球であるHb小胞体を研究している(Fig.1)[2,3]。赤血球の細胞型構造の生理的意義を理解すれば、上述のCell-free HBOCsに副作用が生じたことは容易に理解できる。赤血球は長径約8μmの中窪み円盤状粒子であり、蛋白質Hb(分子量64,500)の高濃度溶液を赤血球膜に内包した構造を持つ。Hb溶液が赤血球膜で覆われている理由は、①本来様々な毒性のあるHbの逸脱、血管壁との直接的な接触の抑制、②腎臓の糸球体からの漏出など血管外漏出を防ぎ血中滞留時間を長くする、③35%濃厚Hb溶液の高い粘度と膠質浸透圧の抑制(赤血球は膠質浸透圧を示さない)、④Hb機能維持のための各種リン酸化合物などエネルギー分子、解糖-並びに還元-酵素系の保持、⑤血管内皮弛緩因子〔NO、一酸化炭素(CO)〕との反応性の制御などの役割もある。また、⑥血液(血球分散系)は非ニュートン流体で、体内循環とくに末梢血管内における特色ある流動形式と生理作用が特性である。

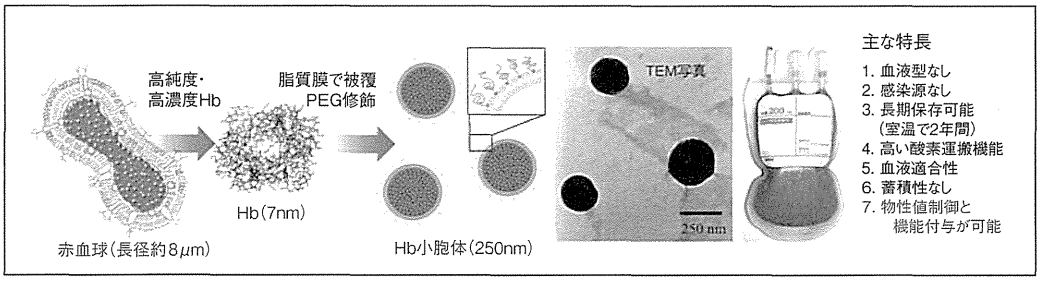

Fig.1. 人工赤血球(Hb小胞体)製剤の概要

有効期限切れ赤血球から精製して得られた高純度高濃度ヘモグロビン溶液をリン脂質小胞体(リポソーム)の中に内包させる。

高純度・高濃度Hb溶液(濃度35%以上、約3万個のHb分子)を脂質分子二層膜(厚さ5nm)で包んだ小胞体(平均粒径250nm)は、脂質成分とHbが分子間相互作用(二次的相互作用:疎水的相互作用、静電的相互作用、水素結合など)だけで形成している分子集合体である。原料のHbは日本赤十字社から提供されるNAT(核酸増幅)検査済みの献血由来の有効期限切れ赤血球由来であるが、精製に際しHbにCOを結合させて安定化し、60℃で10時間の加熱処理とウィルス除去膜処理を組み合わせることで、感染に対する安全性を確保できる。COは光解離によって除去する。約1,500本のポリエチレングリコール(PEG)を粒子表面に配置することにより小胞体粒子間の凝集抑制と分散安定度の向上の効果が得られ、更に脱酸素化して容器に封入することにより溶液のまま室温にて長期保存が可能になった。「ナマモノ」の血

液から高純度Hb溶液を単離し、これを人工赤血球という安定な「物質」に再生したといえる。微粒子表面の性質は、生体適合性を決定する要素である。PEG修飾と負電荷脂質の導入により補体活性や凝固系の活性、あるいは阻害を誘導しない微粒子を構成している。

輸血代替としての人工赤血球投与の可能性

私が大学院生だったときに慶應義塾大学医学部の小林紘一先生のグループと行っていた実験が、ラットの循環血液量の90％を人工赤血球に置換するというものであった[4]。麻酔したラットの頚動脈から1 mL脱血、頚静脈から1 mLの人工赤血球を投与し、ヘマトクリット5％以下まで交換した。人工赤血球は赤血球と同様に膠質浸透圧を持たないので、5％アルブミン溶液を併用した。アルブミンのみで血液希釈をすると代償機能により最初は心拍出量に1.5倍程度の増大がみられるがその後は低下し、全例が90％交換を待たずに死亡する。これに対して人工赤血球/アルブミンで交換すると、全例が生存した。当時、血液がここまで希釈される状況は臨床的にはあり得ないだろうと考えていた。しかし出血が酷く止血手段が無い状況(uncontrolled hemorrhage)では、輸液を絶えず注入することになり、ヘマトクリットが低下する。米国の映画「ブラックホーク・ダウン」では、敵地で大腿動脈を損傷した負傷兵の止血ができず輸液(晶質液)を受け続けるものの、最後は貧血状態となって命を落としていく生々しいシーンがあった。Seishiらの最近の研究では[5]、ラットのuncontrolled hemorrhage modelを作成し、人工赤血球を投与し続けることにより延命されることを確認している。他方、循環血液量の40～50%を急速脱血してショック状態としたラット、ウサギ、ビーグル犬に等量の人工赤血球を投与することにより蘇生でき、血行動態、血液ガス組成などは、脱血液を投与した対照群と同等に推移し、全例が生存できることを確認している(Fig.2)[6,7]。このように、人

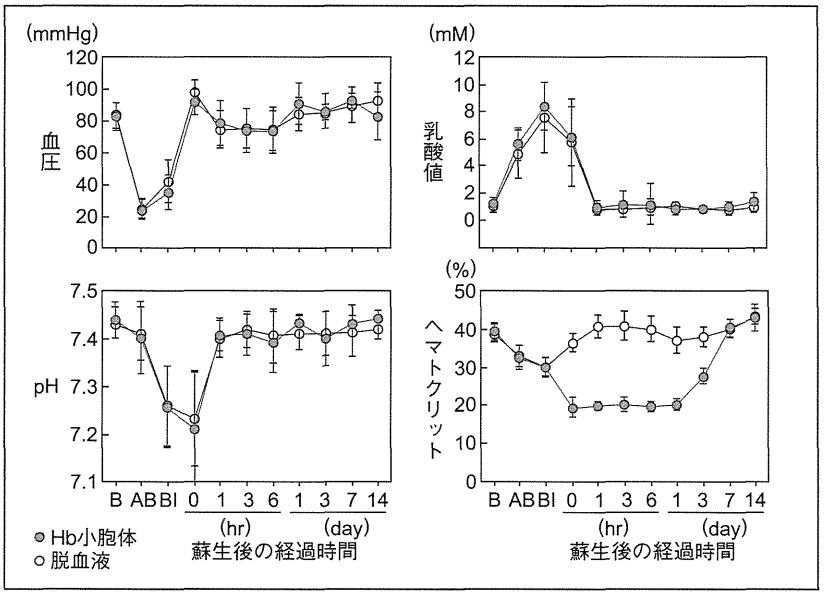

Fig.2. 出血性ショック状態にあるラットの蘇生試験

循環血液量(56mL/kg)の50%を急速脱血してショック状態とし15分経過後に人工赤血球(Hb小胞体)を遺伝子組換えアルブミンに分散させた溶液を同量投与した。6時間後に覚醒させてその後14日間観察した。脱血液の投与と同等の回復を示し、低下したヘマトクリットも1週間以内に回復した。Hb小胞体は細網内皮系に捕捉され、分解されて糞尿中に排泄される。B：ベースライン、AB：脱血後、BI：投与直前　(文献6より引用)

