Porcine Extrahepatic Vascular Endothelial Asialoglycoprotein Receptor 1 Mediates Xenogeneic Platelet Phagocytosis In Vitro and in Human-to-Pig Ex Vivo Xenoperfusion

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Background. Asialoglycoprotein receptor-1 (ASGR1) mediates capture and phagocytosis of platelets in pig-to-primate liver xenotransplantation. However, thrombocytopenia is also observed in xenotransplantation or xenoperfusion of other porcine organs than liver. We therefore assessed ASGR1 expression as well as ASGR1-mediated xenogeneic platelet phagocytosis in vitro and ex vivo on porcine aortic, femoral arterial, and liver sinusoidal endothelial cells (PAEC/PFAEC/PLSEC). **Methods.** Porcine forelimbs were perfused with whole, heparinized human or autologous pig blood. Platelets were counted at regular intervals. Pig limb muscle and liver, as well as PAEC/PFAEC/PLSEC, were characterized for ASGR1 expression. In vitro, PAEC cultured on microcarrier beads and incubated with non-anticoagulated human blood were used to study binding of human platelets and platelet-white blood cell aggregation. Carboxyfluorescein diacetate succinimidyl ester–labeled human platelets were exposed to PAEC/PFAEC/PLSEC and analyzed for ASGR1-mediated phagocytosis. **Results.** Human platelet numbers decreased from 102 ± 33 at beginning to 13 ± 6 × 10^3/μL ($P < 0.0001$) after 10 minutes of perfusion, whereas no significant decrease of platelets was seen during autologous perfusions (171 ± 26 to 122 ± 95 × 10^3/μL). The PAEC, PFAEC, and PLSEC all showed similar ASGR1 expression. In vitro, no correlation was found between reduction in platelet count and platelet-white blood cell aggregation. Phagocytosis of human carboxyfluorescein diacetate succinimidyl ester–labeled platelets by PAEC/PFAEC/PLSEC peaked at 15 minutes and was inhibited ($P < 0.05$ to $P < 0.0001$) by rabbit anti-ASGR1 antibody and asialofetuin. **Conclusions.** The ASGR1 expressed on aortic and limb arterial pig vascular endothelium plays a role in binding and phagocytosis of human platelets. Therefore, ASGR1 may represent a novel therapeutic target to overcome thrombocytopenia associated with vascularized pig-to-primate xenotransplantation.

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Xenotransplantation of porcine organs and cells may help to expand the pool of available donor organs for clinical transplantation. However, despite considerable progress over the past decades, immune responses and other barriers prevent clinical application. The use of organs stemming from genetically modified pigs has prevented hyperacute rejection, antibody-mediated or cellular acute rejection, at least in animal models.[1-3] Nevertheless, other issues related to physiological and immunological incompatibilities, including thrombotic microangiopathy and systemic consumptive coagulopathy, need to be solved.[4-6] The latter are characterized by platelet activation, aggregation, and sequestration leading

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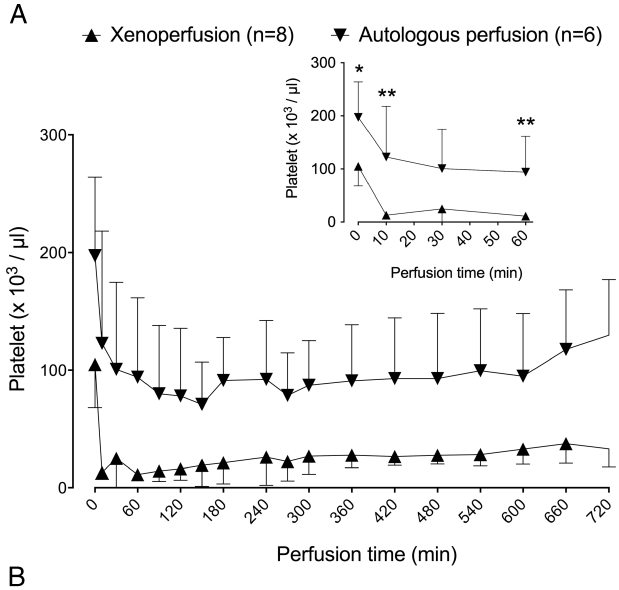


B

Unpaired t-Test (area under the curve over the whole perfusion period)	Significant?	Two-tailed p value
Xenoperfusion vs. Autologous perfusion	**	0.002

FIGURE 1. Platelet counts during ex vivo perfusion of porcine forelimbs with heparinized human or autologous pig blood. Forelimbs amputated from large white pigs of both sexes, weighing 30 to 40 kg, were connected to an extracorporeal perfusion circuit, and then xenogeneic or autologous perfusions were performed. Perfusate blood samples were collected at regular time intervals and platelets counted using a Sysmex analyzer. A, A rapid loss of platelet counts was observed in xenogeneic, but not in autologous perfusions. The inset shows the enlarged graph for the first 60 minutes of perfusion with indication of significant differences between autologous versus xenoperfusion (one-way ANOVA with Bonferroni correction for individual time points; $*P \leq 0.05$, $**P \leq 0.01$). Xenogeneic perfusions (xeno) (n = 8) and autologous perfusions (auto) (n = 6). B, Statistical analysis was carried out for area under the curve of the whole 720 minutes perfusions using the unpaired 2-tailed Student *t* test ($**P \leq 0.005$). ANOVA, analysis of variance.

to a dysregulation of the coagulation system and eventually to organ dysfunction.[7]

