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Beta-radiation reduces the reactivity of extra cellular matrix proteins in intravascular brachytherapy (IVBT), resulting in decreased platelet adhesion

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ABSTRACT

Background: Intravascular Brachytherapy as a tool to reduce restenosis is thought to alter vascular wall biology and vessel wall protein function. Platelet accumulation is also indeed important in the genesis of restenosis.
We examine the in vitro effects of beta-radiation on the certain vessel wall extra cellular matrix proteins. We hypothesized that vessel wall (proteins) had become less prone to thrombosis.
Methods: We examined platelet adhesion to 20-Gy beta radiation treated extra cellular matrix proteins under flow conditions. Platelet flow adhesion was evaluated or quantified by image analysis, aggregation size analysis using the Watershed program and real-time fluorescence images of thrombus formation. The effect of beta radiation on vWF was further showing by measuring the binding of domain-specific antibodies to radiation treated vWF.
Results: 20-Gy beta radiation significantly decreased platelet adhesion to extra cellular matrix protein; vWF and collagen Type III and had no effect on the adhesion upon fibrinogen and fibronectin. The beta-radiation affected mostly the AI, A2 and A3 domains of the vWF molecule on the surface, whereas the D′–D3 and B–C1 domains on the surface remain unaffected and suggesting a significant decrease in vWF binding capacity to the GPIb, heparin and collagen ligands.
Conclusion: Beta radiation treatment can alter the reactivity of the certain vessel wall extra cellular matrix proteins, in particular vWF and collagen. The vessel wall may become less prone to platelet adhesion, which results in decrease thrombus formation. It might help to reduce the onset of acute coronary occlusion after the intervention.

1. Introduction

Exciting results from the trials of anti-proliferatives drug-eluting stents have resulted in a decrease of instant restenosis [1,2]. Intravascular Brachytherapy (IVBT) is still a safe and effective method of prevention of restenosis [3–6]. It has been shown to be favorable in comparison to placebo. IVBT has been recognized as a treatment modality for reducing coronary restenosis after angioplasty and stent-implantation procedures [7–10]. Single doses in excess of 7–56 Gy intracoronary catheter-based beta-radiations have clearly shown a reduction the genesis of restenosis in the animal and clinical models [11,12].

After vascular injury, the first occurring step is the adherence of platelets to the endothelium and the exposed media [13]. As endothelial denudation the platelet adhesion has been observed both by light and scanning electron microscopy after arterial intimal damage and restenosis [14]. Jaster et al have shown that platelet activation during brachytherapy is not caused by irradiation but by the procedure of catheter based VBT [15]. However, their experiments did not study the effect of radiation on the thrombogenicity of the vessel wall. We developed in our laboratory an in vitro flow system [16–27] to mimic the effects of beta radiation and study of platelet adhesion to endothelial extra cellular matrix proteins. We have reported platelet adhesion to photodynamic therapy (PDT)-treated matrix proteins before [20]. The PDT reduces platelet adhesion to vWF and fibrinogen. In contrast,

Abbreviations: GP, glycoprotein; vWF, von Willebrand factor; IVBT, Intravascular brachytherapy; PDT, photodynamic therapy.

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collagen becomes increasingly prone to platelet adhesion [20]. Here we have studied how beta radiation alters the reactivity of the certain vessel wall proteins for platelets.