工赤血球は、自然災害や有事の大量需要に対して、血液型に関わらず、いつでも何処でも必要時に投与して延命ができる酸素輸液剤として期待できる。術前血液希釈、術中出血分の補充的投与の可能性も十分に考えられる。

ところで、ショックの蘇生は全身的な再潅流傷害を生起するが、興味深いことに、COを結合させた人工赤血球を投与したところ、再潅流傷害を低減させた[8]。蘇生初期においては酸素よりも循環血液量の回復が優先され、またCOが細胞保護効果を示したと考えている。COは酸素の200倍の結合力があることが知られているが、体内では圧倒的に酸素が多いため、意外と短時間でCOを解離し呼気を通して排泄され、人工赤血球は酸素を結合し、酸素運搬体に変化する(Fig.3)。勿論COは有害なガスなので至適投与量が存在すると考えられるが、毒をもって毒を制する治療法の可能性の一例になろう。

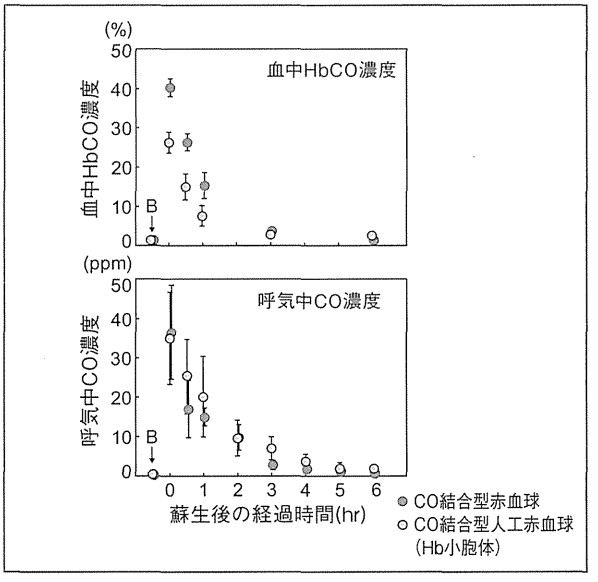

Fig.3. COを結合した人工赤血球(Hb小胞体)または赤血球を投与した後のCOレベルの推移

Fig.2の実験モデルと同様に循環血液量の50%を急速脱血してショック状態とし15分後にCOを結合したHb小胞体あるいは赤血球を投与した。血中HbCO濃度は直ぐに低下し3時間で3%以下になる。平行して呼気中にCOが現れ、減少していくことをガスクロマトグラフィーから検出した。呼吸が保たれていれば、投与したCOは確実に排泄されることが明らかになった。また、この際、COは細胞保護効果を示すことが解っている。B:ベースライン。

更に胸部外科手術では、人工心肺(体外循環回路)の補充液としての利用が期待できる。体外循環回路の小型化も進み、成人患者の場合は補充液に晶質液を使用すれば十分である。しかし小児患者の場合は、全血液量に比較して体外循環回路の容量がまだ大きいため、無輸血充填(血漿増量剤の充填)とした場合に、術中のほんの数時間の血液希釈でも酸素欠乏により脳に障害を与え、術後の知能発達に影響を及ぼすことが報告された。そこでYozuら(慶應義塾大学医学部外科)は、人工赤血球(Hb小胞体)を充填液とすれば脳への酸素供給が維持できるのではと考え、動物実験で人工赤血球(Hb小胞体)を用いた体外循環を実施したところ、脳の機能が保護されることを具体的に明らかにしている[9]。

輸血では対応のできない疾患の治療、外科的治療への応用の可能性

細動脈レベルの血管(たとえば内径20～100μm)を光学顕微鏡でみると、赤血球(長径約8μm)が血管の中心側を流れ(軸集中：centralization)、管壁近傍に血漿層が形成されていることに気付く。この血漿層はplasma layerあるいはRBC-free layerなどと呼ばれる。血管に分岐がある場合には、軸集中した赤血球は血流の速い支流に多く流れていき、遅い方の支流は血漿が多くなる場合がある(血漿分離：plasma skimming)。また、細動脈は更に無数の毛細管に分岐されるが、毛細管全てに一様に血液が流れていない。ある毛細管は血流が速く赤血球が多く流れているかと思うと、ある毛細管は赤血球がたまにしか流れていない。また毛細管内のヘマトクリットは、採血液のヘマトクリットよりも明らかに小さい(Fåhraeus効果)。1996年にProf. Intaglietta の研究室に留学した際にこれらの現象を目の当たりにして驚いたが、ヘモレオロジーの教科書にすべて書いてある事である。では、粒子径が僅か250nmの人工赤血球(Hb小胞体)はどのように流れるのか。答えは、人工赤血球は血漿層と一緒に流れることになる(Fig.4)[10]。実はこれが虚血性疾患における酸素輸送には極めて適している。赤血球が通過出来ない狭窄血管でも、血漿の流れが残っていれば、血漿と一緒に通過して酸素を運搬出来るし、上述のplasma skimmingは、虚血性組織の周辺血管で顕著になると考えられるが、plasma skimmingのplasmaと一緒に流れて虚血性組織に酸素をより多く運搬することになる。

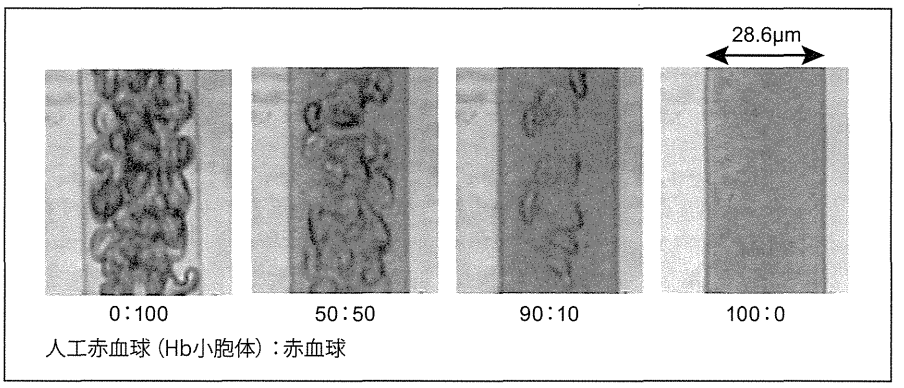

Fig.4. 人工赤血球(Hb小胞体)と赤血球混合系の細管内流動の様子

赤血球のみ(左)では、管壁近傍に血漿層が形成されていることが解る。Hb小胞体の含量を多くしていくと、赤血球が軸側を流れている様子が解る。光学顕微鏡ではHb小胞体粒子一つ一つを見る事が出来ないが、血漿層はHb小胞体が分散しているため次第に濁度が上昇する。右端はHb小胞体のみを流動させた場合。管内がHb小胞体で満たされている。中心流速1mm/s。(文献10より引用)

