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A novel vWF A3-GPI modified EPCs to enhance its adhesion ability to collegen☆

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Abstract

Objective: To gain purified recombination protein rGPI-vWF A3 which can guide endothelial progenitor cells (EPCs) gathering to the internal surface of the injured vessels theoretically and analyze its bioactivity in vitro. **Methods:** The plasmid vector of vWF A3 fusion protein which contained GPI structure was constructed and recombination protein rGPI-vWF A3 was expressed and its bioactivity was analyzed by ELISA and FCM. **Results:** Fusion protein vWF A3-GPI was expressed and purified by immunoaffinity chromatography and it could stably anchored on the surface of EPCs and showed its biological activities of collagen conjunction. **Conclusion:** These findings represent a novel strategy in EPC transplantation treatment for vessel injury.

Keywords: vWF A3-GPI; Protein; endothelial progenitor cells; Collagen

1. Introduction

The homing of endothelial progenitor cells (EPCs) to injured vessels requires adhesion

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molecules on the vascular endothelium adjacent to the tissue. There is evidence for circulation-naive EPCs and transplantation EPCs homing into vessels [1–3]. It has been demonstrated that in diabetes mellitus, expression of cell adhesion molecules such as vascular cell adhesion molecules (*e.g.*, stromal cell-derived factor-1α (SDF-1α) and vascular endothelial growth factor (VEGF)) is actively downregulated in the endothelium [4, 5]. EPCs may be unresponsive to granulocyte-colony stimulating factor (G-CSF) and fail to upregulate expression of cell adhesion molecules [6].

One of the earliest responses of injured vessels is deposition of the extracellular matrix and platelet adhesion to it [7–9]. In vascular lesions, this adhesion is specific for von Willebrand factor-A3 (vWF A3) binding collagen. This suggests that the extracellular matrix could serve as a receptor to target EPCs to injured vessels by expressing vWF A3 onto the surface of EPCs [10–12]. Nevertheless, autologous EPCs are primary cells and gene transfection is not ideal. We therefore applied a novel strategy to insert the collagen ligand vWF A3 into primary EPCs instead of genetic manipulation. This technology, termed "cell-surface painting", makes use of the unusual capacity of purified glycosylphosphatidylinositol (GPI)-anchored (gly-piated) proteins to reintegrate into the plasma membrane of any target cell([13–18], reviewed in [19]). Based on this biochemical property, it is possible to express exogenous proteins at the cell surface without the need for transfection.

We investigated if GPI-linked vWF A3 (vWF A3-GPI)-painted primary isolated rat EPCs would mediate tight adhesion to collagen and if the cell surface painted with vWF A3-GPI would retain its native function *in vitro*.

2. Materials and methods

The investigation was confirmed with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication number 85-23, revised 1996). Approval of the study protocol was granted by the Ethics Review Board of the Third Military Medical College (Chongqing, China).

2.1. Animals, cells and reagents

Wistar male rats were obtained from the Third Military Medical College. EPCs were isolated by density gradient centrifugation with Ficoll separating solution. Cells were plated on fibronectin-coated 24-well plates ($1\times10^6/cm^2$) in 0.5 ml Dulbecco's modified Eagle's medium

(DMEM) supplemented with 20% fetal calf serum (FCS), VEGF (50 ng/ml), basic fibroblast growth factor (bFGF; 5 ng/ml), epidermal growth factor (EGF; 10 ng/ml), penicillin (100 U/ml), and streptomycin (100 U/ml). Four days after culture, nonadherent cells were removed by thorough washing with phosphate-buffered saline (PBS). The medium was changed every three days. CHO was grown in DMEM supplemented with 10% FCS, 5 U/ml (penicillin, 5 μg/ml streptomycin, and 10 μg/ml neomycin).