Thrombocytopenia has been observed previously in pig-to-primate xenotransplantation models, especially in pig-to-baboon liver transplantation[8] and during ex vivo porcine liver perfusion with human blood.[9] Although initial hepatic function, including coagulation, was proven to be satisfactory, the development of thrombocytopenia is currently precluding pig liver xenotransplantation, even as a "bridge" to allotransplantation.[10] Platelet activation and aggregation are critical events that might precede the development of thrombocytopenia and/or sequestration of platelets in xenografts.[11] However, platelet phagocytosis by endothelial cells through receptor-ligand interactions cannot be excluded. Recently, it has been shown that porcine Kupffer cells and liver sinusoidal endothelial cells (LSEC) bind and phagocytose human platelets in ex vivo perfusion systems.[12] Many scavenger receptors have been reported on LSEC that facilitate the clearance of particulate, molecular, and cellular "waste" from the circulation,[13] one of them being the asialoglycoprotein receptor-1 (ASGR1). It functions as a recycling receptor, which mediates capture and phagocytosis of Galβ1-4– and GalNAcβ1-4–terminating glycoproteins.[14,15] Other functions of ASGR1 that have been put forward include clearance of asialoglycoproteins[16] and apoptotic cells.[17] Asialoglycoprotein receptor-1 may also participate in cell-cell, cell-matrix, or intramatrix interactions.[18] The receptor is expressed on porcine Kupffer cells, hepatocytes,[19] and on porcine LSEC (PLSEC). Finally, ASGR1 on PLSEC mediates phagocytosis of human platelets.[20] Asialoglycoprotein receptor-1–mediated human platelet binding depends on the availability of carbohydrate binding sites on ASGR1 as well as exposed carbohydrate ligands on platelets, such as terminal Galβ1-4 and GalNAcβ1-4 residues. Recently, it has been shown that, compared to porcine platelets, human platelets have 4 times more Galβ1-4GlcNAc and GlcNAcβ1-4GlcNAc ligands for ASGR1.[21]

In summary, thrombocytopenia after liver xenotransplantation and ex vivo xenoperfusion is a well-recognized phenomenon. In contrast, little is known on platelet phagocytosis mediated by other endothelial sites. Here, we report that ex vivo xenoperfusion of amputated porcine forelimbs with human blood using extracorporeal perfusion[22] leads to an immediate loss of human platelets from circulation. We therefore examined ASGR1 expression on porcine vascular endothelial cells (EC) including aortic EC (PAEC), femoral arterial (PFAEC), and PLSEC. In addition, the role of ASGR1 for binding and phagocytosis of human platelets was tested in vitro.

MATERIALS AND METHODS

Ex Vivo Perfusion Model

Forelimbs of nontransgenic, large white pigs of both sexes, weighing 30 to 40 kg, were used to perform ex vivo perfusion with 500 mL of whole, heparinized (Liquemin, 10,000 IU/500 mL) anticoagulated human blood (xenoperfusion) or autologous blood (autologous perfusion), for up to 12 hours as described by Bongoni et al.[22] Biopsies and blood samples were collected at regular time intervals and blood cell counts performed using an automated hematology analyzer (Sysmex KX21N; Sysmex Suisse AG, Horgen, Switzerland) to measure changes in platelet counts.

Endothelial Cell Isolation and Culture

Porcine vascular EC (PAEC and PFAEC) were isolated using a previously described method.[23] The PLSEC isolation from porcine liver was performed according to a previously described method.[20,21]

Platelet Isolation and Carboxyfluorescein Diacetate Succinimidyl Ester Labeling

Freshly isolated human platelets were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) using CellTrace CFSE Cell Proliferation Kit (Life Technologies) according to manufacturer's protocols.

Immunofluorescence

Porcine ECs on 8-well Lab-Tek chamber slides (Milian Suisse SA, Vernier, Switzerland) and slides with 5 µm cryosections of porcine limb muscle were fixed in 3.7% formaldehyde or acetone, respectively, for 5 minutes and then incubated with endothelial specific antibodies: rat anti-pig CD31 (R&D Systems, Abingdon, United Kingdom), goat antihuman VE-cadherin (cross-reacting with porcine VE-cadherin, Santa Cruz Biotechnologies Inc., Dallas, TX), rabbit antihuman ASGR1 (cross-reacting with porcine ASGR1;[20] GenWay Biotech, San Diego, CA), and mouse antihuman

CD41 (FITC-labeled; Dako Schweiz AG, Baar, Switzerland) for 2 hours at room temperature or overnight at 4°C. Thereafter, slides were washed and exposed to fluorescence conjugated secondary antibodies: donkey anti-rat immunoglobulin (Ig) G Cy3 (Jackson Immunoresearch, West Grove, PA), donkey anti-goat IgG Alexa 488 (Molecular Probes, Leiden, The Netherlands), and sheep antirabbit IgG Cy3, (Sigma, St. Louis, MO). The slides were analyzed using a confocal laser-scanning microscope (LSM710; Zeiss, Jena, Germany).

In Vitro Human Blood Clotting Assay

The PAEC were cultured on collagen-coated Biosilon microcarrier beads as described previously by Kohler et al and Wuensch et al.[24,25] Beads with confluent PAEC were incubated with non-anticoagulated whole human blood. For more details, see **SDC**, http://links.lww.com/TP/B100).

In Vitro Platelet Phagocytosis—Cell Enzyme-Linked Immunosorbent Assay

Analysis of human platelet phagocytosis by porcine vascular EC was performed based on the assay described by Paris et al.[20] In brief, 4×10^7 CFSE-labeled human platelets were added to wells on 96-well plates containing confluent EC. For inhibition assay, EC were preincubated with polyclonal rabbit anti-ASGR1 antibody, polyclonal rabbit IgG as isotype control (Sigma) or asialofetuin (Sigma) for 30 minutes before addition of human platelets. The fluorescence of CFSE was acquired at 492 nm (reference wavelength 517 nm) using a Tecan Infinite M1000 microplate reader.

