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In stent restenosis and thrombosis assessment after EP224283 injection in a rat model

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ABSTRACT

Objective: After stent implantation, platelet aggregation and thrombus formation are thought to play a key role in the early phase of in-stent restenosis (ISR). Drug-eluting stents have reduced ISR, but are associated with healing-related issues or hypersensitivity reactions, leading to an increased risk of late acute stent thrombosis. EP224283 is a new dual-action antithrombotic molecule combining a GPIIbIIIa antagonist and a factor Xa inhibitor. We investigated its efficacy on restenosis in a rat model of ISR and on platelet adhesion.

Methods and results: Rat aortas were stented and the animals received either EP224283 or vehicle subcutaneously every 48 h. At day 7 and day 28 after surgery, the stented aortas were removed and processed for morphometric analysis or protein analysis. At day 28, EP224283 significantly reduced neointima growth (in the range of 20%). Protein analysis revealed that EP224283 reduced cell proliferation pathways: ERK1/2 and Akt were down-regulated and p38 up-regulated. Expression of Ki67 was also reduced. *In vitro* assessment depicted a reduction of platelet activation and platelet adhesion among treated rats.

Conclusion: These results show a beneficial effect of EP224283 on in-stent restenosis and on stent thrombogenicity that may improve results after stent implantation. Further investigations are required to assess the efficacy of a local delivery of EP224283 on both acute thrombosis and ISR.

1. Introduction

Neointima formation is required for vessel healing after stenting to avoid contact between stent struts and flowing blood, but excessive neointima formation narrows the vessel lumen leading to in-stent restenosis (ISR) and increased morbi-mortality. ISR remains a major concern, diagnosed in 20%–30% of patients after coronary stenting [1] and in 40%–50% of patients after infrapopliteal stenting [2]. Drug-eluting stents (DES) reduce coronary ISR to less than 10% [3], but expose patients to late stent thrombosis, because of the alteration of both the structure and the function of the endothelium [4]. Thu, several teams are currently working on new "pro-healing" DES, which are polymer free, release new drugs or are bioasborbable. Those stents would have the potential to reduce ISR without affecting vessel healing and the re-endothelialization process of the stent struts.

ISR involves complex inter-related molecular and cellular mechanisms, which can be divided into an "early" (days to weeks) and a "late" (weeks to months) phase. Stent implantation induces a mechanical injury to the arterial wall, with media dissection and endothelial disruption. The dysfunctional endothelium produces factors promoting thrombus formation, before fibrin and platelet aggregation provide the basis of a local then systemic inflammatory response [5]. During the late phase, vascular smooth muscle cells (VSMC), normally highly differentiated, change their phenotype and become hypertrophic, with a high rate of proliferation and migration. This aberrant VSMC growth associated with coordinated extracellular matrix synthesis leads to neointima hyperplasia and ISR [6].

Platelets are thought to play a key role in ISR, by expressing adhesion molecules inducing inflammatory cell recruitment, and by releasing growth factors such as platelet-derived growth factor (PDGF), which is involved in VSMC phenotype switching [6]. In clinical practice, antagonists of the most expressed platelet

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glycoprotein (GP) receptor, GPIIbIIIa, have been tested to block the final pathways of platelet aggregation. It has improved results and decreases the incidence of major adverse cardiac events in patients undergoing coronary stenting [7]. Platelet GPIIbIIIa inhibitors are now widely used for the treatment of acute coronary syndromes and during percutaneous coronary revascularization. Recently, GPIIbIIIa receptor blocker (abciximab)-coated stents have proved to be safe and effective in the prevention of ISR in human coronary arteries [8], as well as in the prevention of acute stent thrombosis [9].

Acute stent thrombosis is a potentially life-threatening complication after coronary stenting. In experimental studies, heparin-coated stents have reduced the thrombogenicity of stents [10] and have demonstrated antiproliferative properties [11]. Clinical trials report further evidence that heparin coating may provide additional protection against thrombosis [12].

EP224283 is a new dual-action anticoagulant which combines both GPIIbIIIa antagonist (tirofiban) and factor Xa (FXa) inhibitor (idaparinux) properties [13]. As platelets are key mediators of ISR, and in order to avoid sub-acute stent thrombosis, we hypothesized that EP224283, combining both platelet aggregation inhibition and anticoagulant properties, may attenuate neointima formation after stent placement and also reduce the risk of acute thrombosis. In this study, we assessed the potential protective effects of EP224283 against ISR in a rat model and against stent thrombosis in an *in vitro* model.

2. Materials and methods

2.1. Materials

EP224283 and EP37122 were kindly provided by Endotis Pharma (Romainville, France). EP224283 is a new synthetic compound, which combines the GPIIbIIIa antagonist tirofiban and the antithrombin III binding FXa inhibitor idraparinux, connected through biotin moiety, which makes EP224283 effects neutralizable with avidin [13]. The half-life of tirofiban is very short (24 ± 7 min in rats) and its administration can only be performed by continuous intravenous perfusion [14]. Covalent binding of tirofiban to idraparinux (EP224283) confers to tirofiban the half-life of idraparinux (10 h in rats). EP37122, an idraparinux analog was used to identify the tirofiban's activity in EP224283.

