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Soluble saccharides block the inhibition of agonist-induced human platelet aggregation observed after in vitro incubation of human platelet-rich plasma with porcine aortic endothelial cells

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Abstract Platelet aggregation is a prominent feature in the hyperacute process of vascularized allografts and xenografts. In a study of extracorporeal connection of pig kidneys to the blood circulation of human volunteers, we observed in one case considerable destruction of human platelets in the pig kidney without signs of hyperacute rejection or microthrombi formation. In the present study, we have investigated the agonist-induced aggregation of human platelets in mixtures with porcine aortic endothelial cells (PAEC). In vitro incubation of human platelet-rich plasma (PRP) with PAEC inhibited platelet aggregation induced by ADP, collagen and arachidonic acid in a time-dependent manner and partially inhibited adrenalin-induced aggregation. Aggregation of the human platelets could not be induced by high concentrations of ADP (20 μM) to overcome the inhibition capacity of the PAEC. The PAEC inhibiting effect could be transferred by the supernatants of PAEC/PRP and PAEC/PPP incubation mixtures. Preincubation of the PAEC with aspirin, but not with N^G-methyl-L-Arg, reduced the aggregation inhibitory effect. Control experiments mixing human umbilical vein endothelial cells (HUVEC) and human PRP or mixing porcine PRP and PAEC did not elicit any inhibition of ADP-induced platelet aggregation. The aggregation inhibition effect could partially be blocked by preincubation of PRP with soluble Galα1–3Gal, Galα1–3β1–4GlcNAc, lactose, galactose, and glucose, but not by lactosamine, galactosamine, or glucosamine. The Galα1–3Gal disaccharide was most effective in blocking aggregation inhibition, and to a similar extent as its ability to block the human anti-pig lymphocytotoxicity reaction. In conclusion, the data indicate that PAEC, upon stimulation by human anti-pig xenoantibodies in a nondynamic system, inhibits agonist-induced human platelet aggregation, and that this effect is probably at least partially caused by prostacyclin released from the PAEC.

Key words Human platelets · Porcine aortic endothelial cells · Xenotransplantation, anti-pig xenoantibodies · Galα1–3Gal saccharides

Introduction

The scarcity of human organs for transplantation has focused research on the possibility of grafting animal organs, especially from pigs, to humans. The first major barrier that needs to be overcome for a successful transplantation of vascularized pig organs to humans is the hyperacute rejection (HAR) caused by preformed anti-pig xenoantibodies, followed by complement activation, platelet aggregation, and subsequent thrombosis of the graft [1, 11]. The majority of human anti-pig xenoantibodies are directed to Galα1–3Gal saccharide determinants on the endothelial cell surface [19]. However, considerable variability in the specificity of these xenoreactive anti-Gal antibodies has been observed [6, 10]. The endothelial cells, upon stimulation, prevent platelet aggregation by several mechanisms including the production of prostacyclin, and nitric oxide (NO), and the activation of ADPase [1, 4, 7]. The adhesion and aggregation of platelets in HAR is caused by the loss of endothelial cell function due to antibody binding and complement activation, resulting in a procoagulant cell surface, alteration in cell shape, and the formation of intercellular gaps [1, 18], which allows passage of vascular contents into extravascular tissue and adhesion of platelets.

We have, in a clinical trial, connected pig kidneys to the circulation of two volunteer dialysis patients who were pretreated with plasmapheresis to reduce the level of preformed xenoantibodies [3, 16]. In these experiments, a dramatic consumption of human platelets occurred in the pig kidneys. A similar profound platelet consumption has been observed in patients with fulminant liver failure who were treated with extracorporporal pig liver perfusion [5]. In one of our patients, no platelet aggregates or thrombi were found in the capillaries, and electron microscopy revealed an intact endothelial cell lining of the vessels and membrane fragments in the lumen of the vessels [3]. The finding that human platelets were destroyed in the pig kidney without the formation of thrombi led us to investigate the interaction between human platelets and porcine aortic endothelial cells (PAEC). This paper describes how, in a nondynamic system, incubation of human platelet-rich plasma with PAEC inhibits agonist-induced human platelet aggregation and examines the ability of soluble saccharides to block this inhibition effect.

Materials and methods

Platelet preparation

Human blood was collected in citrate (buffered sodium citrate 0.13 M, 1:10 v/v) from healthy human volunteers who had not ingested nonsteroidal anti-inflammatory drugs for at least 10 days. Platelet-rich plasma (PRP) was obtained by 15-min centrifugation at 240 *g* and 22 °C. The PRP was stored in polypropylene tubes at room temperature and all experiments were performed within 120 min. The concentration of platelets ranged from 150 to 220×10^9 per liter. The ADP-induced aggregation capacity of the platelets present in each preparation was recorded at the beginning of each experimental series. Platelet-poor plasma (PPP) was prepared by centrifugation of citrated whole blood for 20 min at 2000 *g* and 22 °C. Porcine PRP and PPP were prepared in a manner identical to that of the human samples.