In Vitro Platelet Phagocytosis—Immunofluorescence Staining

The CFSE-labeled human platelets, 4×10^7, were added to confluent EC on 8-well chamber slides for 60 minutes at 37°C. After fixation, the slides were stained with mouse anti-pig CD107a (AbD Serotec, Kidlington, Oxford, United Kingdom), anti-CD31, anti-VE-cadherin, or anti-ASGR1 and appropriate fluorescence-labeled secondary antibodies. Slides were analyzed using a Zeiss LSM710 confocal laser-scanning microscope.

For further details in Materials and Methods, see **SDC**, http://links.lww.com/TP/B100.

RESULTS

Ex Vivo Perfusion Model of Xenotransplantation-Induced Platelet Loss

A rapid loss of circulating human platelets was observed during xenoperfusion of pig forelimbs with human blood, whereas in autologous pig blood perfusions only a slow decrease of platelet numbers was seen. The average platelet counts were $236.3 \pm 28.9 \times 10^3/\mu L$ in human blood and $301 \pm 67.2 \times 10^3/\mu L$ in pig blood. The counts dropped about 2-fold to $102.2 \pm 32.9 \times 10^3/\mu L$ and $171.3 \pm 26.4 \times 10^3/\mu L$, respectively, after the first run through the perfusion system before getting into contact with porcine tissue. These reductions in platelet counts are explained by the dilution of the blood with hydroxyethyl starch solution (Voluven, Fresenius) which was used for priming of the extracorporeal circuit. After 10 minutes of xenoperfusion with human blood, platelet counts dropped approximately 10-fold, from $102.2 \pm 32.9 \times 10^3/\mu L$ at the beginning to $13.0 \pm 6.1 \times 10^3/\mu L$ ($P = 0.048$). In autologous pig blood perfusions, the respective values after 10 minutes were not significantly lower than at onset ($171.3 \pm 26.4 \times 10^3/\mu L$), namely, $122.7 \pm 95.5 \times 10^3/\mu L$, but significantly ($P = 0.009$) higher than the ones of the respective xenoperfusions. After 60 minutes of perfusion, platelet counts were $11.3 \pm 4.1 \times 10^3/\mu L$ in xenoperfusions and $94.0 \pm 67.4 \times 10^3/\mu L$ in autologous perfusions, respectively, and stayed at these levels throughout the remaining perfusion time (720 minutes in total, Figure 1). Platelet counts, measured as area under the curve over the whole perfusion period, were significantly lower in xenogeneic perfusions ($P = 0.002$, t test) as compared to autologous perfusions.

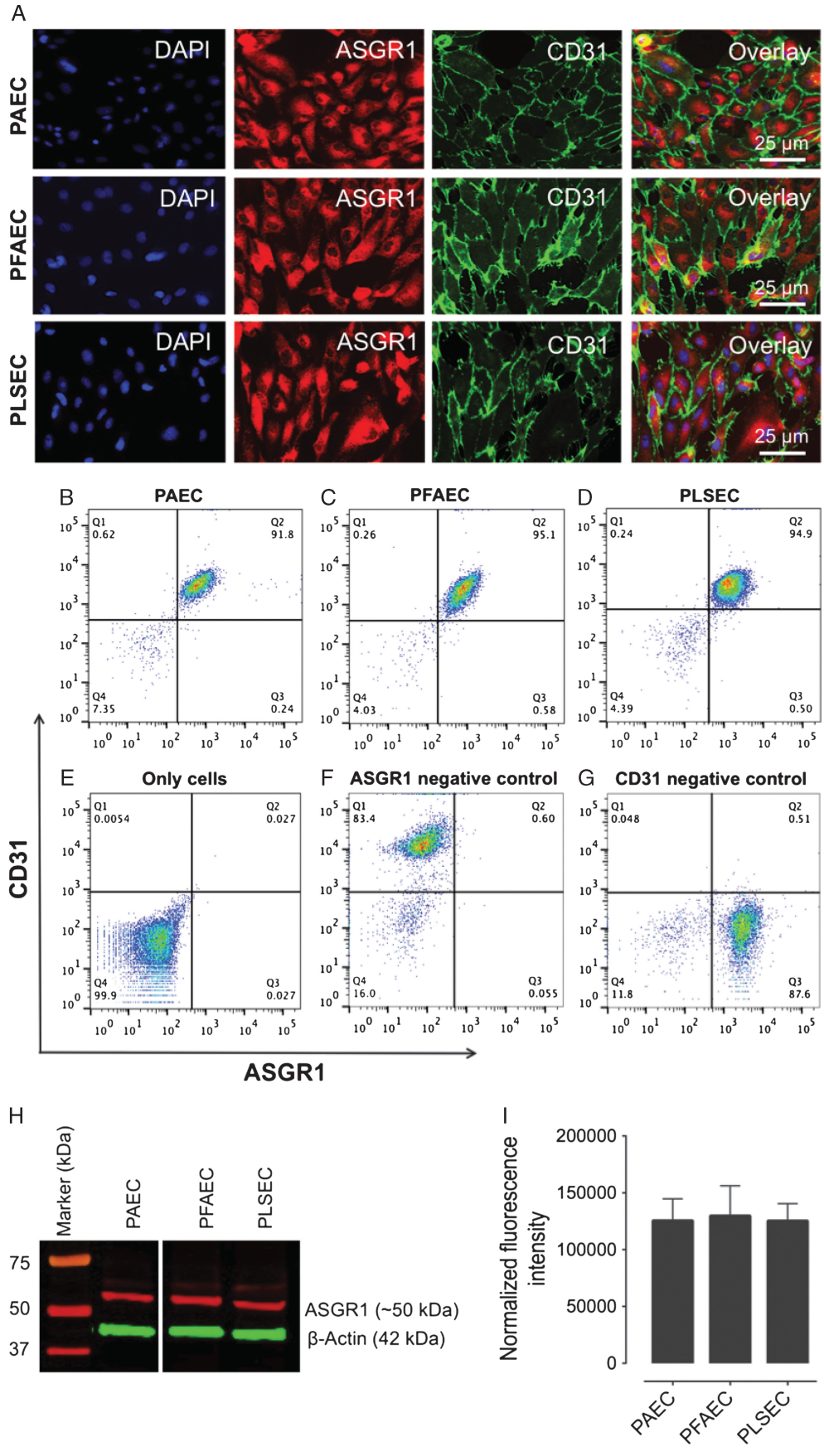
ASGR1 Expression by Porcine Vascular Endothelial Cells