2. Methods

In our vitro experiments, we coat endothelial extra cellular matrix protein vWF, collagen, fibrinogen and fibronectin to glass coverslip by incubation or air-pressure spraying procedure. The interactions between platelet and these proteins are further determined by a blood perfusion system. The flow studies were performed in a well-defined perfusion chamber, which could be reversible sealing of the coverslip to chamber [17,23–25]. A syringe pump was used to aspirate the blood through the coverslip at desired flow rates in the chamber. Both coverslips were perfused in sequence in a random order, thus exposing the control and beta-radiation coverslips to the same blood flow. After perfusion, the coverslips were removed, rinsed, fixed, dehydrated and stained with the May–Grunwald–Giemsa. Platelet adhesion to the coverslip was measured with light microscopy coupled to an image analyzer system. The randomly selected coverslip areas were evaluated. Platelet adhesion was expressed as the percentage of the surface covered with platelets. The platelet adhesion to collagen type III induced aggregate under flow conditions. The aggregation size was evaluated by a morphological analytic program. Another flow chamber was mounted on an inverted-stage Real-time fluorescence microscope. It was suitable for very low light levels and used to visualize directly platelet adhesion and subsequent platelet aggregation, throughout the perfusion period. As platelet adhesion to collagen type III at a shear rate of 1600 s^{-1} was almost completely dependent on vWF, [21] we reasoned that a major damage of beta-radiation was through modification of vWF. So to further study, we performed an ELISA on the normal vWF surface and the beta radiation-treated vWF surface with different domain specific monoclonal antibodies [20].

2.1. The human endothelial extra cellular matrix protein preparation

VWF was prepared from cryoprecipitate using polyethyleneglycol precipitation and gel filtration. Fibronectin was prepared by affinity chromatography from citrated plasma [21,22]. Fibrinogen (Enzyme Research Labs, USA) was further purified by size exclusion chromatography on Sepharose 4B. Placenta collagen type III was purchased from Sigma Chemical Co.

2.2. Coverslips coating

Coverslips were cleaned overnight in a chromium trioxide solution, rinsed with distilled water and coated by 10 μg/ml vWF or 100 μg/ml fibrinogen for 1 h. Each coverslip was then incubated in 1% human albumin to prevent non-specific protein binding.

For fibronectin and collagen coating, spraying technique was done with a retouching airbrush (Badger model 100, IL USA). After the spraying procedure, the coverslips were also incubated for 60 min with 1% human albumin to prevent non-specific protein binding.

2.3. Beta-^{32}P radiation application for the coverslips, which coated by extra cellular matrix proteins

Beta-^{32}P radiation application for the coverslips is performed with an intracoronary centered balloon catheter (Galileo intravascular radiotherapy system, TX USA) [28]. In summary, it consists of four components: 1, the source delivery unit; 2, the centering catheter; 3, the ^{32}P source wire and 4, the inactive wire. The longitudinal distance of the 'full' prescribed dose (100% isodose) coverage, measured by radio chromic films, does about 22 mm constitute the effective radiation length [28,29].

The coverslips, coated with vWF, collagen type III, fibronectin, and fibrinogen, were irradiated in a special coverslip bracket. A beta-^{32}P radiation dose of 20 Gy was used in all experiments. The irradiation time is automatically calculated and controlled by Galileo intravascular radiotherapy system instrument.

2.4. Platelet perfusion procedure

The perfusion studies were performed in a well-defined perfusion chamber, which could be reversible sealing of the coverslip to chamber by vacuum force [23–25]. A syringe pump was used to aspirate the blood through the coverslip at desired flow rates in the chamber. Both coverslips were perfused in sequence in a random order, thus exposing the control and beta-radiation coverslips to the same blood flow.

Blood used for platelet perfusion studies was collected from healthy donors receiving no medication. For vWF and fibrinogen perfusion experiments, blood was anticoagulant 1:10 vol/vol with 110 mM trisodium citrate (pH 7.4). For collagen perfusions, blood was anticoagulant with 1: 10 vol/vol 200 U/ml low molecular weight heparin (Fragmin, Sweden) [25]. The temperature of whole blood was kept on 37 °C. Perfusions were performed at the optimal shear rate for a difference of coated proteins. 300 s^{-1} for fibrinogen and fibronectin, 1000 s^{-1} for vWF and 1600 s^{-1} for collagen [22,26,27,30,31].

2.5. Evaluation of platelet adhesion

After perfusion, the coverslips were removed, rinsed, fixed in 0.5% glutaraldehyde, dehydrated in methanol and stained with the May–Grunwald–Giemsa. Platelet adhesion to the coverslip was measured with light microscopy coupled to an image analyzer system (Optimas 6.0 the Netherlands). Thirty randomly selected coverslip areas were evaluated, and the results from triplicate coverslips were averaged. Platelet adhesion was expressed as the percentage of the surface covered with platelets.