Pig aortic and human umbilical vein endothelial cell suspensions

Primary cultures of PAEC harvested under sterile conditions were prepared from the thoracic aortae of Swedish domestic pigs. The vessel was carefully rinsed with Hank's balanced saline solution supplemented with PEST 100 E/ml and amphotericin B 0.25 μg/ml (Life Technologies, Paisley, Scotland). After rinsing, the aortae were incubated with 1.0 mg/ml collagenase H (Boehringer Mannheim, Mannheim Germany) in PBS at 37 °C for 10 min. After incubation, the collagenase solution, containing detached endothelial cells, was suspended in RPMI 1640 (GIBCO, Paisley, Scotland) culture medium (1:1, v/v) supplemented with 20% fetal calf serum (FCS, GIBCO), 2.0 mM L-glutamine, ECGF/heparin 2.5 ng/ml (Boehringer Mannheim), PEST 100 E/ml, and 0.25 μg/ml amphotericin B. The vessel was rinsed once more with culture medium and the combined collagenase and rinsing suspensions were centrifugated at 130 *g* for 5 min. The cells were resuspended in the same culture medium and seeded out on fibronectin (5 $\mu g/cm^2$, J.R.H Biosciences, Sussex, UK)-coated plastic petri dishes. The cells were grown at 37 °C in a humidified atmosphere of 6.5% CO_2 in air, and nonadherent cells were removed after 1 day when changing medium. The cells were detached using 0.25% trypsin in 0.53 mM EDTA (Life Technologies) and subcultured in plastic flasks. Finally, they were cryopreserved in aliquots at passage 4 in cell culture freezing medium (Life Technologies) for 1–6 months.

After thawing, the cells were washed and cultured in RPMI 1640 supplemented with 20% FCS, 2.0 mM L-glutamine and 0.25 μg/ml amphotericin B. Confluent cultures of PAEC were characterized by their cobblestone morphology and were used between passages 5 and 8. Cells were detached by 0.53 mM EDTA containing either 0.25% trypsin, 200 E/ml collagenase XI, or 200 E/ml collagenase IA-S (Sigma). The endothelial cells were immediately washed in culture medium and reconstituted in serum-free RPMI 1640 supplemented with only L-glutamine. For the platelet aggregation studies, $1–1.5 \times 10^6$ cells/ml were kept at room temperature and used within 1–2 h. Human umbilical vein endothelial cells (HUVEC) [17], a kind gift from B. Risberg (Department of Surgery, Sahlgrenska University Hospital), were cultured to monolayers in E 199 culture media (Life Technologies) supplemented with 20% FCS and penicillin/streptomycin mixture and used between passages 2 and 4. The HUVEC cells were detached and treated in a manner identical to that of the PAEC. The viability of the reconstituted cells was approximately 85% by trypan blue exclusion. All reagents used were endotoxin-free.

Platelet aggregation studies

The platelet aggregation response was studied in a dual-channel platelet aggregometer (Payton) using various agonists. Baselines and maximal transmission were set with PRP and PPP, respectively. A total volume of 450 μl PRP, or mixtures of 325 μl PRP and 125 μl PAEC/HUVEC suspensions, was incubated with 50 μl of the different agonists in 500-μl cuvettes at 37 °C. The amount of ADP used for each PRP batch was calibrated to give a maximal ag-

gregation response, and the final concentration used was in the range of 4.0–8.0 μM. The aggregation response was measured as the amplitude of the optical transmission 1 min after the addition of ADP and presented as percent of transmission maximum (% T-max). In order to rule out the possibility of endogenous ADPase influence, a control experiment with a final concentration of 20 μM ADP was performed. Stock solutions of agonists used were ADP 23.4 mM (Sigma, St. Louis, Mo., USA), arachidonic acid 5 mg/ml (Bio/Data Corporation, Hatboro, Pa., USA), adrenalin 0.1 mM (Sigma), and collagen 2 mg/ml (Diagnostica Stago, Asnieres-Sur-Seine, France).

Supernatants from suspensions of PAEC or from mixtures of PAEC and human PRP/PPP

PAEC suspensions (125 μl) were incubated with 325 μl l of human PRP or PPP for 10 min at 37 °C. The mixtures were centrifuged at 9200 *g* for 1 min and 125 μl of the supernatant was immediately mixed with 325 μl human PRP in the aggregating cuvette; 50 μl ADP (final concentration 4–8 μM) was added to the cuvette after 1 min of incubation. Control supernatants were obtained by incubating PAEC suspensions at room temperature and at 37 °C for 10 min, followed by centrifugation.

Treatment of PAEC with aspirin and N^G-methyl-L-Arg

To block prostacyclin (PGI_2) production, PAEC suspensions were incubated with aspirin (Sigma) at a final concentration of 1.0 mM for 1 h at 37 °C. To block NO synthase, PAEC were incubated with N^G-methyl-L-Arg (Sigma) to a final concentration of 300 μM for 1 h at 37 °C. After incubation, the PAEC suspensions were washed in culture medium and immediately used in the platelet aggregation studies.