2.2. In vivo assay

All animal experiments were performed by a single operator and all study protocols were approved by our local Institutional Animal Care and Use Committee (N°CEEA 212011).

2.2.1. Rat model of in stent restenosis

Adult male Wistar rats (350–400 g, Charles River, France) were fed a normal diet *ad libitum* and separated into a control group, an EP224283 group or an EP37122 group. On the day of implantation, as previously described [15], the rats were anesthetized (ketamine 130 mg/kg and xylazine 14 mg/kg intraperitoneally) and a median laparotomy was performed. The infra renal aorta was clamped after systemic heparinization (300 UI/kg). One bare metal balloon expendable stent (2.5 mm × 12–20 mm, Persillion, Cordis, Johnson & Johnson), 30% oversized as compared to the native aorta, was then deployed at its nominal pressure (8 atm) in the infra renal aorta, through a transverse aortotomy.

For 28 days starting on the stent implantation day, the rats received either subcutaneous (SC) injection of saline/48 h for the control group, EP224283 1.25 mg/kg/48 h SC, or EP37122 10 mg/kg/24 h SC.

Stent length was homogenous between the different groups. A total of 43 rats were enrolled in the study and distributed in three groups as follow:

- for histomorphological analysis at day 28: 9 control rats, 9 EP224283 treated rats and 9 EP37122 treated rats
- for protein expression analysis, at day 7: 5 control rats and 5 EP224283 treated rats, and at day 28: 3 control rats and 3 EP224283 treated rats.

At the end of experimentation, animals were sacrificed, stented arteries were harvested and either perfused with fixative for histological analysis (on day 28), or frozen for protein expression analysis (on days 7 and 28).

2.2.2. Histomorphometrical analysis

As previously described [16], stented arteries were fixed in 4% paraformaldehyde phosphate-buffered solution, dehydrated, and embedded in methylmethacrylate polymer (Technovit 9100 new, Heraeus, Germany). Sections 60 μm thick were cut along the entire embedded stented vessels, stained with Mayer hematoxylin and eosin and examined under light microscopy (Leika DMIL, Germany) with magnification of ×5. Images were digitized and recorded with the use of a video camera (AxioCam ERc 5s, Carl Zeiss Microscopy, Germany). For each stented vessel, 15 sections along the entire length were randomly chosen for the morphometric analysis, performed with a computerized digital microscopic planimetry algorithm by an independent observer blinded to drug regimen. Cross-sectional areas of media, intima, and lumen were measured. Neointimal thickening was expressed as the ratio of the neointima area to the media area (n/m).

2.2.3. Western Blot analysis

Western Blot analyses were performed on frozen (−28 °C) stented aortas harvested 7 or 28 days after stent implantation. After a careful extraction of stent from the arterial wall, the aorta tissue was homogenized and tissue extracts were cleared by centrifugation (10,000 g for 20 min at 4 °C). Twenty or 30 μg of protein were charged in 10% sodium dodecyl sulfate-polyacrylamide electrophoresis gel and transferred to a nitrocellulose membrane, which was incubated with specific antibodies for detection of ERK1/2, phosphorylated ERK1/2, p38 mitogen-activated protein kinase (MAPK), phosphorylated p38MAPK, phosphorylated Akt (pThr^{-308}), total Akt (Cell Signaling, France); p27^{kip}, Ki67, p21 (SantaCruz BiotechnologyInc); heme-oxygenase1 (HO1) and actin (Sigma–Aldrich, France). Immunoreactive bands were visualized using peroxidase conjugated secondary antibody and subsequent ECL detection (Western Lightning® Plus-ECL, PerkinElmer, France), then quantified by densitometric analysis using Multi Gauge V3.0 (FUJIFILM Graphic Systems France S.A.S.).

2.2.4. In vitro thrombogenicity assessments

2.2.4.1. Blood sampling and hematological tests. During 8 days, 4 rats were injected with EP224283 1.25 mg/kg/48 h SC and 4 rats with saline. Before the first injection and then at 4, 24, 48, 72, 96 and 195 h, 500 μl of blood were retro-orbitally collected from two EP224283 treated rats for anti-Xa activity measurement. After 8 days, under general anesthesia, 10 ml of blood were collected from rat (2 ml were used for platelet activation analysis and the rest was used for following platelet adhesion test).

2.2.4.2. Platelet adhesion on cobalt chrome surface. Platelet suspensions with 4×10^5 cells/μl from 3 EP224283 treated rats or 3 control rats were prepared by diluting platelet-rich plasma (PRP) in platelet-poor plasma (PPP), which was obtained by centrifuging the