また、人工赤血球の一つの特徴は、アロステリック因子の含量を換えることにより酸素親和度を自在に調節出来ることがある。酸素結合解離曲線を左方シフトさせた高酸素親和度の人工赤血球は、酸素を離し難くなる。正常の酸素分圧にある組織であれば、この高酸素親和度人工赤血球は酸素を放出せずに通過する。しかし低酸素分圧の組織では酸素を離すことになり、酸素のターゲッティングが可能となる[11]。これまでに有茎皮弁モデルや脳梗塞モデル動物を用い、人工赤血球の虚血性疾患に対する有効性、創傷治癒効果について確認している[12,13]。腫瘍組織の毛細血管は形状がいびつで、赤血球が流れず血漿のみが流れている場合も多い。腫瘍組織酸素分圧が低いことが放射線治療の十分な効果が得られないことの原因とされる。ここに人工赤血球を投与すると腫瘍酸素分圧が上昇するので、その時を狙って放射線照射することにより、腫瘍重量の増大が抑制されることが明らかになっている[14]。

おわりに

このほか、人工赤血球は、移植臓器の保存潅流液としての利用、輸血拒否患者の対応、獣医療領域への利用、レーザー治療のターゲットとしての利用など、様々な可能性が芽生えてきている。問題なのは、現段階で実用化に向けて製薬会社からの支援をなかなか頂けないことである。本製剤が従来に無い範疇の製剤であること、投与量が数リットルに及ぶ大量投与を伴うが安全性が担保されるのか、また現在輸血で何とか間に合っているのに何故その代用物が必要か、など消極的な意見を聞く事もある。今回は安全性評価試験については割愛させて頂いたが、安全性を実証する実に多くの非臨床*in vivo*試験結果が得られており[2,3,15]、次段階に進むべき対象であると考えている。少子高齢化でこのままだと2027年には100万人分の献血液が不足するという予測が日本赤十字社から公表されている。有効期限切れとなった献血液や、不規則抗体を有する献血液は現在廃棄されているが、献血者の善意を無駄にせずにこれらを有効利用することは重要な課題ではないだろうか。更に大規模災害や有事の際の大量需要にどう対応するかを考えれば、やはり血液型がなく備蓄可能な人工赤血球が必要ではないか。また、今回紹介したように輸血では為し得ない疾患の治療や外科的手法にも用いることができる可能性がある。更に、献血技術が十分でない諸国への国際貢献も期待できる。是非とも皆様からのご支援を頂き、人工赤血球を実用化させたい。

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プロムナード

アートな弘前とサイエンスな琉球

弘前大学医学部附属病院麻酔科 医員
斎藤 淳一

弘前大学では琉球大学との交換留学を行っています。平成21年度から開始されたばかりの新しい制度ですが、昨年度は私にチャンスがやってきました。弘前大学と琉球大学の魅力が少しでも伝われればと、この場を借りて書かせていただきます。

弘前大学では年間麻酔管理症例数約3,600件、そのほぼ全例を全身麻酔で行っています。プロポフォール、フェンタニル(あるいはレミフェンタニル)、ケタミンを用いた全静脈麻酔(total intravenous anesthesia : TIVA)と超音波ガイド下末梢神経ブロックが全身麻酔の中心となります。「医療とはサイエンスとアートとの融合」という先代の松木教授の言葉から、麻酔科医各人がサイエンス〔薬物動態学(PK)/薬力学(PD)〕をベースにアート(五感、第六感)を有機的に融合させ、各症例の全身麻酔を行っています。標的濃度調節持続静注(target-controlled infusion : TCI)を用いないのも"アート"へのこだわりかもしれません。弘前大学では麻酔科医の経験とセンスに重きを置いた麻酔管理を行っています。

一方、琉球大学は"サイエンス"に重きを置いた麻酔管理という印象を受けました。TCIを用いた術後持続フェンタニルの患者自己調節鎮痛(patient controlled analgesia : PCA)やPK/PDを意識した麻酔薬の使い方を勉強させていただきました。そんな中、最も感銘を受けたのが揮発性麻酔薬による導入と維持(volatile induction/maintenance of anesthesia : VIMA)での自発呼吸温存気管挿管です。シンプルながら呼吸は安定、循環変動が小さく、患者へのストレスが少ない点が印象的でした。TIVAで管理できない症例はないと思っていますが、TIVAに勝るVIMAを自発呼吸温存気管挿管に見たように思います。他にもTIVAの良さ、ケタミンの良さ、ケタミンと吸入麻酔薬との相互関係等々、琉球大学に行き初めて感じるものがありました。

今後も弘前大学と琉球大学の交換留学が続いていくことを願っています。気候、文化など大きく異なる弘前と琉球ですが、違うからこそ面白いと思います。琉球大学の先生方も寒さを恐れず弘前へ来てください。私が学んだことを一人でも多くの後輩が経験してくれることを期待しています。最後になりましたが、留学の機会を与えて下さった当教室員のみなさん、温かく迎えてくださった琉球大学および関連病院の先生方に御礼申し上げます。

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Carbon monoxide-bound hemoglobin-vesicles for the treatment of bleomycin-induced pulmonary fibrosis

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Lung

ABSTRACT

Carbon monoxide (CO) has potent anti-inflammatory and anti-oxidant effects. We report herein on the preparation of a nanotechnology-based CO donor, CO-bound hemoglobin-vesicles (CO-HbV). We hypothesized that CO-HbV could have a therapeutic effect on idiopathic pulmonary fibrosis (IPF), an incurable lung fibrosis, that is thought to involve inflammation and the production of reactive oxygen species (ROS). Pulmonary fibril formation and respiratory function were quantitatively evaluated by measuring hydroxyproline levels and forced vital capacity, respectively, using a bleomycin-induced pulmonary fibrosis mice model. CO-HbV suppressed the progression of pulmonary fibril formation and improved respiratory function compared to saline and HbV. The suppressive effect of CO-HbV on pulmonary fibrosis can be attributed to a decrease in ROS generation by inflammatory cells, NADPH oxidase 4 and the production of inflammatory cells, cytokines and transforming growth factor-β in the lung. This is the first demonstration of the inhibitory effect of CO-HbV on the progression of pulmonary fibrosis *via* the anti-oxidative and anti-inflammatory effects of CO in the bleomycin-induced pulmonary fibrosis mice model. CO-HbV has the potential for use in the treatment of, not only IPF, but also a variety of other ROS and inflammation-related disorders.

1. Introduction

Idiopathic pulmonary fibrosis (IPF) is a chronic and progressive type of fibrous interstitial pneumonia with an unknown cure, except for lung transplantation. Patients with IPF have an estimated median survival of 2–5 years [1–3]. Pirfenidone (5-methyl-1-phenyl-2-[1H]-pyridone) is currently the only orally administered drug approved for clinical use in the treatment of IPF in both the EU and Japan. Recently, the CAPACITY (Clinical Studies Assessing Pirfenidone in idiopathic pulmonary fibrosis: Research of Efficacy and Safety Outcomes) program showed that pirfenidone has a favorable benefit–risk profile, and, as a result, represents an appropriate treatment option for patients with IPF [4]. On the other hand, this multinational, double-blind, placebo-controlled study (CAPACITY006) also showed that pirfenidone treatment does not completely improve the clinically meaningful effects on forced vital capacity (FVC) and survival benefit [4]. In addition, it is well-known there are some significant side effects associated with the use of

Abbreviation: CO, carbon monoxide; HbV, hemoglobin-vesicles; CO-HbV, CO-bound hemoglobin-vesicles; IPF, idiopathic pulmonary fibrosis; FVC, forced vital capacity; ROS, reactive oxygen species; TGF-β, transforming growth factor-β; CO-RM, CO-releasing molecules; HbCO, carboxyhemoglobin; RBC, red blood cell; PEG, polyethylene glycol; BLM, bleomycin; 8-OH-dG, 8-hydroxy-2′-deoxygenase; NO_2-Tyr, nitrotyrosine; TNF-α, tumor necrosis factor-α; IL-6, interleukin-6; IL-1β, interleukin-1β; Nox4, nicotinamide adenine dinucleotide phosphate oxidase 4; Poldip2, polymerase delta interacting protein 2; EMT, epithelial–mesenchymal transition.