Blocking of human natural anti-Gal antibodies by soluble saccharides

Galα1–3Gal or Galα1–3Galβ1–4GlcNAc and Galβ1–4Glc (lactose) with the reducing end linked to an 8-carbon atom lipophilic chain were from Glycorex (Lund, Sweden). Lactose, *N*-acetyl-lactosamine, galactose, *N*-acetyl-galactosamine, glucose, and *N*-acetyl-glucosamine with nonsubstituted reducing ends were from Sigma. Human PRP samples were mixed with the soluble saccharides to a final concentrations of 10, 5, 1, and 0.1 mg/ml, respectively, as stated in Table 2 and the legend to the figures. After 10 min of incubation, 325 μl of the PRP/saccharide mixtures was transferred to the aggregation cuvette and 125 μl of PAEC was added. ADP was added after the time needed for each PRP/PAEC combination to reach optimal aggregation inhibition.

Mass spectrometric identification of prostacyclin metabolite

The stable prostacyclin metabolite 6-Keto PG F1α, MW 370 (Sigma K 2626) was used as a reference compound for identification of prostacyclin release. Four milliliters of ethanol was added to 1 ml of supernatant from the PAEC/PRP incubation mixture. After centrifugation, the supernatant was dried under N_2, dissolved in 2 ml PBS, and applied to a C_{18} column (Varian Associates, Harbor City, Calif., USA), which had been prewashed with ethanol, hexanol, and water. The bound hydrophobic compounds were eluted with methyl acetate and dried under N_2. The reference compound was analyzed both as a pure compound and after mixing with PPP, followed by preparation in a way similar to that for PAEC/PRP supernatants. Mass spectrometry analysis was performed using a tandem magnetic sector-time of flight instrument (AutoSpec OAFPD Micromass, Manchester, UK) operated in negative electrospray ionization mode (EI) in a mass range of 100–3100 at 10 s/decade, at about 1500 resolution (10% valley), and at a source temperature of 80 °C. The samples were dissolved in methanol:water (4:1, v/v) and introduced into the source housing via a loop injector (50 μl) connected to a micropump at a flow rate of 10 μl/min.

Table 1 Human platelet aggregation response to ADP in the presence of xenogenic and allogenic endothelial cells. Human (H-PRP) or porcine (P-PRP) platelet-rich plasma were mixed with porcine (PAEC) or human (HUVEC) endothelial cell suspensions, followed by the addition of ADP. The effect of preincubating PAEC with aspirin or N^G-methyl Arg, respectively, before mixing with H-PRP were also studied. Results are presented as percent of T-max for PRP (T-max PRP = 100%)

Experimental setup	Platelet aggregation % T-max of PRP Mean ± SD
H-PRP/PAEC (n = 12)	11.7 ± 3.4
H-PRP/HUVEC (n = 5)	92.4 ± 11.6
P-PRP/PAEC (n = 5)	104.8 ± 29.5
H-PRP/PAEC + Aspirin (n = 4)	50.1 ± 10.8*
H-PRP/PAEC + N^G-methyl Arg (n = 3)	11.8 ± 1.2

* $P < 0.01$ compared to the corresponding PRP/PAEC mixture

Statistical analysis

Standard statistical methods were used for calculation of mean value and standard deviation (SD). The differences between means were evaluated with the two-sided, paired Student's *t*-test, and a *P* value below 0.05 was considered statistically significant.

Results

Mixing human PRP with HUVEC (Fig. 1A) or pig PRP with PAEC for 10 min, followed by the addition of ADP, resulted in a normal aggregation response (Table 1) fully comparable with the aggregation response seen in control experiments using only PRP (data not shown). Mixing PAEC with human PRP, followed by the immediate addition of ADP, resulted in a prompt aggregation of the platelets (Fig. 1B). Incubation of the PAEC with human PRP for various time periods prior to the addition of ADP resulted in a time-dependent inhibition of the ADP-induced platelet aggregation (Fig. 1C–E). A complete inhibition of the ADP-induced aggregation occurred after 5–10 min of PAEC/PRP incubation, and no ADP-induced aggregation was seen after 30 min of incubation. The experiments were performed using PRP from ten different human donors and primary PAEC cultures from three different pigs.