As shown by immunofluorescence and confocal microscopy, cultured PAEC, PFAEC, and PLSEC were positive for CD31 and ASGR1 (Figure 2A) as well as positive for VE-cadherin and ASGR1 (**Figure S1A, SDC**, http://links.lww.com/TP/B100). Fluorescence intensity of ASGR1 staining was quantitated using ImageJ software (NIH), and significance was tested using 1-way analysis of variance with Bonferroni correction. No difference in ASGR1 expression was observed between the different types of EC tested ($P > 0.72$; **Figure S1B, SDC**, http://links.lww.com/TP/B100). Expression of ASGR1 on porcine vascular EC was further confirmed using flow cytometry. PAEC, PFAEC, and PLSEC were CD31/ASGR1 double positive for 91.5%, 95.1%, and 94.9% of the total cell numbers, with no difference in ASGR1 expression levels (Figure 2B to D). The respective controls were also tested and are represented in Figure 2E to G. Western blot analysis of whole cell lysates using PAEC, PFAEC, and PLSEC all showed ASGR1 protein expression as a band at ~50 kDa (Figure 2H), with no significant differences in expression levels as analyzed by ImageJ software (Figure 2I).

ASGR1 Protein Expression and Platelet Deposition on Pig Limb Tissue Samples

Porcine limb muscle (Figure 3A to B) and liver (Figure 3C to D) tissue samples were all positive for ASGR1 expression as assessed by immunofluorescence. The fact that staining for VE-cadherin and CD31, respectively, colocalized with ASGR1 in all biopsy sections suggests that ASGR1 is indeed expressed on endothelial cells, corresponding to the observations made in vitro using cultured porcine vascular EC. However, quantitative analysis showed significantly higher ($P < 0.0001$) ASGR1 staining on liver tissue as compared to limb, supposedly due to the known expression of ASGR1 on hepatocytes (Figure 3E).

Muscle tissue biopsies from xenoperfusions were also analyzed for human platelet infiltration and sequestration by staining for CD41, a human platelet marker. As shown in Figures 3F to G, a higher number of human platelets was found in the muscle tissue after 1 hour of perfusion (48 ± 11 platelets per high-power viewing field) as compared with 12 hours of perfusion (1 ± 2, $P < 0.0001$). Furthermore, fluorescence microscopy analysis of tissue samples after 1 hour of perfusion showed that sequestered platelets (CD41, green) were colocalized (yellow) with CD31 (Figure 3H, white arrows) as well as ASGR1 (red, Figure 3I, white



arrows), indicating binding of platelet aggregates to the endothelium via ASGR1.

Platelet Deposition on PAEC in a Microcarrier-Based in Vitro Xenotransplantation Model

PAEC grown on microcarrier beads were incubated with fresh, non-anticoagulated human blood. The mean clotting time in the presence of PAEC was significantly prolonged as compared to microcarrier beads without PAEC (with PAEC, 40.3 ± 8.1 minutes vs 12.8 ± 4.9 minutes without PAEC; 2-tailed *t* test, $P = 0.007$). Blood-bead mixture was sampled after 10 minutes and 20 minutes of incubation and analyzed for platelet-lymphocyte, platelet-neutrophil, and platelet-monocyte staining by flow cytometry. Platelet counts dropped from 277.0 ± 45.1 × 10^3/μL at baseline to 119.1 ± 88.4 × 10^3/μL ($P < 0.01$ vs baseline) after 10 minutes, and 35.0 ± 43.0 × 10^3/μL ($P < 0.01$ vs baseline; $P < 0.05$ vs 10 minutes) after 20 minutes of incubation. Baseline values for percentage of white blood cell (WBC)-platelet aggregates were 12.1% ± 3.8% for lymphocytes, 6.7% ± 3.3% for neutrophils, and 18.6% ± 5.3% for monocytes. No significant changes in CD42a-positive platelet aggregation with lymphocytes (15.9% ± 14.6% and 16.5% ±3.9%) and with neutrophils (14.0% ± 7.3% and 19.3% ± 3.3%) were observed after 10 and 20 minutes of incubation, respectively. In contrast, the percentages of platelet-monocyte aggregates within the CD14-positive monocyte population were significantly increased (41.8% ± 6.0% after 10 minutes, and 44.4% ± 3.9% after 20 minutes, for both $P < 0.0001$ vs baseline) (**Table S1, SDC**, http://links.lww.com/TP/B100). In addition, no statistically significant correlation between reduction in platelet counts and occurrence of platelet-lymphocyte ($r^2 = 0.951$, $P = 0.142$), platelet-neutrophil ($r^2 = 0.993$, $P = 0.053$), or platelet-monocyte ($r^2 = 0.935$, $P = 0.164$) aggregates was observed.