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pirfenidone, which include photosensitivity (more than 50% of patients) [5]. Therefore, the development of drugs designed to suppress the progression of this disease or to improve respiratory function is of great importance.

Since new pathogenic pathways and mediators of IPF are discovered, the progression of IPF appears to result from a complex combination a number of factors, including inflammation, reactive oxygen species (ROS) and transforming growth factor (TGF)-β. Although recent studies have suggested that the repeated administration of drugs with either anti-oxidative or anti-inflammatory properties would be expected to be useful in the treatment of IPF [6,7], the clinical use of these agents for IPF have not been approved worldwide. This is likely because these agents targeted only one of the many pathogenesis pathways, and, because mechanism responsible for the development of IPF is complex, little alleviation occurs. Therefore, a shift in the effective treatment strategy for IPF from agents that block a single functional action to an agent that can address multiple functions is clearly needed.

Carbon monoxide (CO) possesses anti-inflammatory, anti-oxidant and anti-proliferative effects, and has attracted interest as a possible clinically viable medicinal agent [8,9]. Similar to medical gasses that are routinely used in clinical situations, such as nitric oxide and oxygen, clinical applications of CO take the form of inhaled gaseous therapy and the use of CO-releasing molecules (CO-RM) [10,11]. In fact, several studies have demonstrated the efficacy of inhaled CO and CO-RM in preclinical animal models such as disorders related to inflammation and redox [12–14]. In addition, it was reported that inhaled CO and CO-RM also exerts protective effects in the case of several types of lung diseases, including pulmonary hypertension, asthma and ischemia reperfusion [15–17]. Taking these findings into consideration, CO holds enormous potential for use in the treatment of pulmonary disorders, including IPF. However, CO-RM rapidly liberates CO, with a half-life of 1–21 min, which is extremely short in terms of producing a significant therapeutic impact [18]. To achieve a sustainable therapeutic effect of CO, the continuous or repeated administration of CO-RM would be required. In addition, although high serum carboxyhemoglobin (HbCO) levels can cause several toxicity [19], it is difficult to control the serum HbCO levels as the result of inhaled CO and avoid CO intoxication. Therefore, it should be noted that an alternative pathway for the therapeutic delivery of CO to the lungs is essential in the successful clinical application of CO.

Recent developments in nanotechnology-based carriers, namely, Hemoglobin-vesicles (HbV), would offer great potential for effective CO delivery, and could lead to strategies in the development of new CO donors. To date, several preclinical trials have evaluated the histology, biochemical analysis and pharmacokinetic properties after the single or repeated administration of a putative dose of HbV in rodent, pig and monkey [20–24]. The results show that HbV possesses good biological compatibility (low complement activation) and is promptly metabolized (no accumulation in the body) even after a massive single or repeated infusion. Furthermore, the size of HbV is controlled at ca. 250 nm, because it can prevent capillary plugging, renal excretion and vascular wall permeability. Fortunately, CO easily and stably binds to hemoglobin (Hb) in the form of HbV as well as red blood cell (RBC), because the cellular structure of HbV most closely mimics the characteristics of a natural RBC, in which a highly concentrated Hb is encapsulated within a liposome with polyethylene glycol (PEG). In addition, in a previous study, we reported that CO was exhaled within 6 h after administering CO-bound HbV (CO-HbV) to hemorrhagic-shocked rats [25]. These findings led us to the hypothesis that HbV has the potential for use a carrier of CO to the lungs. Given the known therapeutic effects of CO-HbV on IPF, we decided to first evaluate whether CO-HbV could protect against IPF using an IPF animal model of bleomycin (BLM)-induced pulmonary fibrosis. In subsequent experiments, we investigated the reason why CO-HbV functions to suppress the progression of IPF.

2. Materials and methods

2.1. Preparation of HbV and CO-HbV solution

HbVs and CO-HbV were prepared under sterile conditions, as previously reported [26]. In short, the Hb solution was purified from outdated donated RBC, which was provided by the Japanese Red Cross Society (Tokyo, Japan), and the oxyhemoglobin converted into HbCO by bubbling with CO gas. The lipid bilayer was a mixture of 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine, cholesterol, and 1,5-bis-*O*-hexadecyl-*N*-succinyl-L-glutamate (Nippon Fine Chemical Co. Ltd., Osaka, Japan) at a molar ratio of 5/5/1, and 1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine-*N*-PEG (NOF Corp., Tokyo, Japan) (0.3 mol%). The CO-HbV particles were prepared by the extrusion method, and suspended in a physiological salt solution, filter-sterilized (Dismic, Toyo-Roshi, Tokyo, Japan; pore size, 450 nm). By illumination with visible light under an oxygen atmosphere, CO-HbV was converted to HbV. The HbV particles suspended in physiological salt solution bubbled with nitrogen for storage. The average diameters of the HbV and CO-HbV were maintained at approximately 250 nm *via* stepwise extrusion through cellulose acetate membrane filters with a final pore size of 0.2 μm (Fig. 1). The HbV and CO-HbV suspended in physiological salt solution were at [Hb] = 10 g/dL and [lipid] = 9.0 g/dL. The HbCO rate in CO-HbV was nearly 100%, while that in HbV was less than 5%.

2.2. Production of BLM-induced pulmonary fibrosis mice model

All animal experiments were conducted in accordance with the guidelines of Kumamoto University for the care and use of laboratory animals. To create BLM-induced pulmonary fibrosis model mice, Sea-ICR mice (6 weeks, male; Kyudo Co.,Ltd, Saga, Japan) were intratracheally treated with BLM (5 mg/kg; Nippon Kayaku, Tokyo, Japan) in PBS (1 ml/kg) under anesthesia with chloral hydrate (500 mg/kg) as previous report [27]. Saline, HbV, or CO-HbV was administered *via* the tail vein at 30 min before BLM treatment and 24 h after BLM treatment.

2.3. Plasma biochemical parameters

At 7 and 14 days after the HbV injection, BLM-induced pulmonary fibrosis model mice were anesthetized with ether and collected blood. Blood samples were immediately centrifuged (3000 g, 10 min) to produce plasma. The plasma samples were then ultracentrifuged to remove HbV (50,000 g, 30 min), because HbV interferes with some of the laboratory tests [28]. All plasma samples were stored at −80 °C until used. All plasma samples were analyzed by Clinical Chemistry Analyzer (JEOL, JCA-BM6050, Tokyo, Japan).