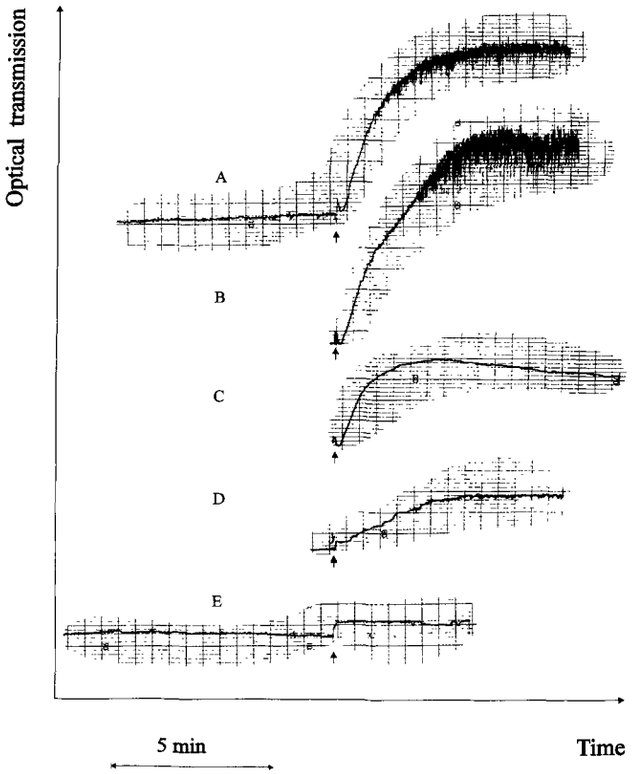

Fig. 1 Aggregation response of human platelets to ADP in the presence of HUVEC and PAEC, respectively: **A** Incubation of human PRP (325 µl) with HUVEC suspension (125 µl) for 10 min, followed by the addition of 50 µl ADP (final concentration 7 µM), results in a normal aggregation pattern. Incubation of human PRP (325 µl) with PAEC suspension (125 µl), followed by the addition of 50 µl ADP (final concentration 4 µM): **B** immediately, **C** after 1.15 min, **D** after 5 min, and **E** after 10 min, results in a time-dependent inhibition of platelet aggregation. *Arrows* indicate time of ADP addition

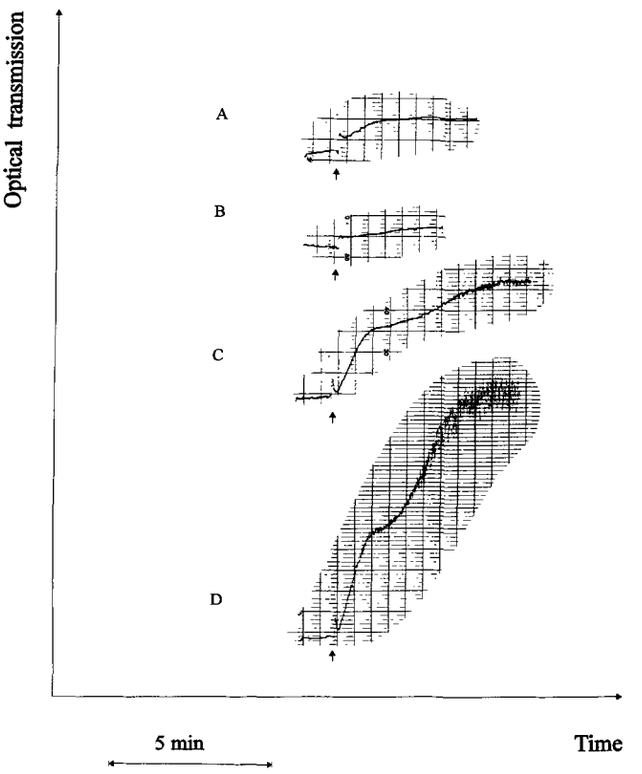

Fig. 2 Aggregation response of human platelets to ADP in the presence of supernatants from: **A** PAEC/PRP, **B** PAEC/PPP, **C** PAEC cultured at 37 °C, and **D** PAEC cultured at room temperature. **A** Incubation of human PRP (325 µl) for 1 min with PAEC/PRP supernatant (125 µ, preincubated for 10 min at 37 °C, followed by centrifugation), followed by the addition of 50 µl ADP. **B** Human PRP incubated with PAEC/PPP supernatant, followed by the addition of ADP, gave a similar aggregation as for incubating PRP with PAEC. Incubation of human PRP with supernatants from PAEC suspensions kept at **C** 37 °C and **D** room temperature, followed by the addition of ADP, shows that PAEC kept at 37 °C but not at room temperature produce a soluble factor that partly inhibits the ADP-induced aggregation. Final ADP concentration was 7 µM in all experiments. *Arrows* indicate time of ADP addition

Detaching the PAEC from the culture flasks using collagenase/EDTA or trypsin/EDTA gave similar results.

No spontaneous aggregation of the platelets occurred during 10 min of preincubation of human PRP with PAEC or HUVEC. An anti-aggregation effect of PAEC on human platelets was also found when collagen and arachidonic acid were used as agonists (data not shown). Using adrenaline as an agonist, an aggregation occurred, but the rate of aggregation was much slower and the % T-max was reduced compared to the control. To test whether the anti-aggregation effect was caused either by a product secreted or dependent upon a direct interaction, supernatants from either PAEC/PRP or PAEC/PPP mixtures or PAEC alone were added to human PRP in the aggregation cuvette, followed by the addition of ADP after 1 min of incubation. The results showed that the anti-aggregation effect could be transferred by the supernatants from both PAEC/PRP and PAEC/PPP mixtures (Fig. 2A, B) and that a basal secretion of this product occurred when PAEC were kept at 37 °C but not at room temperature (Fig. 2C, D). If ADP was added after 10 min or later, the inhibition did not occur, indicating that the transferred component(s) was (were) unstable.