Beads were sampled after 20 minutes of incubation and analyzed for platelet deposition on PAEC/microcarrier beads. Immunofluorescence staining of microcarrier beads/PAEC for the endothelial marker CD31 and for the platelet marker CD41 showed that CD41-positive platelet deposition was colocalized with CD31 on microcarrier beads, suggesting that after 20 minutes of incubation, human platelets adhered to PAEC (**Figure S2, SDC,** http://links.lww.com/TP/B100).

Phagocytosis of Carboxyfluorescein Diacetate Succinimidyl Ester—Labeled Human Platelets by Porcine Vascular Endothelial Cells

Freshly isolated human platelets were labeled with CFSE and incubated with cultured PAEC, PFAEC, and PLSEC in vitro. Within minutes, human platelets bound to all types of EC, with a peak of fluorescence intensity at 15 minutes. There was nonstatistically significant difference in fluorescence intensity between different types of EC ($P \geq 0.056$). From 30 to 60 minutes, the fluorescence declined to approximately 50% of the maximum (Figure 4A).

As shown by overlay immunofluorescence staining for CD31 (red), CFSE-labeled human platelets adhered to the surface of EC after 30 minutes of incubation (Figure 4B). After 45 minutes, however, CFSE-labeled human platelets were internalized by EC, and colocalization of fluorescence for ASGR1 and the lysosomal marker CD107a was observed (Figure 4C).

Inhibition of Platelet Phagocytosis by Blocking of the ASGR1 Receptor

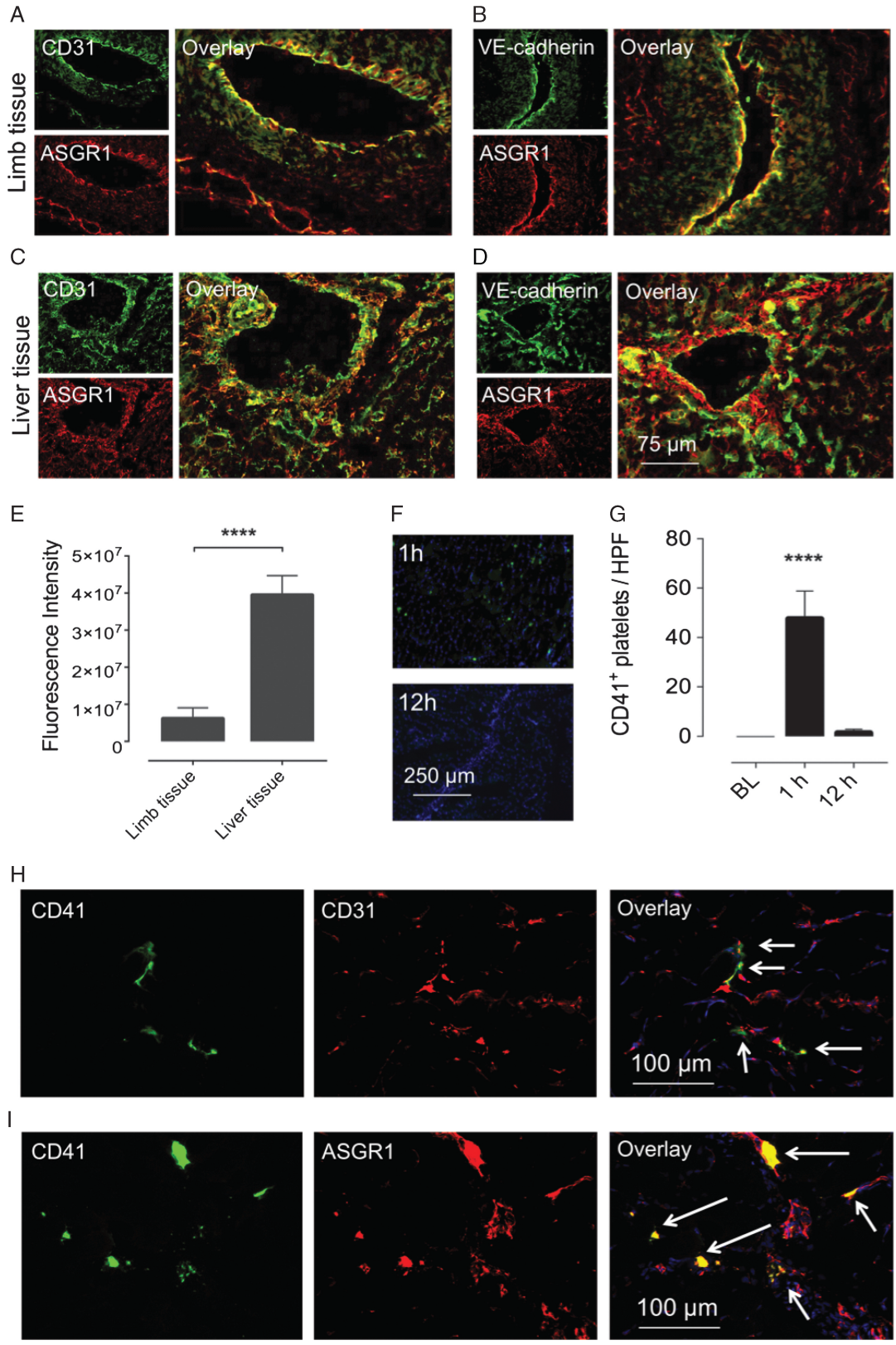
To confirm involvement of ASGR1 in human platelet phagocytosis, we examined the effect of blocking the ASGR1 receptor. Preincubation of PAEC, PFAEC, and PLSEC with polyclonal rabbit anti-ASGR1 antibody led to a significant, dose-dependent reduction of platelet binding. At 2 μg/mL, significance levels were $P < 0.01$, $P < 0.05$, and $P < 0.01$ for PAEC, PFAEC, and PLSEC, respectively. At 5 μg/mL, the significance of the reduction of platelet binding was $P < 0.0001$ for all tested EC. Use of a nonspecific isotype control (polyclonal rabbit IgG) had no effect on platelet binding (Figure 5A). In addition, asialofetuin, a glycoprotein lacking terminal sialic acid, was tested as a competitive ligand for ASGR1 in platelet binding assays. Also, for asialofetuin, a clear, dose-dependent reduction of platelet binding was observed, with $P < 0.01$ to < 0.001 for 2 μg/mL and $P < 0.001$ to < 0.0001 for 5 μg/mL (Figure 5B).