2 2. Cloning of vWF A3-GPI and GPI-linked red fluorescent protein (RFP-GPI)

vWF A3 gene fragments were amplified from the total RNA of human umbilical vein endothelial cells by reverse transcription-polymerase chain reaction (RT-PCR). RNA LA PCR kit Ver. 1.1 (Takara Bio, Japan) was used with the following RT-PCR primers; FW-vWF A3 (5′- CGC TCG AGA TGA TTC CTG CCA GAT TTG CCG GGG TGC TGC TTG CTC TGG CCC TCA TTT TGC CAG GGA CCC TTT GTT CCC CTG CAC CTG ACT GC-3′) and RV-vWF A3 (5′-ATG TCG ACC CTA ACA AAT CCA GA GCA-3′). GPI gene fragments were amplified from the total RNA of human umbilical mononuclear cells by RT-PCR. The RT-PCR primers were: FW-GPI (5′-ATG TCG ACG ACT ACA AGG ACG ACG ATG ACA AGT CTA GAC CAA ATA AAG GAA GTG GA-3′) and RV-GPI (5′-TAG GTA CCC TAA GTC AGC AAG CCC ATG -3′). Amplified gene fragments encoding vWF A3 and GPI, respectively, were introduced into the *Xho*I-*Sal*I and *Sal*I-*Kpn*I sites and cloned into *Xho*I-*Kpn*I sites of the cloning plasmid pcDNA 3.1(-) (Invitrogen, USA) and confirmed by DNA sequencing analysis. The 5′-terminus of the vWF A3 gene fragment was tagged with a sequence encoding secretion peptide derived from the RT-PCR primer FW-vWF A3. The 5’-terminus of the GPI gene fragment was tagged with a sequence encoding encoding DDDDK derived from the RT-PCR primer FW-GPI. The resulting plasmid was named pcDNA3.1(-)-vWF A3-GPI.

RFP gene fragments were amplified from plasmid pDsRed2-1 by PCR with the following primers; FW-RFP (5′-CGC TCG AGA TGA TT C CTG CCA GAT TTG CCG GGG TGC TGC TTG CTC TGG CCC TCA TTT TGC CAG GGA CCC TTT GTG CCT CCT CCG AGA ACG-3′) and RV-RFP (5′- CGG TCG ACC AGG AAC AGG TGG TGGC-3′). Its PCR product was digested and subcloned into the *Xho*I-*Kpn*I sites of pcDNA3.1 (-)-vWF A3-GPI.

2.3. Transfection of GPI-anchored proteins into CHO producer cells

CHO cells were stably transfected by the calcium phosphate procedure and G-418-resistant CHO clones were established. Expression of GPI-linked (glypiated) constructs was analyzed by flow cytometry in a FACStar (Becton Dickinson, Erembodegen, Belgium) using mouse-anti-Flag monoclonal antibody M2 (Sigma, Buchs, Switzerland) and PE-conjugated goat anti-mouse monoclonal antibody (Milan Analytica AG, La Roche, Switzerland).

2.4. Purification of GPI-anchored proteins

Twenty 15-cm dishes of confluent CHO transfectants expressing vWF A3-GPI or RFP-GPI were washed twice with PBS. Cells were resuspended in 15 ml of ice-cold homogenization buffer (20 mmol/L sucrose, 1 mmol/L $MgCl_2$, 20 mmol/L Tris-HCl pH 7.4, 20 μg/ml benzamidine, 4 μg/ml antipain, 0.25 μg/ml leupeptin). Cells were kept on ice for 10 min and then homogenized using a tight-fitting glass dounce homogenizer (25 strokes). The homogenate was spun for 5 min at 500×*g* and the supernatant retained. To maximize yield, pelleted nuclei were further washed twice with homogenization buffer and the supernatants added to the first harvest. The final volume of the supernatants was adjusted to 50 ml and Brij78 (Fluka, Buchs, Switzerland) was added to a final concentration of 0.5%. Supernatants were solubilized for 5 h at 4 ℃. After centrifugation (30 min, 5 000×*g*), the two GPI-linked molecules were separately affinity-purified on an anti-Flag-coupled agarose column (Sigma) and extensively washed with 20 mmol/L Tris-HCl pH 7.0 containing 0.5% Brij78 followed by PBS. Purified proteins were eluted with 0.1 mol/L glycine pH 2.8. Fractions of 500 μl were collected and neutralized with 60 μl 1 mol/L Tris-HCl pH 8.0. Purified proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by silver staining and Western blotting using anti-Flag mAb. Aliquots were stored at −80 ℃; sequential thawing and freezing was avoided.