2.4. Histological and immunohistochemical analyses

The whole lungs were removed and fixed with 10% phosphate buffered formalin. The tissue was then dehydrated at room temperature through a graded ethanol series and embedded in paraffin. The prepared tissues were cut into 4-μm-thick sections for histological and immunohistochemical evaluation. Hematoxylin and Eosin (HE) stain and Masson's trichrome stain were performed as previously described [27]. The immunostaining for 8-hydroxy-2′-deoxygenase (8-OH-dG) and nitrotyrosine (NO_2-Tyr) were performed as described in a previous report with minor modifications [29]. In short, the primary antibody reaction was conducted below 4 °C overnight, and the secondary antibody reaction at room temperature for 90 min. In addition, the primary antibody containing NO_2-Tyr (Millipore, Tokyo, Japan, cat#: AB5411) and 8-OH-dG [15A3] (Santa Cruz, California, USA, cat#: sc-66036) was diluted 50 fold prior to use. The secondary antibodies for 8-OH-dG and NO_2-Tyr were Alexa Fluor 488 goat anti-rabbit IgG (H + L) (Invitrogen, Eugene, USA, cat#: AB11008) and Alexa Fluor 546 goat anti-rabbit IgG (H + L) (Invitrogen, Eugene, USA, cat#: AB11010), respectively. In each case the secondary antibody was diluted 200 times before use. After the reaction, the slide was observed using Microscope (Keyence, BZ-8000, Osaka, Japan).

2.5. Determination of hydroxyproline level in lung tissues

On day 14 after BLM administration, the left lung was removed and hydroxyproline content was determined as described previously [30]. The absorbance was measured at 550 nm to determine the amount of hydroxyproline.

2.6. Measurement of lung mechanics and FVC

Measurement of lung mechanics and FVC were performed with a computer-controlled small-animal ventilator (FlexiVent; SCIREQ), as described previously [31]. Mice were mechanically ventilated at a rate of 150 breaths/min, using a tidal volume of 8.7 ml/kg and a positive end-expiratory pressure of 2–3 cm H_2O. Total respiratory system elastance and tissue elastance were measured by the snap shot and forced oscillation techniques, respectively.

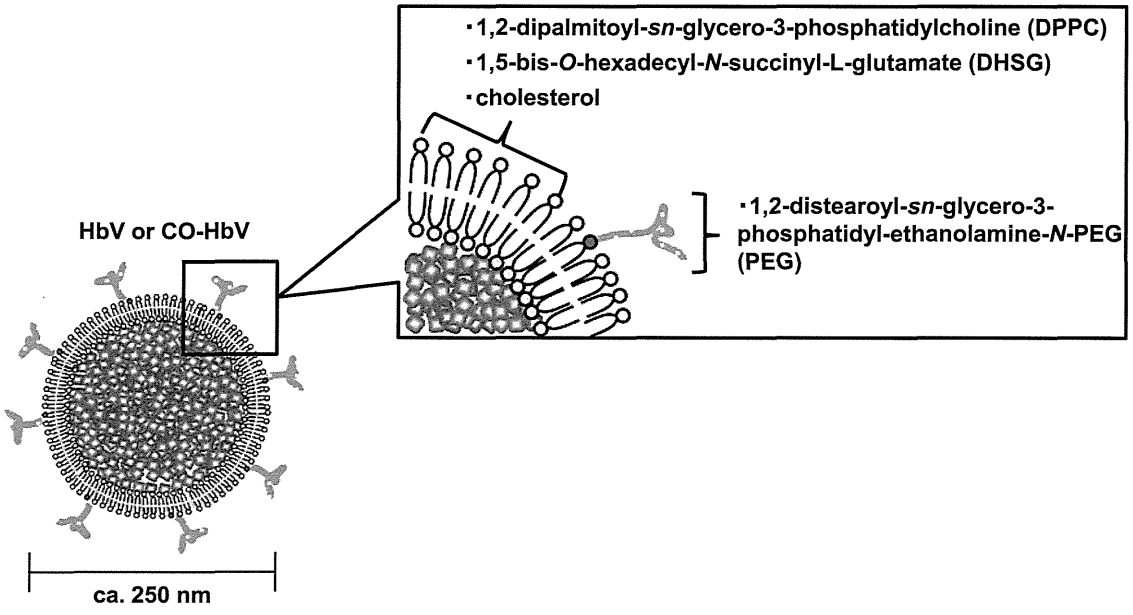

Fig. 1. Structures of hemoglobin-vesicles and its lipid components.

2.7. Counting of cells in bronchoalveolar lavage fluid (BALF)

At days 3 after the BLM administration, BALF was collected as described previously [27]. Total cell number was counted using a hemocytometer. Cells were stained with Diff-Quick reagents (Kokusai Shiyaku, Kobe, Japan), and the ratios of alveolar macrophages, neutrophils, and lymphocytes to total cells were determined. More than 200 cells were counted for each sample.

2.8. Quantification of tumor necrosis factor (TNF)-α, interleukin-6 (IL-6), interleukin-1β (IL-1β) and activated TGF-β1 in lung tissue

At days 7 and 14 after the BLM administration, whole lungs were removed and homogenized in 0.5 ml of buffer (PBS, 1% protease inhibitor cocktail, 10 mM EDTA, 0.05% Tween-20). After centrifugation at 21,000 g for 10 min at 4 °C (twice), the supernatants were recovered. The amount of TNF-α, IL-6, IL-1β on Day 7 and activated TGF-β1 on Day 14 in the supernatant was measured by ELISA kit (TGF-β1 ELISA kit; R&D Systems Inc., Minneapolis, USA, IL-6, TNF-α and IL-1β ELISA kit; Biolegend, San Diego, USA).

2.9. Western blotting analysis

At day 7 after the BLM administration, whole lungs were removed and homogenized in a homogenization buffer composed of 70 mmol/l sucrose, 10 mmol/l HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 210 mmol/l mannitol, 1 mmol/l EDTA, 1 mmol/l EGTA (ethylene glycol tetraacetic acid), pH 7.5, 200 mmol/l dithiothreitol, and 1% protease inhibitor cocktail. The homogenate was centrifuged at 720 g for 5 min at 4 °C, and the supernatants were recovered. The supernatants were centrifuged at 10,000 g for 5 min at 4 °C and further centrifuged at 100,000 g for 1 h. The resultant pellet is referred to as the crude membrane fraction. After measurement of the protein content, each sample was mixed in a loading buffer (2% sodium dodecyl sulfate, 62.5 mmol/l Tris–HCl and 1% 2-mercaptoethanol). These samples (40 mg) were run on 12.5% sodium dodecyl sulfate polyacrylamide gels, followed by electrophoretic transfer to nitrocellulose membranes. The membranes were blocked by treatment with 5% skimmed milk in PBS for 1 h at room temperature and then incubated with rabbit polyclonal anti-human nicotinamide adenine dinucleotide phosphate oxidase 4 (Nox4) [H-300] (Santa Cruz, California, USA, cat#: sc-30141, 1:500) antibodies, goat polyclonal anti-human $p22^{phox}$ [C-17] (Santa Cruz, California, USA, cat#: sc-11712, 1:600) antibodies, rabbit polyclonal anti-human polymerase delta interacting protein 2 (Poldip2) (Abgent, California, USA, cat#: AP7626b, 1:200) antibodies, or mouse monoclonal anti-human β-actin antibody (1:5000) overnight at 4 °C. The membranes were washed with 0.05% Tween-20 (T-PBS), and a horseradish peroxidase–conjugated anti-goat IgG antibody (Santa Cruz, California, USA, cat#: sc-2768, 1:5000), an anti-rabbit IgG antibody (Santa Cruz, California, USA, cat#: sc-2004, 1:5000), and an anti-mouse IgG antibody (Santa Cruz, California, USA, cat#: sc-2005, 1:10,000) were then used for the detection of the target proteins. SuperSignal Western blotting detection reagents (Thermo Scientific, Rockford, IL) were used for immunodetection.