DISCUSSION

During ex vivo xenoperfusion of porcine limbs with heparinized human blood, human platelets disappeared within minutes from the circulating blood. This phenomenon is in line with the observation of xenograft-induced thrombocytopenia and represents a major problem in pig-to-primate xenotransplantation.[8] Transplantation of organs from α1,3-galactosyltransferase gene-knockout (GalTKO) or GalTKO and human CD46 transgenic pigs also led to thrombocytopenia throughout the posttransplant period.[11] Determination of the mechanisms responsible for this thrombocytopenia is necessary for the development of preventive strategies. In this study, we have shown the expression of ASGR1, a known platelet binding C-type lectin receptor, in PAEC as well as PFAEC. In addition to platelet activation and aggregation, we showed, by specific inhibition using polyclonal anti-ASGR1 antibody, that human platelet binding and uptake by PAEC and PFAEC is mediated via ASGR1.

FIGURE 2. Expression of ASGR1 on PAEC, PFAEC, and PLSEC in vitro. A, Cultured PAEC (upper row), PFAEC (middle row), or PLSEC (lower row) were analyzed for expression of ASGR1 as well as the endothelial specific marker CD31 by immunofluorescence staining and fluorescence microscopy. PAEC, PFAEC, and PLSEC were all positive for CD31 and ASGR1. Nuclei stained with DAPI. One representative of at least 3 independent experiments is shown. B, PAEC, C, PFAEC, and D, PLSEC were analyzed for expression of ASGR1 and CD31 by flow cytometry. One representative of 3 independent experiments is shown. E, Representative image of PAEC only, without any antibody staining. F, ASGR1/CD31 staining on PAEC in the presence of both ASGR1 and CD31 primary antibodies and only the secondary antibody against anti-CD31. G, ASGR1/CD31 staining on PAEC in the presence of ASGR1 and C31 primary antibodies but only the secondary antibody against anti-ASGR1. These controls were used to test non-specific binding. H, Western blot analysis of ASGR1 protein and β-actin from whole cell lysate using PAEC, PLAEC, and PLSEC cultures. Representative data of 3 independent experiments are shown. I, Quantitative analysis of Western blot bands of ASGR1/β-actin from whole cell lysates of PAEC, PFAEC and PLSEC by ImageJ software. Shown are mean values ± standard deviations of 3 high-power viewing fields each of at least 3 independent experiments. No significant differences in ASGR1 expression levels were observed as tested by 1-way ANOVA with Bonferroni correction.

Asialoglycoprotein receptor-1, a component of the Ashwell-Morell asialoglycoprotein receptor, is located on hepatocytes, macrophages, and LSEC, and mediates binding and removal of circulating glycoproteins carrying Galβ1-4– or GalNAcβ1-4–terminating epitopes.[21,26] Asialoglycoprotein receptor-1 was previously considered a hepatocyte-specific protein,[27] but has also been shown to occur extrahepatically in thyroid,[28] small and large intestines,[29,30], and testis.[31] Its existence in renal proximal tubular epithelial cells has also been studied,[32] whereas it has never been described to occur



