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Polyethylene Glycol Diisocyanate Decreases Platelet Deposition After Balloon Injury of Rabbit Femoral Arteries

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Abstract

Background—Platelet deposition after angioplasty remains problematic and may contribute to intimal hyperplasia and restenosis. We proposed that polyethylene glycol diisocyanate (PEG-DISO), a polymer that rapidly forms covalent linkages with amine residues on proteins, could mask thrombogenic vascular wall proteins from platelets, thereby abrogating acute platelet deposition.

Methods and Results—To test this hypothesis, we isolated the femoral arteries of 10 New Zealand White rabbits and injured them with 3 passes of a 2F Fogarty catheter which was inserted through a distal arteriotomy. Immediately after balloon injury, ¹¹¹indium-labeled autologous platelets were infused peripherally and the injured femoral arteries were randomly treated for 1 minute with a PEG-DISO solution in one artery and a control solution of the phosphate buffered saline vehicle in the contralateral artery. Following treatment, reflow was initiated. The vessels were harvested after 1 hour and radioactivity was quantified in a gamma counter. Platelet counts were standardized by weight and expressed as platelets/mg (mean ± SEM). Platelet deposition onto arteries treated with PEG-DISO was $(1.2 \pm 0.5) \times 10^6$ platelets/mg compared to $(5.6 \pm 4.2) \times 10^6$ platelets/mg onto the contralateral control arteries treated with vehicle ($P < 0.005$). Scanning electron micrographs of the injured vessel segment confirmed qualitatively less platelet deposition on the treated segments than on the control segments.

Conclusion—Treatment with PEG-DISO significantly inhibited platelet deposition after vascular injury. These data support the hypothesis that treatment with PEG-DISO masks surface adhesive proteins from platelet receptors *in vivo* and that the resulting molecular barrier significantly reduces platelet deposition onto the damaged vessel wall for at least one hour. The formation of a molecularly thin barrier to platelet deposition may thus be a novel and effective treatment to abrogate acute intravascular thrombosis and may have value in the treatment of restenosis.

Keywords

thrombosis; angioplasty; platelet deposition inhibitor; poly(ethylene glycol)

Introduction

Percutaneous transluminal coronary angioplasty (PTCA) is a successful means of revascularizing patients with significant coronary artery disease. This procedure is limited by two major complications, a 2–8% risk of acute vessel closure and a 30–50% incidence of restenosis at the site of PTCA [1,2]. These complications have been significantly reduced with the now predominant use of intracoronary stenting, but restenosis continues to be a major limitation [3,4]. PTCA causes vascular injury which leads to platelet adhesion and platelet aggregation [5]. This deposition in turn elicits the release of prothrombotic, inflammatory and growth stimulating substances [6]. The ensuing platelet thrombus contributes to the major complications of PTCA [7].

Attempts to limit this platelet adhesion and activation after vascular injury have met with mixed success. Aspirin decreases acute vessel closure but not restenosis [8]. Abciximab, a glycoprotein IIb/IIIa receptor inhibitor, has been shown to inhibit not only acute complications, but also to decrease clinical restenosis at six months [9,10]. This therapeutic platelet inhibition comes at the cost of a small increase in the risk of bleeding complications, although this risk can be minimized by changes in heparin dosing and careful management of vascular sheaths and heparin dosing [11,12].

Polyethylene glycol diisocyanate (PEG-DISO) is a polymer with a polyethylene glycol backbone and reactive isocyanate groups at each end. It rapidly reacts with amine groups on proteins to covalently attach PEG via urea linkages. Therefore, if applied to an injured vessel surface, PEG-DISO could bind and mask the exposed adhesive ligands from luminal contact with platelets and other cells. PEG modification of proteins in solution is widely used in the pharmaceutical industry to increase the plasma half-life and decrease the immunogenicity of protein and peptide therapeutics [13]. We have previously shown that PEG-DISO decreases platelet deposition onto injured human vessel surfaces in an *ex vivo* perfusion model [14]. In other related previous work we have shown that amine-reactive PEG can react with adsorbed proteins to block acute platelet adhesion from flowing blood onto a variety of biomaterial surfaces and adsorbed type I collagen [15].

Because of this promising preliminary data, we hypothesized that forming a molecular barrier with PEG-DISO would mask the adhesive ligands exposed after vascular injury *in vivo* and thereby inhibit platelet deposition and activation.

Methods

Twelve New Zealand White rabbits were sedated with xylazine (5 mg/kg i.m.) and atropine (0.01 mg/kg) followed in fifteen minutes by general anesthesia with ketamine (40 mg/kg i.m.) and acetylpromazine (0.1 mg/kg i.m.). This anesthetic regimen was supplemented with additional doses of ketamine (1.5 mg/kg i.v.) and xylazine (0.8 mg/kg i.v.) as needed. All procedures were performed in compliance with the regulations of the University of Pittsburgh Institutional Animal Care and Use Committee.