2.10. Detection of superoxide

Dihydroethidium was used to evaluate lung superoxide concentrations *in situ*, as described in detail elsewhere [32]. After the reaction, the slide was observed under a microscope (Keyence, BZ-8000, Osaka, Japan).

2.11. Statistics

Statistical analyses were performed using the analysis of variance. A probability value of $p < 0.05$ was considered significant.

3. Results

3.1. Evaluation of the optimal dosing of CO-HbV in BLM-induced pulmonary fibrosis

Fig. 2A shows a schematic summary of the experimental protocols used in the study. Pulmonary fibrosis was induced in the mice by a single intratracheal administration of BLM (at day 0) and confirmed 14 days later. In order to determine the optimal dosing of CO-HbV in BLM-induced pulmonary fibrosis, histopathological analysis (HE stain and Masson's trichrome stain) and hydroxyproline levels were evaluated after the administration of CO-HbV at 30 min prior to the BLM treatment and day 1 after the BLM treatment at doses of 250, 500, 1000 and 1400 mg Hb/kg. As shown in Fig. 2B and C, pulmonary fibrosis was suppressed as the result of the administration of CO-HbV and suppression was dose-dependent. The maximum ameliorative effect was achieved at a concentration of 1000 mg Hb/kg.

We also evaluated the toxic effects of CO-HbV administration in BLM-induced pulmonary fibrosis mice. No evidence of the development of signs of hypoxia or abnormal behavior was found when CO-HbV as administered at a dose of 1000 mg Hb/kg. Furthermore, no changes in of serum laboratory parameters reflecting hepatic, renal and pancreatic function were found at 7 and 14 days after CO-HbV administration, except for an elevation in cholesterol levels, compared to the saline treatment in BLM-induced pulmonary fibrosis mice (Table 1). Based on these results, we concluded that the optimal dose of CO-HbV was 1000 mg Hb/kg.

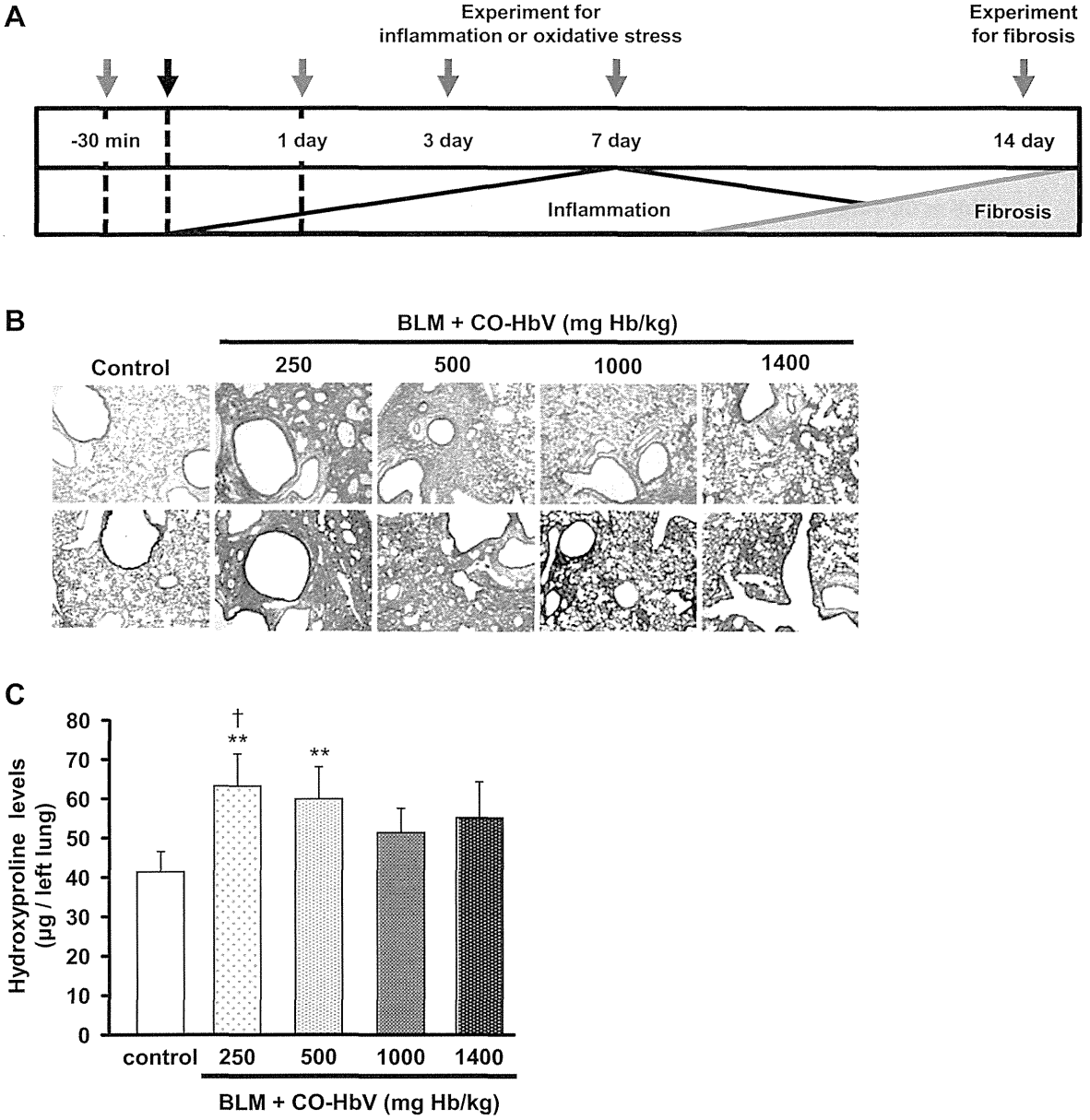

Fig. 2. CO-HbV affects BLM-induced pulmonary fibrosis in a dose-dependent manner. (A) Outline of the experimental design. Mice were treated with bleomycin (BLM, 5 mg/kg) once on day 0. They were also administered by CO-HbV *via* the tail vein at 30 min before BLM treatment and 24 h after BLM treatment. (B) Histopathologic evaluation at after CO-HbV treatment on day 14 in BLM-induced pulmonary fibrosis mice. Sections of pulmonary tissues were prepared on day 14 and subjected to hematoxylin and eosin staining (upper panels) and Masson trichrome staining (lower panels). (C) Hydroxyproline levels in left lung at after CO-HbV treatment on day 14 in BLM-induced pulmonary fibrosis mice. The pulmonary hydroxyproline level was determined on day 14 as described in the "Materials and methods" section. Each value represents the mean ± s.d. ($n = 5–6$). $^{**}P < 0.01$ versus control. $^{\dagger}P < 0.05$ versus CO-HbV.

3.2. Effect of CO-HbV on BLM-induced pulmonary fibrosis

To assess the effect of CO-HbV on the development of BLM-induced pulmonary fibrosis, BLM-induced pulmonary fibrosis mice were treated with saline, HbV (1000 mg Hb/kg) or CO-HbV (1000 mg Hb/kg) at 30 min prior to the BLM treatment and 1 day after BLM treatment. Mice that were administered saline or HbV showed massive weight loss in response to the BLM treatment, whereas weight loss was suppressed in the case of CO-HbV administration (Fig. 3A). Histopathological analysis (HE stain and Masson's trichrome stain) demonstrated that the BLM administration induced severe lung damage in the saline group (Fig. 3B). In addition, the BLM treatment significantly increased the hydroxyproline content of the lung as compared with the control group (Fig. 3C). These phenomena were all significantly suppressed by the CO-HbV treatment, but these effects were negligible in the case of the HbV treatment.