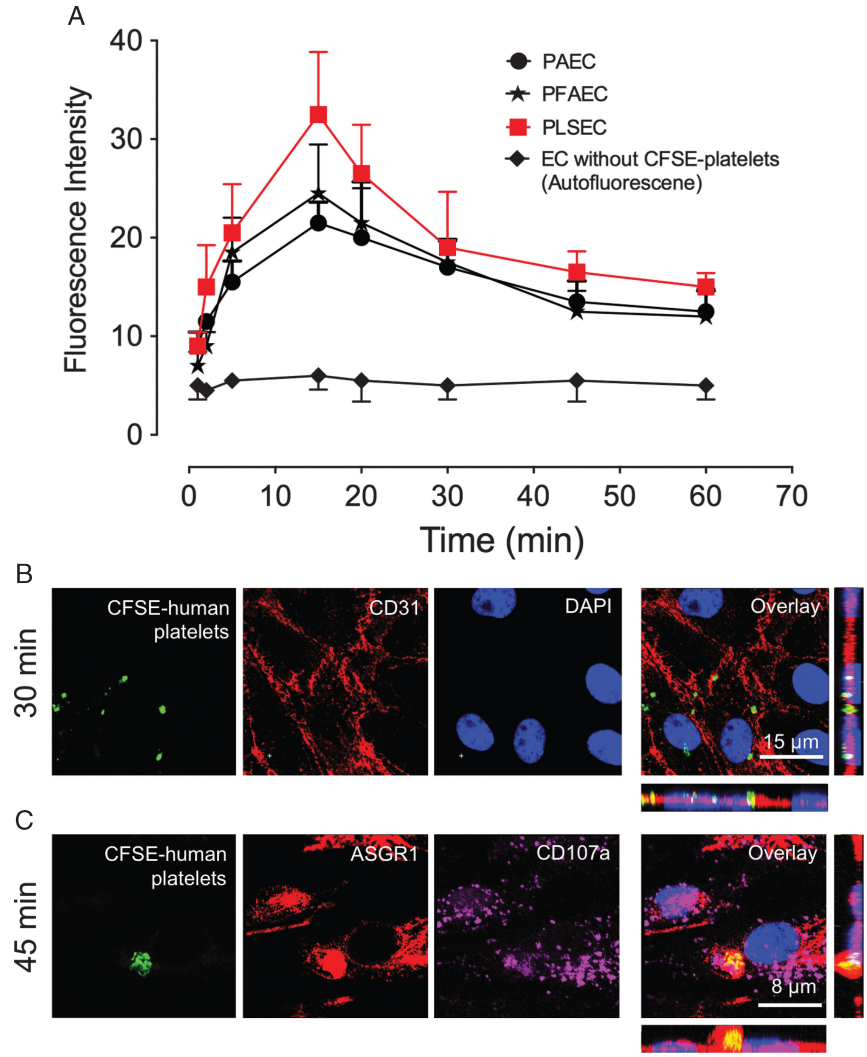


FIGURE 4. Human platelet phagocytosis and degradation by porcine vascular EC. (A) CFSE-labeled human platelets were incubated with porcine vascular EC for different time points as shown on the *x* axis, followed by detection of CFSE fluorescence intensity on a plate reader. All ECs were also measured without CFSE-labeled platelets to determine the level of autofluorescence. Data are expressed as mean ± SD of 3 independent experiments. (B) Confocal microscopy analysis of CFSE-labeled human platelets by PAEC in vitro. After 30 minutes of incubation, CFSE-labeled platelets (green fluorescence) adhered to PAEC that were positive for CD31 (red fluorescence). Side views are shown as z panels to the right of and below the overlay image. (C) Prolonged incubation of CFSE platelets for 45 minutes resulted in internalization into PAEC. Shown are PAEC stained for ASGR1 (red) and the lysosomal marker CD107a (purple). Side views are shown as z panels to the right and below the overlay image. In all fluorescence staining experiments, nuclei were stained with DAPI. One representative of 3 independent experiments is shown.

on endothelial cells outside the liver. Our data now show that in addition to LSEC, extrahepatic vascular endothelial cells also express ASGR1. In vitro, porcine vascular (aortic and femoral arterial) endothelial cells, characterized by expression of the endothelial specific markers VE-cadherin and CD31, were shown to express ASGR1 by immunofluorescence staining, flow cytometry, and Western blot. Taken together, these assays demonstrate that the ASGR1 protein is present on PAEC as well as PFAEC.

The asialoglycoprotein receptor is composed of 2 subunits, a major subunit (ASGR1) and a minor subunit (ASGR2),[33,34] both of which are necessary to form a functional trimer of 2 ASGR1 and 1 ASGR2.[35,36] Staining of limb muscle tissue sections confirmed the presence of ASGR1 in vascular

FIGURE 3. ASGR1 expression on biopsy samples from ex vivo perfusions. A and B, Pig limb skeletal muscle biopsies, collected before perfusion from the contralateral extremity as baseline samples, and (C and D) liver tissue samples were stained for expression of ASGR1 and endothelial markers (CD31 and VE-cadherin). Results show that ASGR1 protein is expressed in vascular endothelial cells that are positive for CD31 or VE-cadherin, as well as in hepatocytes. Representative data of 3 independent experiments are shown. E, Quantitative analysis of ASGR1 staining on limb and liver tissue samples by ImageJ software. Shown are mean values ± standard deviations of 3 high-power viewing fields each of at least 3 independent experiments. Significance was tested using the unpaired 2-tailed Student *t* test (****$P \leq 0.0001$). F, Biopsy samples, collected at different xenoperfusion time points, were stained for platelets using anti-CD41 antibody to study binding and sequestration. G, CD41-positive human platelet binding and sequestration was high at 1 hour perfusion and then declined to baseline levels at 12 hours xenoperfusion. The amount of platelets was expressed as $CD41^+$ platelets per HPF. Significance was tested by 1-way ANOVA with Bonferroni correction (****$P \leq 0.0001$). H and I, Staining for colocalization of human platelets with pig CD31 and/or ASGR1 on 1h xenoperfused pig limb biopsy samples. Human platelets stained with CD41 were colocalized with porcine (H) CD31 and (I) ASGR1. HPF, high-power viewing field.