Arterial injury

Bilateral common femoral arteries were exposed and isolated between airtight ligatures. An arteriotomy was made distally in the superficial femoral artery, and a 2F embolectomy catheter (Baxter Healthcare Corp., Edwards Division, Santa Ana, CA) was advanced proximally into the common femoral artery. The embolectomy catheter was inflated and withdrawn through the isolated femoral segment 3 times and then removed. A 20 gauge IV catheter was then inserted through the arteriotomy and into the isolated common femoral artery segment. This segment was then cleared of blood by flushing with 3 ml of saline and

allowing the solution to drain distally through the loosened distal ligature. Immediately after balloon injury, a 0.2 g/mL solution of PEG-DISO (MW 3400, Shearwater Polymers, Huntsville, AL) dissolved in phosphate buffered saline at pH 8.0 (PBS) was prepared. One arterial segment was randomly chosen to be treated with the PEG-DISO and the contralateral arterial segment was treated with the PBS vehicle in a blinded manner. These solutions (0.5 ml) were infused into the isolated arterial segment via the IV catheter with both proximal and distal ligatures tightly closed and allowed to incubate for one minute. The isolating common femoral ligatures were then released, allowing reperfusion. The IV catheter was then removed and the distal superficial femoral artery was ligated proximal to the arteriotomy site allowing continued distal flow through the deep femoral artery branch. After injury and treatment of the femoral arteries, as described above, ^{111}In -labeled platelets, previously produced by labeling autologous platelets from blood samples obtained before surgery with ^{111}In -oxine, were injected via an ear vein catheter, and allowed to circulate for 1 hour. After the one hour incubation, the animals were sacrificed with an overdose of pentobarbital (50 mg/kg iv). The femoral artery segments were harvested and flushed with saline and then fixed with 2.5% glutaraldehyde. A segment of uninjured, untreated carotid artery was harvested as a sham-injured control.

Platelet labeling

Blood was drawn from a central ear artery into citrate buffer (0.013 mol/L final concentration). Platelets were separated by centrifugation and labeled with ^{111}In oxine (Mallinckrodt Medical, Inc., St. Louis, MO) using the method of Johnson et al. [16]. Labeled platelets were resuspended in rabbit plasma and reinjected as described above. Vessel segment radioactivity was quantified in a gamma counter (Wallac Inc., Gaithersburg, MD).

Scanning electron microscopy

Samples for scanning electron microscopy were dehydrated in a series of ethanol washes, CO_2 critical point dried, and sputter coated. Samples were kept desiccated until analyzed under the scanning electron microscope (JOEL Series 300 Scanning Electron Microscope).

Visualization of vessel reactivity

To visualize the reactivity of a damaged blood vessel lumen with an amine-reactive polyethylene glycol molecule similar to PEG-DISO, two rabbit femoral arteries were isolated and cut axially so that a flat arterial segment was formed. The luminal surface of each arterial segment was scraped three times with a spatula to mimic the damage done in the previously described, *in vivo* balloon procedure. One segment was incubated for one minute with 0.2 g/mL of a polyethylene glycol molecule that was terminated with fluorescein on one end and N-hydroxysuccinimide on the other end (fluorescein-PEG-NHS, MW 5000, Shearwater Polymers). The other arterial segment was incubated for one minute with the PBS vehicle control. Fluorescein-PEG-NHS was used instead of DISO-PEG since the latter molecule cannot be modified in a stable manner with a fluorescein group. The amine reactivity of isocyanate and N-hydroxysuccinimide are similar according to the producer (Shearwater Polymers). After the incubation period, the arterial segments were rinsed vigorously and imaged with confocal microscopy.

Data analysis/statistics

Platelet counts in the arterial segments were standardized by weight and expressed as platelets/mg of wet tissue. The data are shown as mean $\pm$ the standard error of the mean (SEM). The data were not normally distributed, therefore significance was determined using the Wilcoxon signed rank test. The sample size was determined by the number of animals

necessary to show a 75% inhibition of platelet deposition with an $\alpha = 0.05$ and a power of 80%.

Results

The femoral arteries of 12 rabbits were injured and treated. There were two thrombotic occlusions of the injured femoral artery segments and both occurred in vehicle-treated segments. Due to the magnitude of the difference in platelet deposition after total thrombotic occlusion and after non-occlusive balloon injury, the platelet deposition data from these occluded vessels were not comparable to the others. Therefore, these animals were excluded from further analysis, leaving a total of 10 animals (20 arterial segments) for the final analysis of platelet deposition.

Platelet deposition onto arteries treated with PEG-DISO was $1.2 \pm 0.5 \times 10^6$ platelets/mg wet tissue compared to $5.6 \pm 4.2 \times 10^6$ platelets/mg wet tissue onto the contralateral control arteries treated with vehicle ($P < 0.005$) (Fig. 1). Treatment with PEG-DISO also decreased platelet deposition qualitatively when assessed by scanning electron microscopy (Figs. 2 and 3). There were less platelets visible on the treated vessel surface than on the control surface. The platelets on the control surface also had spread extensively and extended pseudopodia, indicating activation on this surface. This evidence of activation was generally not seen in the few platelets deposited on the treated vessel surface. Those platelets were more rounded and smooth with few pseudopodia.