Moreover, to evaluate possible changes of respiratory function and lung mechanics associated with pulmonary fibrosis, we measured FVC and elastance. Based on data obtained using a computer-controlled ventilator, FVC clearly decreased in the BLM-treated mice and that this decrease was significantly suppressed by treatment with CO-HbV (Fig. 3D). The changes in lung mechanics associated with pulmonary fibrosis are characterized by an increase in elastance. Total respiratory system elastance (elastance of the total lung, including the bronchi, bronchioles, and alveoli) and tissue elastance (elastance of the alveoli) increased following BLM treatment, effects that were partially restored by the

Table 1
Plasma clinical chemistry test results in control mice and BLM-induced pulmonary fibrosis mice after saline, HbV and CO-HbV administration.

	Control	Day 7			Day 14		
		BLM + saline	BLM + HbV	BLM + CO-HbV	BLM + saline	BLM + HbV	BLM + CO-HbV
AST	34.8 ± 3.5	67.3 ± 18.5	73.7 ± 20.3	74.0 ± 24.3	61.7 ± 35.0	53.8 ± 7.3	52.9 ± 16.8
ALT	23.9 ± 3.6	46.3 ± 14.7	62.0 ± 17.3	61.0 ± 28.6	43.1 ± 21.0	33.0 ± 7.9	37.0 ± 15.1
ALP	317.9 ± 66.2	315.7 ± 106.4	302.8 ± 67.3	334.0 ± 75.8	313.8 ± 39.2	357.8 ± 54.0	324.3 ± 65.1
BUN	22.7 ± 3.7	28.3 ± 3.3	27.8 ± 5.0	30.0 ± 5.4	22.2 ± 3.3	24.1 ± 3.1	22.6 ± 1.8
CRE	0.10 ± 0.03	0.14 ± 0.02	0.10 ± 0.03	0.13 ± 0.03	0.10 ± 0.02	0.11 ± 0.01	0.10 ± 0.01
CK	105.6 ± 35.7	130.3 ± 55.6	105.2 ± 48.3	96.5 ± 29.9	224.9 ± 255.8	69.4 ± 22.1	70.3 ± 15.2
LDH	152.6 ± 42.1	301.4 ± 135.2	341.0 ± 63.6	258.7 ± 66.1	263.0 ± 141.5	278.2 ± 90.5	237.4 ± 45.0
AMY	2052.1 ± 318.2	2739.7 ± 459.7	2619.7 ± 457.1	2480.3 ± 347.4	2036.9 ± 335.1	2520.0 ± 394.6	2292.4 ± 295.7
T-CHO	125.6 ± 24.1	119.1 ± 25.9	160.7 ± 25.1	143.0 ± 23.8	127.7 ± 13.9	118.4 ± 16.4	131.3 ± 23.9

AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; BUN, urea nitrogen; CRE, creatinine; CK, creatine kinase; LDH, lactate dehydrogenase; AMY, amylase; T-CHO, total cholesterol.

administration of CO-HbV (Fig. 3E and F). These results suggested that CO-HbV could be therapeutically beneficial for the treatment of BLM-induced pulmonary fibrosis.

3.3. *Effect of CO-HbV on BALF cells, and inflammatory cytokines and chemokine levels in lung tissue*

It is well-known that the inflammation plays an important role in the pathogenesis of IPF, in view of the presence of interstitial and alveolar inflammatory cells as well as the expression of inflammatory cytokines in the lungs of patients with IPF [33,34]. We postulated that the inhibition of pulmonary fibrosis by CO-HbV might contribute to the anti-inflammatory effect of CO [8,9]. As an indicator of inflammation, the cells in BALF were analyzed. As a result, the administration of BLM resulted in a significant increase in the number of inflammatory cells (total cells: Fig. 4A), alveolar macrophages (Fig. 4B) and neutrophils (Fig. 4C) on days 3 after BLM administration. The CO-HbV treatment significantly reduced all types of cells in the BALF.

We also examined the effect of CO-HbV on TNF-α, IL-6 and IL-1β levels in the lung tissue of BLM-induced pulmonary fibrosis at days 7. As shown in Fig. 5, the levels of TNF-α (Fig. 5A), IL-6 (Fig. 5B) and IL-1β (Fig. 5C) in lung tissue were increased by BLM were significantly decreased as the result of the CO-HbV treatment. These data suggest that CO-HbV exerts an anti-inflammatory action against BLM-induced pulmonary damage, and consequently ameliorates BLM-induced pulmonary fibrosis.

3.4. *Effect of CO-HbV on ROS in lung tissue*

A number of studies have suggested that the cellular redox state and the balance of oxidants/antioxidants play a significant role in the progression of pulmonary fibrosis in animal models and also possibly in human IPF [35]. To evaluate the effect of CO-HbV on ROS induced by the BLM treatment in the lung, immunostaining of 8-OH-dG and NO_2-Tyr, an oxidation product derived from nucleic acids and proteins, in lung sections were performed on day 3 after the BLM administration. As shown in Fig. 6A, the accumulation of 8-OH-dG (upper) and NO_2-Tyr (lower) in lung tissue increased in the BLM-treated mice as compared to control mice, while CO-HbV clearly suppressed the levels of these oxidative stress markers in the lungs.

Recent reports have suggested that ROS generation by the Nox family NADPH oxidases, especially Nox4, might be implicated in the pathogenesis of IPF [36,37]. In order to evaluate the ROS derived from Nox4, we examined superoxide production in lung tissue. As a result, the BLM treatment showed an obvious increase in superoxide production, Nox4 activity, while CO-HbV treatment suppressed superoxide production (Fig. 6B). However, no difference in the protein expression of Nox4 between saline and CO-HbV was found, as evidenced by immunostaining and western blotting analysis (Fig. 6C and D). Although very little is known concerning the pathway of Nox4 activity, it is well known that $p22^{phox}$ and Poldip2 are important regulators of Nox4 activity [38]. Thus, we next determined the protein expression of $p22^{phox}$ and Poldip2 at 7 days after BLM administration. Similar to the increase in the protein expression of Nox4, the protein expression of $p22^{phox}$ was also increased by BLM treatment (Fig. 6E). On the other hand, the protein expression of Poldip2 was decreased by the BLM treatment (Fig. 6F). Interestingly, no change was found in the expression of both $p22^{phox}$ and Poldip2 between the saline and CO-HbV treatment (Fig. 6E and F). These results indicate that CO derived CO-HbV suppressed the superoxide production generated by Nox4 without any detectable changes in the protein expression of Nox4, $p22^{phox}$ and Poldip2, indicating that CO suppressed Nox4 activity *via* a currently unknown pathway.

3.5. *Effect of CO-HbV on active TGF-β1 levels in lung tissue*

TGF-β1 has been reported to play pivotal roles in the progression of pulmonary fibrosis, including fibroblast proliferation and collagen deposition [39]. To reveal the mechanism underlying the suppressive effect of CO-HbV on BLM-induced pulmonary fibrosis, the levels of active TGF-β1 in lung tissue on day 14 were determined. As shown in Fig. 7, the level of active TGF-β1 was increased in the BLM-treated mice, while CO-HbV decreased the level of active TGF-β1 to the same level as the control group.