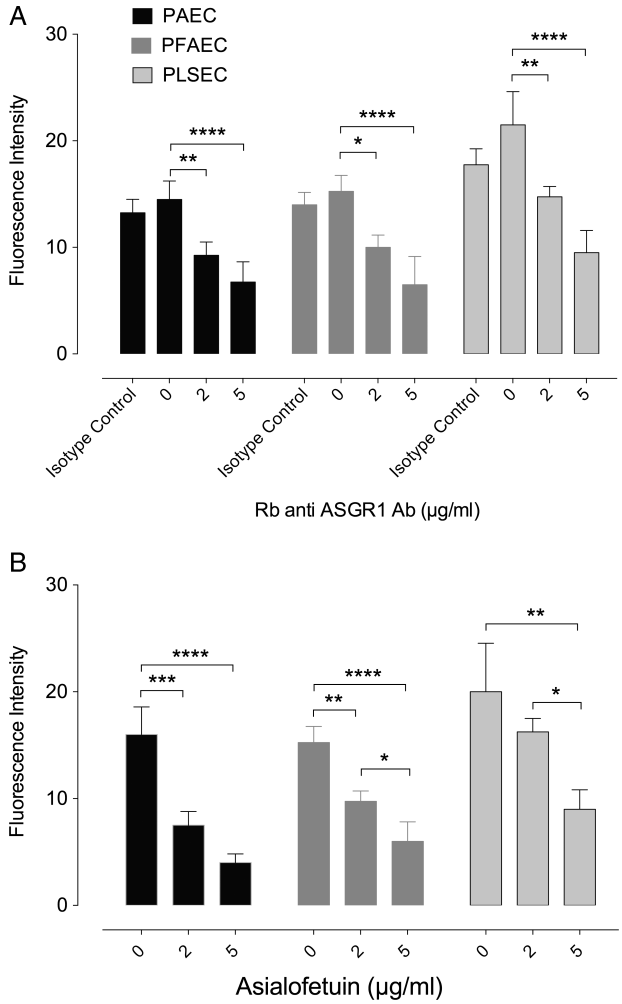


FIGURE 5. Inhibition of human platelet binding and phagocytosis. PAEC, PFAEC, and PLSEC were preincubated with increasing concentrations (0-5 μg/mL) of either (A) rabbit polyclonal anti-ASGR1 antibody or polyclonal rabbit IgG as isotype control, or (B) asialofetuin, for 30 minutes, followed by incubation with CFSE-labeled human platelets. After 15 minutes of incubation, human platelet binding was measured using a fluorescence plate reader. Significance was tested using one-way ANOVA with Bonferroni correction (*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, ****$P \leq 0.0001$). Data are expressed as mean ± SD of 3 independent experiments.

endothelial cells. In addition to PLSEC, other liver cells including hepatocytes express ASGR1 and thus comparative analysis showed significantly higher ASGR1 staining on liver than limb tissue samples. Furthermore, muscle tissue biopsies from porcine limbs, ex vivo xenoperfused for 1 hour with human blood, stained positive for CD41, indicating human platelet sequestration. Staining for CD41 colocalized with ASGR1 protein, suggesting ASGR1-dependent human platelet binding and sequestration.

Platelet activation, aggregation, and phagocytosis can occur through multiple mechanisms in xenoperfusion or xenotransplantation. In addition, platelets have the potential to influence the immune responses through mechanisms involving endothelial cells and leukocytes. In a recent study, high amounts of platelet-platelet and platelet-WBC aggregates were found and linked to low apparent platelet counts.[7,11] In the present study, we also observed platelet-WBC aggregation after 10 and 20 minutes of incubation of whole, non-anticoagulated human blood with PAEC grown on microcarrier beads. These data suggest that platelet aggregation with WBC might be a common phenomenon in pig-to-primate xenotransplantation or xenoperfusion. However, the reduction in platelet counts showed no statistically significant correlation with the percentage of platelet-WBC aggregation. Therefore, other mechanisms, particularly receptor-mediated adhesion to EC, may contribute to the reduction in platelet counts. This was supported by detection of co-localized deposition of CD41-positive platelets with PAEC/CD31 on microcarrier beads that are covered with a monolayer of PAEC, after incubating with human blood. Furthermore, our in vitro results using freshly isolated human platelets and porcine vascular EC suggest that, at least in part, receptor-ligand interactions are involved in the clearance of human platelets in vitro and ex vivo in pig-to-human limb xenotransplantation models.

In vitro, binding of human platelets to EC was observed and peaked after 15 minutes incubation as reported previously using PLSEC and CFSE-labeled human platelets.[20,21] Thereafter, the CFSE fluorescence elicited by the labeled platelets gradually declined and at 45 minutes was colocalized with CD107a, a marker for lysosomes. These findings are in line with the finding that the receptor forms a complex with galactose-terminated glycoproteins, which are internalized and transported to lysosomes, as indicated by colocalized deposition of CFSE platelets, ASGR1, and CD107a. The receptor and ligand dissociate, and the receptor is recycled back to the cell surface.[37]

Asialoglycoprotein receptor-1 mediates endocytosis of glycoproteins from which the terminal sialic acid residues have been removed. The presence of higher Galβ1-4 or GalNAcβ1-4 ligand concentrations on human as compared to porcine platelets may facilitate porcine ASGR1-mediated human platelet phagocytosis.[21] Platelet aggregation and phagocytosis by PLSEC has also been reported in pig-to-baboon liver xenotransplantation and can be prevented by blocking integrin adhesion pathways.[38] Indeed, preincubation of PAEC with polyclonal anti-ASGR1 antibody significantly reduced human platelet binding. Also, blocking of ASGR1 using asialofetuin, a competitive ligand for ASGR1, significantly reduced platelet uptake by EC in a dose-dependent manner, supporting involvement of the porcine ASGR1 in human platelet phagocytosis. Generation of ASGR1 knockout pigs, with or without introduction of the human ASGR1 and on top of GalTKO and expression of human regulatory proteins for complement and coagulation, may therefore help to overcome the problem of thrombocytopenia in pig-to-human xenotransplantation.

In conclusion, we found expression of functional ASGR1 in extrahepatic porcine vascular endothelial cells. ASGR1 on the vascular endothelium of porcine organs may therefore mediate binding and phagocytosis of human platelets during pig-to-human xenotransplantation.

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