Fluorescence visualization of a damaged femoral segment reacted with fluorescein-PEG-NHS demonstrated some regional variation in the ability of the amine-reactive polymer to attach (Fig. 4). This fluorescence was not attributable to the vessel wall, as seen in the control segment.

Discussion

Treatment with PEG-DISO successfully inhibited platelet deposition in the rabbit femoral artery injury model. This decrease in platelet accumulation supports our hypothesis that formation of a molecular barrier with PEG-DISO can mask the adhesive ligands exposed after vascular injury and thereby inhibit platelet deposition and activation. By inhibiting platelet thrombus formation, PEG-DISO may offer a means to decrease the complications of coronary interventions and vascular surgery attributable to acute vascular thrombosis.

This inhibition of platelet deposition occurred after a one-minute treatment duration. The rapid reaction kinetics of the isocyanate reaction allow treatment of the injured vessel within a time frame sufficiently brief to avoid ischemia. This should allow the use of PEG-DISO after interventions in vascular beds that tolerate ischemia poorly.

By nonspecifically masking the adhesive ligands on the vessel surface, PEG-DISO can inhibit diverse ligand-receptor interactions, including platelet and possibly leukocyte adhesion. This may allow it to inhibit the formation of the prothrombotic and inflammatory milieu that promotes the hyperplastic response to vascular injury. By acting locally at the site of injury, systemic inhibition of platelet function is not necessary, and the inherent risk of bleeding complications from systemic antiplatelet agents can be avoided.

Visualization of the attachment of a fluorescent, similarly reactive polyethylene glycol molecule qualitatively demonstrated the presence of some spatial variation in vessel reactive sites. This variation may explain the why the treatment did not completely block platelet adhesion. Whether the variation in vessel reactivity is due to a variation in surface protein (and thus lysine) concentration or due to mass transfer considerations, such as fluid transport

into crevices, is not clear. Furthermore, it has not been demonstrated which vascular surface protein modification might be responsible for the reduction in platelet deposition. Collagen seems like a likely candidate, and previous experimentation by our group in which type I collagen was modified with PEG-DISO *in vitro* demonstrated that the thrombogenicity of this protein could be markedly reduced [15]. It is also possible that other prothrombotic surface proteins might be modified, such as von Willebrand factor or tissue factor.

Previous studies have shown that formation of a polymer barrier at the luminal surface decreases thrombus formation and inhibits the neointimal response to injury [17]. This work used PEG-diacrylate, which requires light activation to initiate polymerization and forms a thicker, macroscopic barrier on the order of 20 μm . Further studies showed that coverage of the damaged arterial surface with a degradable form of the PEG-diacrylate polymer which persisted for only 24–48 hours was sufficient to inhibit the response to injury in a rabbit carotid injury model [18]. This implies that early inhibition of platelet deposition may passivate the injured arterial wall and limit the response to injury.

At present it is unclear how long the attached PEG in our study would persist at the site of injury *in vivo*. We hypothesize that as the surface is remodeled by enzymatic activity, PEG-modified protein fragments might be released into the blood. Alternatively, the urea bond which links PEG to the modified surface protein may hydrolyze at a rate greater than the surface remodeling rate, leading to the release of PEG alone into the blood with subsequent clearance in the kidneys [13,19]. Further investigation is ongoing to evaluate this and to evaluate the effect of such a barrier on intimal hyperplasia. The acute benefits, however, appear striking and may be of value in the setting of both coronary interventions and vascular surgery.

In summary, treatment with PEG-DISO significantly inhibited platelet deposition after vascular injury. These data support the hypothesis that treatment with PEG-DISO masks surface adhesive proteins from platelet receptors *in vivo* and that the resulting molecular barrier significantly reduces platelet deposition onto the damaged vessel wall for at least an hour. The rapid kinetics of the isocyanate reaction give this treatment potential for application in vascular interventions. PEG-DISO treatment may offer advantages over systemic anti-platelet therapy because of its selective treatment of the site of vascular damage and its broad coverage of adhesive ligands. The formation of a molecularly thin barrier to platelet deposition may thus be a novel and effective treatment of acute vascular thrombosis.

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Fig. 1.
¹¹¹In-labeled platelet deposition after balloon injury of the femoral artery and treatment with vehicle control (n = 10), or PEG-DISO, (n = 10). Platelet deposition on the uninjured carotid artery (Sham, n = 10) is also shown for comparison. Data are shown as mean ± SEM. *P < 0.005.

Fig. 2. Scanning electron micrographs of a femoral artery that has been balloon-injured and treated with PEG-DISO (A. 750 $\times$, B. 2000 $\times$).

Fig. 3. Scanning electron micrographs of a femoral artery that has been balloon-injured and treated with the vehicle control saline solution (A. 750 $\times$, B. 2000 $\times$).

Fig. 4. Confocal micrographs of A) a fluorescein-PEG-NHS treated, scrape-damaged rabbit femoral artery in vitro and B) a vehicle control treated, scrape-damaged artery.