2.5. Cell adhesion assay

Ninety-six-well plates were coated overnight at 4 ℃ with 100 μl/well of pepsin-digested bovine type I, III or IV collagen (Sigma, USA) at 20 μg/ml in PBS. vWF A3-GPI- or RFP-GPI-transfected cells ($0.2/0.4/0.6/0.8/1.0/1.2\times10^4$) in 100 μl of

culture were added per well. After 15 min at 37 ℃, unbound cells were removed by 4–6 washes with pre-warmed culture. Bound cells were evaluated by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazoli um bromide (MTT) hydrolysis using Cell Counting Kit-8. One-hundred microliters of WST-8 (Dojindo, Japan) were added per well and incubated for 2 h at 37 ℃. The extent of reduction of WST-8 to formazan was quantified by measuring optical density at 450 nm.

2.6. Phosphatidylinositol-specific phospholipase-C (PI-PLC) cleavage of vWF A3-GPI or RFP-GPI

In order to assay the activity of rhHAPO, cell attachment assay was performed. 96-well plates were coated with 10 μg/ ml fibronectin (FN) (Sigma) in PBS at 4 ℃ overnight. The plates were then treated with 0.5% BSA for 30 min at room temperature to block nonspecific binding sites. Immediately prior to adding ECV304 cells, wash wells 3 times with 0.1 ml PBS. All cells were harvested with 5 mmol/L EDTA in PBS, resuspended in Dulbecco's modified Eagle's medium with 2% fetal bovine serum, at 2×10^5/ml. Then, 100 μl were added to each well and rhHAPO was added to make a series of final concentrations (0, 5, 10, 50, 100, 500, or 1 000 ng/ml). The plates were incubated for 30 min at 37 ℃, 5% CO_2 and then washed twice with PBS to remove the unbound cells. The adherent cells were fixed with 3% formaldehyde, 30% ethanol in PBS containing 0.5% crystal violet for 15 min. After two gentle washes in PBS, add 0.1 ml DMSO to lyse cells and read absorbance at 570 nm on a microtiter plate reader.

2.7. Cell-surface painting with vWF A3-GPI or RFP-GPI

For cell surface painting with vWF A3-GPI or RFP-GPI, target cells were washed twice, resuspended in culture medium to $\leq10^7$ cells/ml, and incubated with 0.4 μg/ml purified GPI-anchored proteins for 120 min at 37 ℃ with occasional mixing. Cells were washed extensively and the efficiency of painting measured by FACS analysis.

2.8. Purified protein binding collagen

Ninety-six-well plates were coated with type III collagen overnight at 4 ℃. After discarding the solutions and washing with PBS containing 0.05% Tween 20, blocking reagent (BlockAce; Dai-Nippon Pharmaceuticals, Osaka, Japan) was incubated overnight at 4 ℃ in the collagen-coated

well. Plates were intensively washed with PBS-T. Plates were then incubated with 100 μl/well of serial dilutions of vWF A3-GPI or RFP-GPI in DMEM at 37 ℃ for 1 h. After washing with PBS-T, wells were reacted with anti-Flag monoclonal antibody (26503, R&D Systems). Bound antibodies were detected by enzyme-linked immunosorbent assay (ELISA) with horseradish peroxidase (HRP)-conjugated anti-mouse IgG rabbit poly-clonal antibodies (Dako) with H_2O_2 and o-phenylenediamine. Collagen binding was determined by subtracting the absorbance at 660 nm from that at 490 nm.

2.9. Statistical analysis

Values are presented as *mean*±*SD*. One-way Anova was carried out for comparison between two measurements. $P<0.05$ was considered significant.