In order to test if the inhibition of ADP-induced aggregation could be due to degradation by cellular ADPase, the concentration of ADP was increased to a final concentration of 20 µM without obtaining an aggregation response (Fig. 3A). In order to identify possible mechanisms responsible for the inhibition of aggregation, PAEC suspensions were pretreated with aspirin to block cyclo-oxygenase and with N^G-methyl-L-Arg to

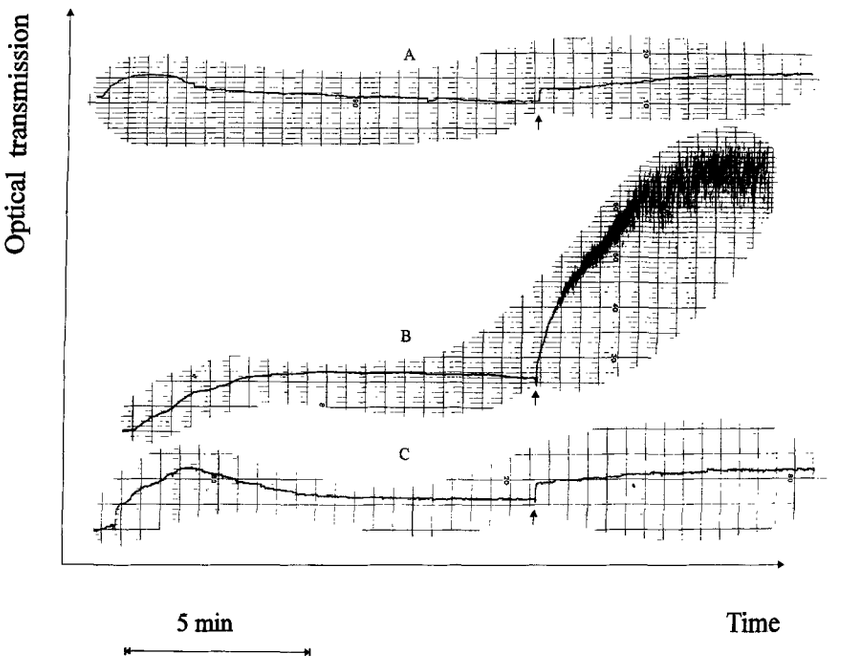

Fig. 3 A Human PRP mixed with untreated PAEC, followed by the addition of ADP to a final concentration of 20 μM. The higher ADP concentration was used to counteract an eventual degradation of ADP by cellular ADPase. **B** The effect of preincubating PAEC with 1.0 mM aspirin and **C** with 300 μM N^G-methyl-L-Arg. Human PRP (325 μl) was mixed with the preincubated PAEC suspensions (125 μl) and incubated for 10 min at 37 °C, after which 50 μl ADP was added (final concentration 7 μM) at times indicated by *arrows*

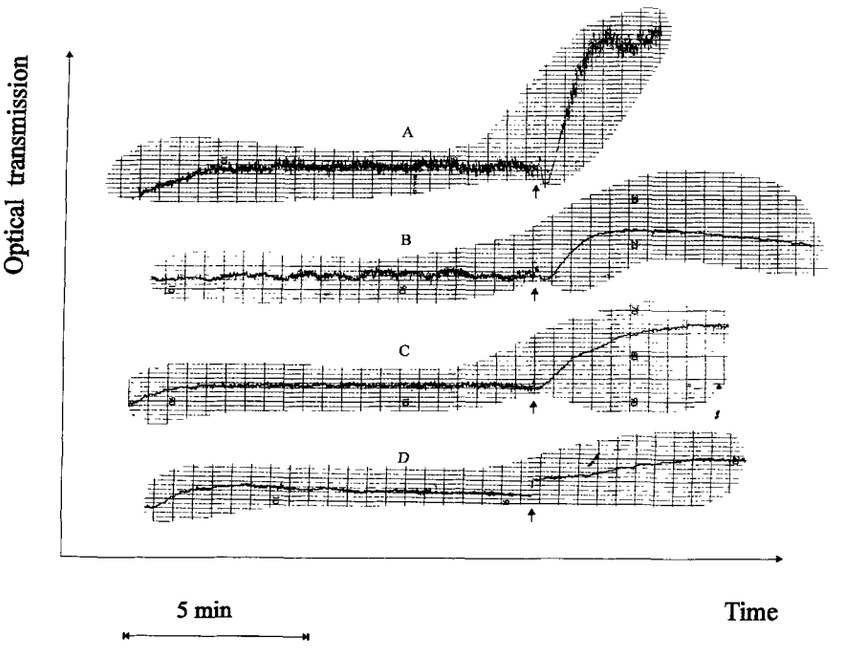

Fig. 4 Effect of Galα1–3Gal disaccharides on the aggregation response of human platelets to ADP in the presence of PAEC. **A** A control experiment incubating 450 μl PRP with Galα1–3Gal (10 mg/ml) for 10 min, followed by the addition of 50 μl ADP. After mixing 325 μl PRP with Galα1–3Gal **B** 10 mg/ml, **C** 1 mg/ml, and **D** 0.1 mg/ml for 10 min, 125 μl of PAEC was added and incubated for 10 min, followed by the addition of 50 μl ADP (final concentration 7 μM) at times indicated by *arrows*. Galα1–3-Gal disaccharide concentrations of 10 mg/ml and 1 mg/ml reduced the inhibition effect of the aggregation response induced by the PAEC