4. Discussion

In present study, we evaluated the therapeutic effects of CO-HbV on IPF and investigated the impact of CO on the pathogenesis of IPF using a BLM-induced pulmonary fibrosis mice model. Three major findings were uncovered in the investigation. First, CO-HbV suppressed the progression of pulmonary fibril formation and improved respiratory function. Second, the mechanism underlying the suppressive effect of CO-HbV on BLM-induced pulmonary fibrosis can be attributed to the anti-oxidative and anti-inflammatory effects of CO. Furthermore, ROS generation was decreased as the result of the inhibition of the activity of the NADPH oxidase family, which is an important role in the pathogenesis of IPF, with no detectable changes in its protein expression. Finally, it can be concluded that HbV has considerable potential for effectively delivering CO to the lungs, suggesting that CO-HbV has promise for use as an effective CO donor.

Guidance on the diagnosis and management of IPF updated by the American Thoracic Society (ATS), European Respiratory Society (ERS), Japanese Respiratory Society (JRS) and Latin American

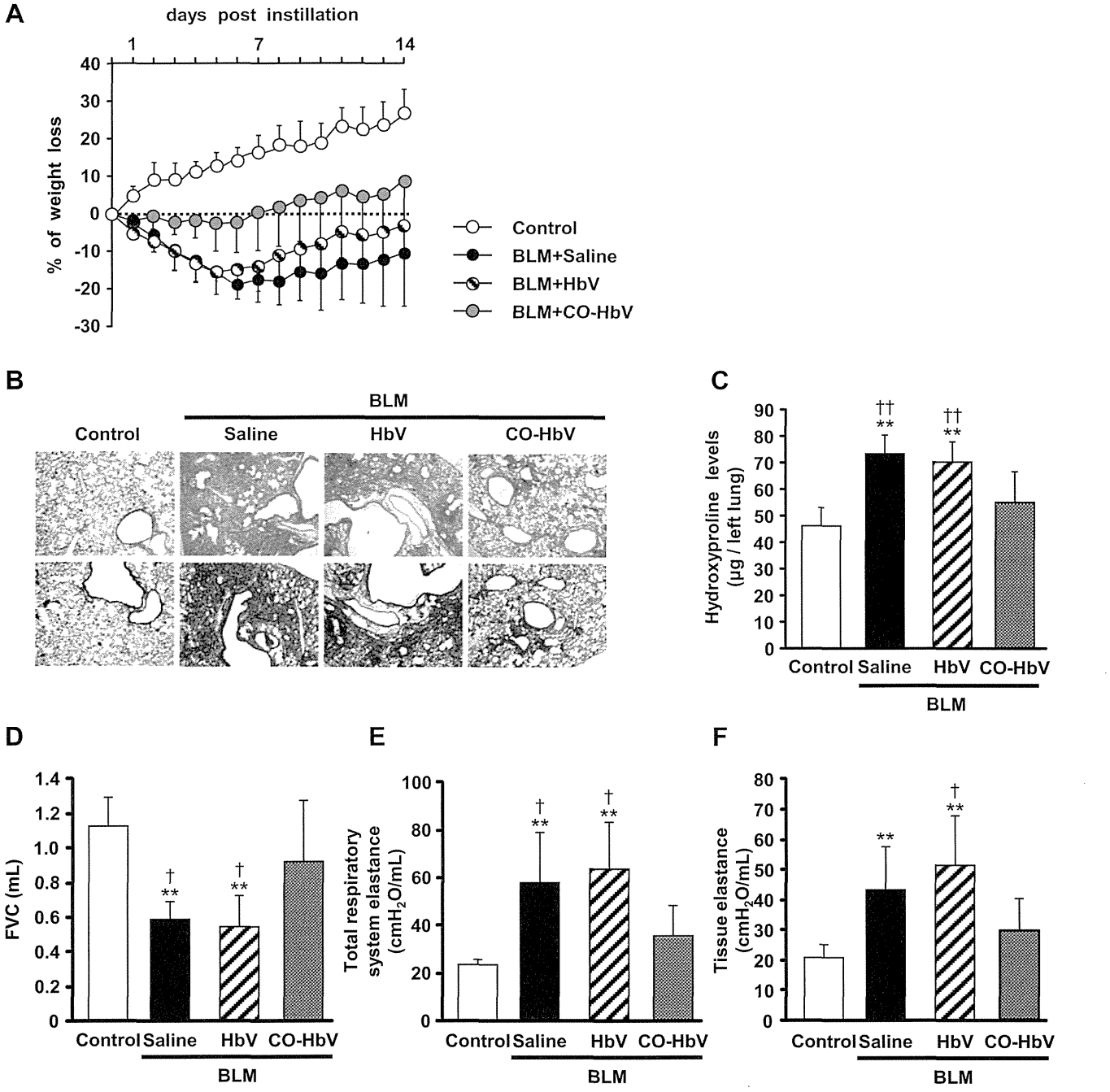

Fig. 3. Effects of CO-HbV against bleomycin-induced pulmonary fibrosis and alterations in lung mechanics. (A) The weight differences during 14 days after BLM treatment. Mice were treated with bleomycin (BLM, 5 mg/kg) once on day 0. They were also administered with saline, HbV (1000 mg Hb/kg) or CO-HbV (1000 mg Hb/kg) *via* the tail vein at 30 min before BLM treatment and 24 h after BLM treatment. Each value represents the mean ± s.d. ($n = 4–5$). (B) Histopathologic evaluation at after saline, HbV or CO-HbV treatment on day 14 in BLM-induced pulmonary fibrosis mice. Sections of pulmonary tissue were prepared on day 14 and subjected to hematoxylin and eosin staining (upper panels) and Masson trichrome staining (lower panels). (C) Hydroxyproline levels in left lung at after saline, HbV or CO-HbV treatment on day 14 in BLM-induced pulmonary fibrosis mice. The pulmonary hydroxyproline level was done on day 14 as described in Fig. 2 legend. Each value represents the mean ± s.d. ($n = 3–7$). $^{**}P < 0.01$ versus control. $\dagger\dagger P < 0.01$ versus CO-HbV. (D–F) The lung mechanics and respiratory functions at after saline, HbV or CO-HbV treatment on day 14 in BLM-induced pulmonary fibrosis mice. Forced vital capacity (D), total respiratory system elastance (E) and tissue elastance (F) were determined on day 14 as described in the "Materials and methods" section. Each value represents the mean ± s.d. ($n = 4–5$). $^{**}P < 0.01$ versus control. $\dagger\dagger P < 0.01$ versus CO-HbV. $\dagger P < 0.05$ versus CO-HbV.

Thoracic Association (ALAT) gave a 'weak no' recommendation to pirfenidon therapy, which is only drug approved for clinical use. Use of the drug can produce side effects (photosensitivity) and its effect on reducing pulmonary issues is small [40]. Therefore, it is important to examine the effect of candidate drugs on the progression of pulmonary fibrosis, lung mechanics as well as side effects. In the present study, severe pulmonary fibrosis induced by BLM was dramatically suppressed by intravenous CO-HbV administration (Fig. 3B and C). Furthermore, CO-HbV suppressed a BLM-induced increase in lung elastance and a decrease in FVC (Fig. 3D–F), indicating that CO-HbV could be beneficial for the treatment of patients with IPF. In addition, there were no changes of serum laboratory parameters reflecting hepatic, renal and pancreatic function for the experimental period after CO-HbV