3. Results

3.1. vWF A3-GPI: an collagen-binding molecule

The fusion protein vWF A3-GPI binds with high affinity to collagen. Our aim was to direct primary EPCs into injured intima using this ligand, without the need for constitutive expression of vWF A3 and viral transfection, which could lead to detrimental effects *in vivo* in the long term. To achieve this, we equipped the plasma membrane of primary EPCs with vWF A3-GPI. This strategy, called painting, can be performed in standard medium and does not interfere with EPCs function [2, 4, 9–11]. vWF A3-GPI was constructed using the GPI anchor derived from the decay accelerating factor (DAF). An inserted Flag epitope enabled affinity purification of the construct. As a control, we generated RFP-GPI without the collagen domain from red fluorescent protein (RFP).

CHO cells were stably transfected with vWF A3-GPI and RFP-GPI and cell surface expression was determined by FACS analysis (Fig.1). CHO clones showing high expression levels were selected for the purification of the glypiated proteins. To confirm that the newly generated molecules were indeed inserted into the plasma membrane of the cell *via* a GPI anchor, we incubated the cells with phosphatidylinositol-specific phospholipase C (Fig. 1), which specifically cleaves most GPI-linked proteins [8, 12], including vWF A3-GPI and RFP-GPI.

We assayed collagen-binding property of the vWF A3-GPI-transfected cells (vWF A3-GPI-CHO). Fig. 2 shows that vWF A3-GPI-CHO specifically bound to collagens in a dose-dependent