block NO production. Preincubation of PAEC with aspirin before mixing with human platelets (Table 1, Fig. 3B) resulted in a 50% reduction in the inhibition of ADP-induced aggregation while blocking NO production (Table 1, Fig. 3C) did not affect aggregation inhibition. This indicates that the inhibition effect is due to a compound produced in the cyclo-oxygenase metabolic pathway of the PAEC.

Based on the present knowledge, the most likely compound would be prostacyclin. We therefore analyzed the supernatant from PAEC/PRP incubations for the presence of the stable prostacyclin metabolite 6-

Table 2 Effect of soluble saccharides' ability to block the inhibition of ADP-induced aggregation of human platelets in the presence of xenogenic porcine endothelial cells. Human PRP was preincubated for 10 min with each saccharide, followed by the addition of PAEC suspension, and the aggregation of the platelets was initiated by the addition of ADP. Disaccharides (Galα1-3Gal, lactose, lactosamine) and monosaccharides (galactose, galactosamine, glucose, glucosamine) were tested in 10 mg/ml and 5 mg/ml, respectively. Results are presented as percent of T-max for PRP (T-max PRP = 100%)

Experimental setup	Platelet aggregation % T-max of PRP Mean ± SD
PRP/PAEC (n = 16)	18.8 ± 6.4
PRP + Galα1-3Gal/PAEC (n = 14)	36.4 ± 13.1*
PRP + Lactose/PAEC (n = 10)	30.1 ± 15.3**
PRP + Lactosamine/PAEC (n = 5)	21.3 ± 11.5
PRP + Galactose/PAEC (n = 7)	32.9 ± 19.7
PRP + Galactosamine/PAEC (n = 9)	13.5 ± 8.3
PRP + Glucose/PAEC (n = 9)	34.6 ± 20.1**
PRP + Glucosamine/PAEC (n = 6)	16.7 ± 9.0

* $P < 0.001$; ** $P < 0.05$ compared to the corresponding PRP/PAEC mixture

Keto PG F1α. Mass spectrometry analysis (data not shown) showed a distinct peak at m/z 369 in the PAEC/PRP supernatant that was present only in trace amounts in the control samples. This peak is the molecular ion peak of 6-Keto PG F1α with loss of a proton (M-1). This confirms the suggestion that the major product responsible for the inhibition of ADP-induced platelet aggregation is prostacyclin produced by PAEC due to an interaction with human plasma. However, the possibility that other mediators of the arachidonic pathway may also contribute to the effect cannot be ruled out.

One explanation could be that the PAEC are stimulated by preformed, naturally occurring human anti-pig xenoantibodies present in the sera of all humans. The majority of these xenoantibodies are directed to the carbohydrate antigen epitope Galα1–3Galβ1–3GlcNAc [19]. We therefore tested whether soluble Galα1–3Gal, Galα1–3Galβ1–3GlcNAc, and other saccharides were able to block the PAEC-induced inhibition of human platelet aggregation (Fig. 4, Table 2). Galα1–3Gal, Galα1–3Galβ1–3GlcNAc, lactose, galactose, and glucose were all able to partially block the inhibition of ADP-induced aggregation of PAEC/PRP mixtures. Galα1–3Gal and Galα1–3Galβ1–3GlcNAc saccharides, in concentrations of 10 mg/ml and 1.0 mg/ml, gave a similar blocking effect while a concentration of 0.1 mg/ml had no blocking effect. Lactosamine, galactosamine, and glucosamine did not affect the inhibition of ADP-induced human platelet aggregation (Table 2). The capacity of the saccharides to block the inhibition of ADP-induced aggregation was found to vary between sera from different individuals. In general, the Galα1–3Gal saccharide blocking effect was statistically highly significant ($P < 0.001$) and the mean % T-max obtained was 36% of the control aggregation experiment (Table 2). This is in the same range as the ability of soluble Galα1–3Gal saccharides to block the anti-pig lymphocytotoxicity effect of human sera [15].