2.6. The aggregation size analysis, using Watershed program

The platelet adhesion to collagen type III induced really aggregate under flow conditions. The aggregation size was analyzed by the Watershed program provided by the Optima software [24,32]. The Watershed technique separates confluent or overlapping objects based on color intensity levels. This technique was reproducible with a variation coefficient of 9.8%. Platelet aggregation size on collagen type III surface was subdivided into 6 groups; [1] contact and spread platelet with an area of $< 8\ \mu m^2$, [2] aggregated with an area between 8–40 μm^2, [3] aggregated with an area between 40–200 μm^2, [4] aggregated with an area between 200–400 μm^2, [5] aggregated with an area between 400–600 μm^2, [6] aggregated with an area $>600\ \mu m^2$.

2.7. Real-time fluorescence images of thrombus formation

The flow chamber was mounted on an inverted-stage microscope equipped with an epifluorescent illumination and a Pioneer DVD-recorder (Fig. 1). It was suitable for very low light levels and used to visualize directly platelet adhesion and subsequent platelet aggregation, throughout the perfusion period. Whole blood was labeled by mepacrine (Sigma), warmed to 37 °C, aspirated through this chamber. The perfusion images were recorded with a time interval of 80 ms and automatically evaluated.

2.8. An ELISA study of control vWF surface and beta radiation-treated vWF surface with domain specific monoclonal antibodies

We performed an ELISA on the normal vWF surface and the beta radiation-treated vWF surface with domain specific monoclonal antibodies.

20 Gy beta-radiation treated vWF coverslip and control coverslip were incubated with monoclonal antibodies directed against specific domains of the vWF molecule (RAG-35, 40, RU-1, RU-5, antiD′–D3, and vWF9) or polyclonal antibody. Then the antibody binding upon vWF was quantified as follows: coverslips were incubating for 1 h with the peroxidase-labeled anti-mouse or rabbit antibody and coloring with H_2O_2-phenylenediamine (OPD) buffer. Coloring was stopped by 3 N H_2SO_4. The optical density was measured at 492 nm. Results were expressed as the percentage of the control.

2.9. The vWF domain specific monoclonal antibodies made by our laboratory

Recombinant A2, A3, D′–D3 of human vWF, was expressed and purified. To further delineate how beta-radiation affected the vWF protein, antibodies, which directed against specific vWF domain, were made in our laboratory, Utrecht. Monoclonal antibody RU1 directed against the A2 repeat, monoclonal antibody RU5 directed against the A3 repeat and a polyclonal rabbit antibody directed against the D′–D3 region were prepared in our laboratory. For production of monoclonal antibody RU1 and RU5 ($IgG_{2a,k}$) hybridoma cells were injected in mice, and ascites was collected. IgG was purified on a protein G-Sepharose column, and Fab fragments were generated using an ImmunoPure-Fab kit (Pierce, Rockford, IL). RU5-Fab was further purified with affinity chromatography in our laboratory Utrecht.

2.10. Other vWF domains specific monoclonal antibodies

Monoclonal Antibody RAG-35, directed against the A1 domain, is a generous gift of Dr. van Mourik, (Sanquin research, Amsterdam, Netherlands). RAG-40, which directed against the D4 domain, is also a gift of Dr. van Mourik. vWF9, which inhibits interactions between VWF and GPIIbIIIa or αvβ3, is a generous gift of dr. Denis, (INSERM U143, Le Kremlin-Bicetre, France;). Polyclonal anti-vWF peroxides labeled antibody was obtained from Dako Co, Denmark.

2.11. Statistical analysis

All perfusions were performed in triplicate for each condition. The results are presented as the mean from experiments with blood from 3 different donors, unless indicated otherwise. One-way Analysis of Variance (ANOVA) and Tukey–Kramer Multiple Comparisons Test evaluation included calculation of mean values, standard error and nonparametric test for comparison of every two groups. All reported P values are two-sided. P values below 0.05 were considered to indicate statistical significance. All analyses were performed with the use of GraphPad, Prism and Instat software (San Diego USA.).