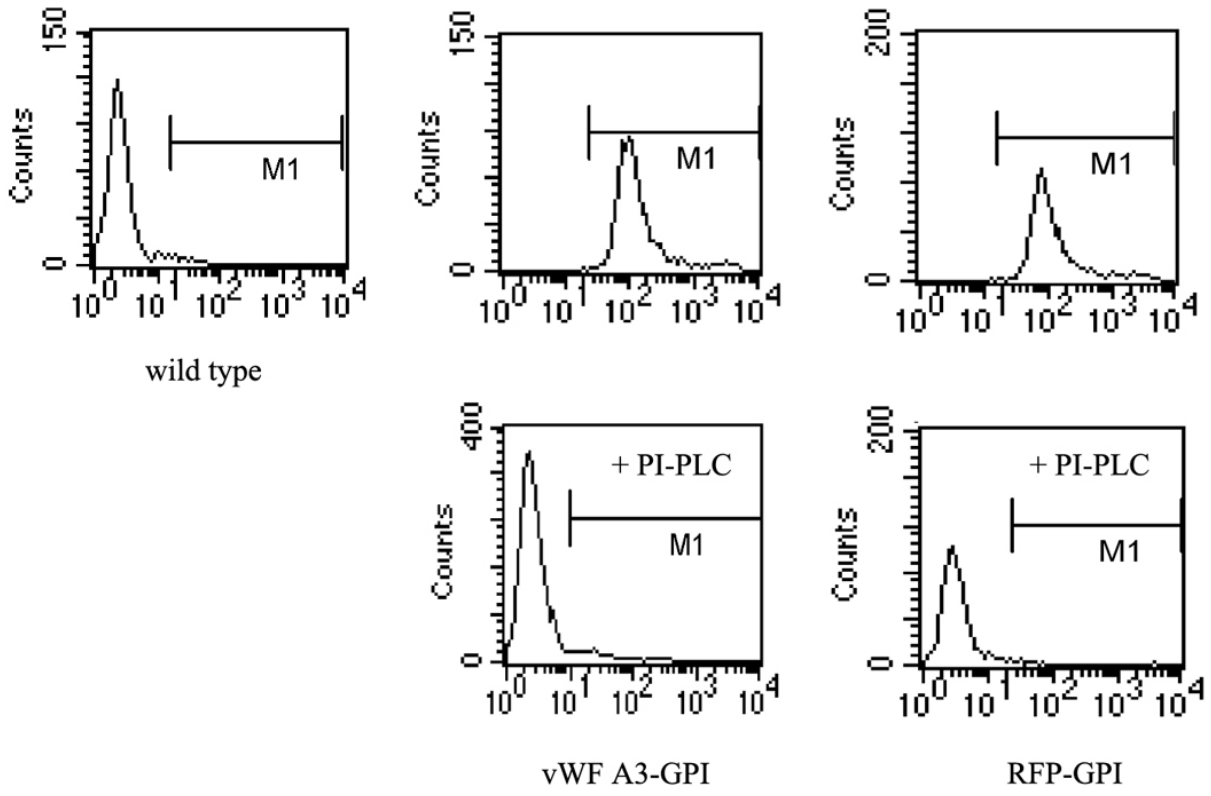

Fig. 1. GPI-linked molecules expressed on CHO cells. vWF A3-GPI and RFP-GPI were stably transfected into CHO producer cells. The GPI-anchored proteins were then cleaved by PI-PLC treatment for 2 h. Cell surface expression of the fusion proteins was examined by FACS staining using an anti-Flag mAb.

manner, proving that vWF A3 retained its collagen-binding function. vWF A3-GPI-CHO showed the highest affinity to type III collagen, medium affinity to type I collagen, and the lowest to type IV. In contrast, no binding of RFP-GPI-CHO was observed to any type of collagen(data not shown). These results indicate that the cells expressing a GPI-linked form of vWF A3-GPI specifically adhere to major collagen types, preferentially to type III collagen that is known to play a major role in tissue remodeling after injury.

3.2. Painting of primary lymphocytes with vWF A3-GPI

vWF A3-GPI and RFP-GPI were purified by affinity chromatography from transfected CHO cell lysates. SDS-PAGE analysis of purified proteins revealed the predicted apparent molecular masses of 60 kDa and 68 kDa for vWF A3-GPI and

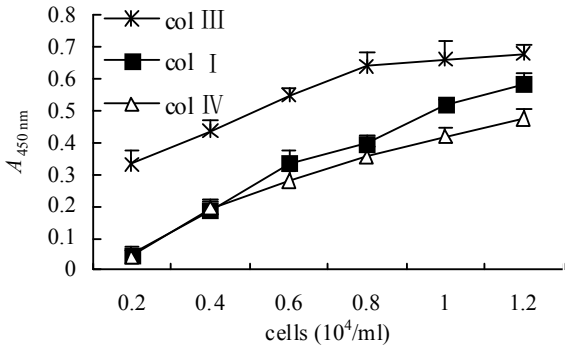

Fig. 2. Specific adhesion of vWF A3-GPI-transfected cells to collagen. vWF A3-GPI-expressing CHO cells were incubated in wells previously coated with 20 μg/ml type I, III and IV collagen and bound cell was evaluated by MTT hydrolysis using Cell Counting Kit-8.

RFP-GPI, respectively (Fig. 3). For cell surface painting, affinity-purified glypiated proteins were incubated with cell lines and primary cells in culture medium. Painting of vWF A3-GPI and RFP-GPI was comparable on rat and mouse EPCs (Fig. 4A). This demonstrates that cell surface painting is applicable and efficient for many cell types. Insertion of the GPI-linked molecules into the plasma membrane was observed as early as 1 h following incubation at 37℃ reaching a plateau at 2 h. Incubation for prolonged periods (up to 4 h) did not increase the painting efficiency (data not shown). Reduced cell surface expression was