Discussion

The vascular endothelium has a unique position between the blood and the tissue, and has antithrombotic properties that could become prothrombotic if activated. These functions are mediated by a very complex system of mediators. The interaction between endothelial cells (mainly porcine and bovine) and human platelets has been studied extensively. These studies have mainly used washed platelets, omitting the xenoantibodies, and focused on the endothelial function and pathogenesis of arteriosclerosis, thrombosis, and vasospasm in the autologous situation [2, 4, 8, 9, 12, 13, 21]. In the hyperacute rejection of discordant vascularized xenografts, the process is initiated by the preformed xenoantibodies and complement that activate the endothelial cells to become prothrombotic and to disrupt the endothelial cell lining, exposing the subendothelium and resulting in interstitial edema, hemorrhage, and thrombosis of the graft [1, 11, 19]. Xenogenic activation of endothelial cells leads to the release of heparan sulfate [1, 18], loss of cellular ADPase [14], and retraction of endothelial cells [18].

In this study we have shown a time-dependent inhibition of agonist-induced platelet aggregation when human PRP is mixed with PAEC. The inhibition effect was reduced by preincubation of human PRP with soluble saccharides, with Galα1–3Gal terminating saccharides being the most effective. Lactose, galactose, and glucose also reduced the aggregation inhibition but to a lesser degree. Lactosamine, galactosamine, and glucosamine were nonreactive. The Galα1–3Galβ1–3GlcNAc trisaccharide showed the same effect as the Galα1–3Gal disaccharide, indicating that amino groups located on the terminal or subterminal sugar residues prevent the saccharide from blocking agonist-induced platelet aggregation while an amino group further away from the terminal part does not. The ability of Galα1–3Gal terminating saccharides to reduce the anti-aggregating effect is in the same range as its ability to inhibit lymphocytotoxicity to pig cells [15]. Possible reasons for not achieving a greater reduction include low-affinity antigen/antibody interaction, variability in specificity between the saccharides and the antibodies, or multivalent interaction between the antibodies and the cell.

Studies on the ability of different soluble saccharides to block the binding of human xenoantibodies to pig cells have shown a great variation in antibody binding pattern and saccharide blocking effect. Sandrin et al. [20] showed that human anti-pig antibodies react pre-

dominantly with terminal galactose residues when the ability of different carbohydrates to inhibit the hemagglutination reaction of pig erythrocytes was examined. Galactose decreased the hemagglutination titer of a human serum pool by 75% and galactosamine decreased the titer by 50% at saccharide concentrations of 300 mM (54 mg/ml galactose and 65 mg/ml galactosamine, respectively). Parker et al. [10] tested the specificity of xenoreactive antibodies, focusing especially on the IgM isotype, for α-galactosyl structures. Approximately 30% of the xenoreactive anti-Galα1–3Gal IgM did not recognize melibiose (Galα1–6Glc) or other α-galactosyl sugars. A substantial variability was found in the specificity of the xenoreactive anti-Galα1–3Gal IgM for α-galactosyl terminating sugars, and the concentration of Galα1–3Gal saccharide needed to block the binding of xenoreactive antibodies to the cell surface was in the range of 20–200 mM (6.8–68 mg/ml) for a complete blocking of the xenoreactive IgM. Kujundzic et al. [6] found considerable variability in the ability of human AB sera from 75 donors to lyse pig kidney (PK 15) cells. A highly significant positive correlation was found between cytotoxic activity and surface-bound IgG and IgM, as well as a "tendency for positive correlation" between cytotoxicity and anti-αGal IgG titers but no correlation between cytotoxicity and anti-αGal IgM or IgA titers [6].

Another explanation for the failure to achieve a complete reduction in the inhibition may be a slight activation of the complement cascade since complement activation has been shown to be able to induce prostacyclin formation in cultured endothelial cells [22].

Indometacin-treated PAEC have been shown to block agonist-induced aggregation of washed platelets, and stimulation with bradykinin has resulted in an inhibition effect due to the release of NO [12]. In our study, mixing human PRP with nontreated PAEC resulted in a complete inhibition of agonist-induced platelet aggregation that could be blocked by aspirin pretreatment of the PAEC (Table 1, Fig. 2A). This suggests that stimulation of PAEC by xenoantibodies may not be sufficient for induction of NO production. Using washed human platelets, it was shown that by mixing the platelets with PAEC, HAEC [14], or aspirin-treated HUVEC [8], agonist-induced platelet aggregation was inhibited due to the ADPase activity of the endothelial cells. A rapid and spontaneous aggregation response of human platelets has been found when mixing human PRP and PAEC [14]. We did not observe a similar result in our experimental setup. This discrepancy may be explained by slightly different experimental conditions, such as a variation in xenoantibody titers and complement levels, resulting in fast prothrombotic changes in the PAEC.

It has to be borne in mind that, in contrast to the autologous and allogenic situations, the role of the anti-aggregating properties of porcine endothelial cells in vitro, most likely achieved by preformed xenoantibodies in the test system, may not be relevant for the in vivo rejection process of a vascularized, discordant xenograft. Prostacyclin is short-lived and quickly washed away in the circulation. Prothrombotic factors, such as the loss of heparan sulfate and ADPase, EC retraction, exposure of the subendothelium, and formation of complement-activation derived components, may become dominant after a few minutes, resulting in thrombosis of the capillaries.