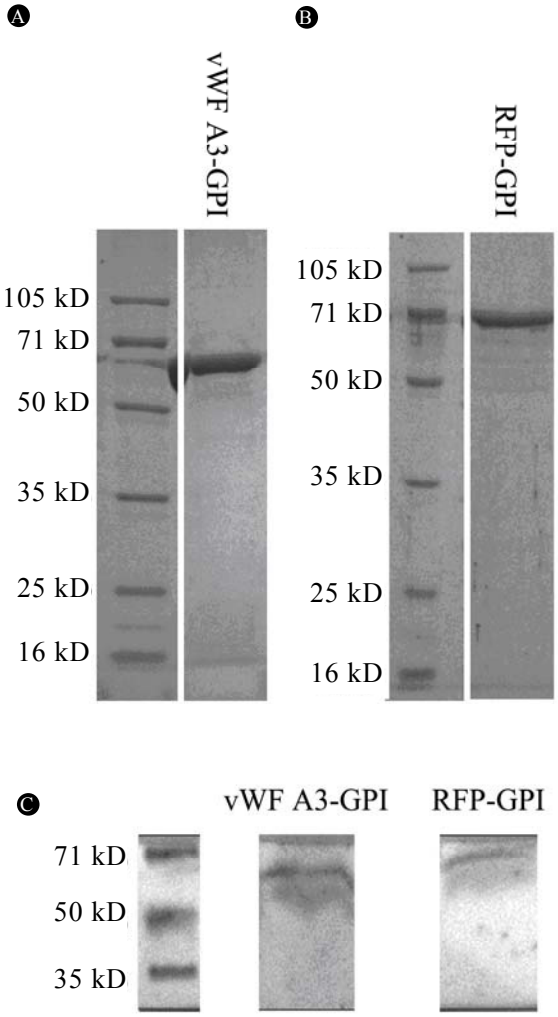

Fig. 3. Purification of glypiated vWF A3 and RFP. GPI-linked proteins from transfected CHO cells lysates were affinity-purified using an anti-Flag mAb column. Purified proteins were analyzed by SDS-PAGE (A) and silver staining (B) or Western blotting using an anti-Flag mAb (C). The molecular masses of standard proteins are indicated on the left.

observed when the painting procedure was performed at either 4℃ or room temperature (data not shown). The expression of cell surface-painted molecules remained stable at the cell surface for up to 12 h, and decreased thereafter relative to the rate of cell division (Fig. 4B).

3.3. vWF A3-GPI-painted EPCs adhere to collagen

Primary rat EPCs+ painted with vWF A3-GPI specifically adhere to coated collagen but not to BSA as a control protein. Unpainted EPCs were unable to bind to either substrate. The adhesion of vWF A3-GPI painted rat EPCs gradually augmented with increasing collagen concentration. Maximal binding was reached at 1 μg/ml of coated collagen. EPCs painted with vWF A3-GPI, but not RFP-GPI, specifically bound to collagen. Painted cells also bound to immobilized anti-FLAG mAb, demonstrating that plasma membrane insertion of the chimeric molecules was efficient. Untreated EPCs adhered weakly to immobilized anti-FLAG mAb. Similar results were obtained for painted mouse EPCs cells (data not shown). In conclusion, cell surface painting is an efficient method to insert vWF A3-GPI into the plasma membrane of various cell types, including primary cells. See Fig. 5.

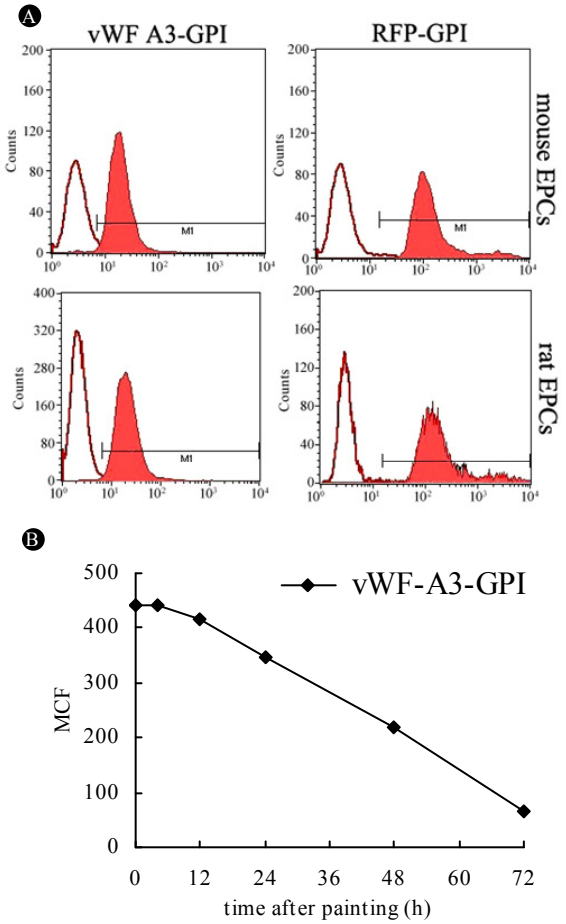

Fig. 4. Cell surface painting with purified vWF A3-GPI and RFP-GPI. A: Mouse EPCs were incubated for 2 h in the presence (filled histograms) or absence (open histograms) of purified vWF A3-GPI (left panel) or RFP-GPI (right panel), respectively. After extensive washing of the cells, plasma membrane-inserted glypiated proteins were quantified by FACS analysis using the anti-Flag mAb M2. B: Kinetics of the surface expression of painted GPI-anchored proteins. Rat EPCs were painted with affinity-purified vWF A3-GPI, washed and surface expression of the chimeric proteins was analyzed by flow cytometry at different time points after cell culture.