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References

1. Bach FH, Robson SC, Winkler H, Ferran C, Stuhlmeier KM, Wrighton CJ, Hancock WW (1995) Barriers to xenotransplantation. Nature Med 1: 869–873
2. Benistant C, Achard F, Marcelon G, Lagarde M (1993) Platelet inhibitory functions of aortic endothelial cells. Effect of eicosapentaenoic and docosahexanoic acids. Atherosclerosis 104: 27–35
3. Breimer ME, Björck S, Svalander CT, Bengtsson A, Rydberg L, Karlsen-Lie K, Attman P-O, Aurell M, Samuelsson BE (1996) Extracorporeal ("ex vivo") connection of pig kidneys to humans. I. Clinical data and studies of platelet destruction. Xenotransplantation 3: 328–339
4. Broekman MJ, Eiora AM, Marcus AJ (1991) Inhibition of human platelet reactivity by endothelium-derived relaxing factor from human umbilical vein endothelial cells in suspension: blockade of aggregation and secretion by an aspirin insensitive mechanism. Blood 78: 1033–1040
5. Chari RS, Collins BH, Magee JC, Dimaio JM, Kirk AD, Harland RC, McCann RL, Platt JL, Meyers WC (1994) Treatment of hepatic failure with ex vivo pig liver perfusion followed by liver transplantation. N Engl J Med 331: 234–237
6. Kujundzic M, Koren E, Neethling FA, Milotic F, Kosec M, Kujundzic T, Martin M, Cooper DKC (1994) Variability of anti-αGal antibodies in human serum and their relation to serum cytotoxicity against pig cells. Xenotransplantation 1: 58–65

7. Marcus AJ (1990) Thrombosis and inflammation as multicellular processes. Pathophysiological significance of transcellular metabolism. Blood 76: 1903–1907
8. Marcus AJ, Safler LB, Haijar KA, Ullman HL, Islam N, Broekman J, Eiora AM (1991) Inhibition of platelet function by an aspirin-insensitive endothelial cell ADPase. J Clin Invest 88: 1690–1696
9. Nosaka S, Nakayama K, Hashimoto M, Sasaki T, Gu K, Saitoh Y, Kin S, Yamauchi M, Masumura S, Tamura K (1996) Inhibition of platelet aggregation by endocardial endothelial cells. Life Sci 59: 559–564
10. Parker W, Lateef J, Everett ML, Platt JL (1996) Specificity of xenoreactive anti-Galα1–3Gal IgM for α-galactosyl ligands. Glycobiology 6: 499–506
11. Platt JL, Bach FH (1991) The barrier to xenotransplantation. Transplantation 52: 937–947
12. Radomski MW, Palmer RMJ, Moncada S (1987) The anti-aggregating properties of vascular endothelium: interactions between prostacyclin and nitric oxide. Br J Pharmacol 92: 639–646
13. Radomski MW, Palmer RMJ, Moncada S (1987) Comparative pharmacology of endothelium-derived relaxing factor, nitric oxide and prostacyclin in platelets. Br J Pharmacol 92: 181–187
14. Robson SC, Kaczmarek E, Siegel JB, Candinas D, Koziak K, Millan M, Hancock WW, Bach FH (1997) Loss of ATP diphosphohydrolase activity with endothelial cell activation. J Exp Med 185: 153–163
15. Rydberg L, Hallberg E, Björck S, Magnusson S, Strokan V, Samuelsson BE, Breimer ME (1995) Studies on the removal of anti-pig xenoantibodies in the human by plasmapheresis/immunoadsorption. Xenotransplantation 2: 253–263
16. Rydberg L, Björck S, Hallberg E, Magnusson S, Sumitran S, Samuelsson BE, Strokan V, Svalander CT, Breimer ME (1996) Extracorporeal ("ex vivo") connection of pig kidneys to humans. II. The antipig antibody response. Xenotransplantation 3: 340–353
17. Rydholm H, Boström S, Hansson G, Risberg B (1994) Endothelial prostacyclin production, synergistic effect between adrenergic stimulating and blocking drugs. Thromb Res 76: 333–341
18. Saadi S, Platt JL (1995) Transient perturbation of endothelial integrity induced by natural antibodies and complement. J Exp Med 181: 21–31
19. Sandrin M, McKenzie IFC (1994) Galα1–3Gal, the major xenoantigen(s) recognised in pigs by human natural antibodies. Immunol Rev 141: 169–190
20. Sandrin MS, Vaughan HA, Dabkowski PL, McKenzie IFC (1993) Anti-pig IgM antibodies in human serum react predominatly with Gal(α1–3)Gal epitopes. Proc Natl Acad Sci 90: 11391–11395
21. Siney L, Lewis MJ (1992) Endothelium-derived relaxing factor inhibits platelet adhesion to cultured porcine endocardial endothelium. Eur J Pharmacol 229: 223–226
22. Suttorp N, Seeger W, Zinsky S, Bhakdi S (1987) Complement complex C5b-8 induces PGI_2 formation in cultured endothelial cells. Am J Physiol 253: C13–21