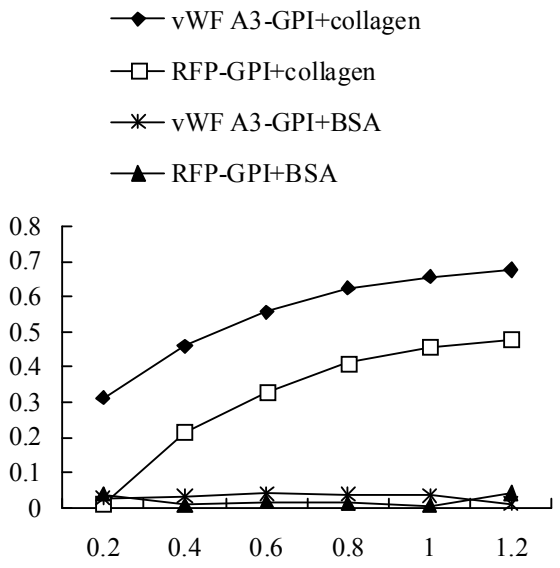

Fig. 5. Painted EPCs adhere to collagen.

4. Discussion

We present evidence that novel molecular vWF A3-GPI painted EPCs can specifically bind to collagen *in vitro*.

The ultimate goal for EPC transplantation therapy is the homing of primary effector cells into vessels [20–23]. Successful transfection of primary cells is a challenging goal because of the low transfection efficiency seen with current methods. Constitutive expression may not be necessary for the desired function of the exogenously expressed protein (*e.g.*, homing molecule). As an alternative method for expressing vWF A3 in primary cells, we painted cells with a GPI-anchored form of vWF A3. Studies in humans with parasitic infections or mice transgenic for DAF have provided evidence that insertion of dissociated GPI-anchored proteins into cell membranes occurs *in vivo*. Addition of a glypiation signal to cDNA permits expression of the protein as a GPI-anchored form [19].

Painting offers several advantages over conventional gene transfection or viral infection methods. First, it is applicable to any type of cell, including primary cells, which are difficult to transfect. The efficiency of painting is high, and reveals homogenous insertion of the glypiated protein into the plasma membrane. Second, painting is rapid (maximal insertion is reached after 90 min of incubation of the GPI-linked protein with cells) and protein expression on the surface is immediate. Painting is carried out under normal culture conditions and does not require special treatment of cells. Third, painting is not restricted to a minimal number of cells. The introduced proteins remain stable on the cell surface for several hours, enabling them to mediate biological functions. Cell surface-painted molecules have been shown to retain full biological functions [15, 24–27].

Therefore, according to previous findings, we designed a novel vWF A3-GPI molecule and used it

to modify EPCs. We expected modified EPCs to target injured vessels and enhance rehabilitation of the vessel.

The cloned vWF A3-GPI was demonstrated by western blotting. Predicted apparent molecular masses of 60 kDa and 68 kDa for vWF A3-GPI and RFP-GPI, respectively, were presented.

We assayed the collagen-binding of vWF A3-GPI-transfected cells. vWF A3-GPI-CHO was specifically bound to collagens in a dose-dependent manner. Binding of RFP-GPI-CHO to any type of collagen was not observed. These results indicate that cells expressing a GPI-linked form of vWF A3-GPI specifically adhere to major collagen types (preferentially to type III collagen).

In summary, our study demonstrated that painting primary cells with a high-affinity ligand (the extracelluar matrix) offered a promising strategy for treatment of the damaged vasculature.

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