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Laser-induced primary and secondary hemostasis dynamics and mechanisms in relation to selective photothermolysis of port wine stains

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Background: Superficial vascular anomalies such as port wine stains are commonly treated by selective photothermolysis (SP). The endovascular laser–tissue interactions underlying SP are governed by a photothermal response (thermocoagulation of blood) and a hemodynamic response (thrombosis). Currently it is not known whether the hemodynamic response encompasses both primary and secondary hemostasis, which platelet receptors are involved, and what the SP-induced thrombosis kinetics are in low-flow venules.

Objectives: To (1) define the role and kinetics of primary and secondary hemostasis in laser-induced thrombus formation and (2) determine which key platelet surface receptors are involved in the hemodynamic response.

Methods: 532-nm laser-irradiated hamster dorsal skin fold venules were studied by intravital fluorescence microscopy following fluorescent labeling of platelets with 5(6)-carboxyfluorescein. Heparin and fluorescently labeled anti-glycoprotein Ib- α (GPIb α) and anti-P-selectin antibodies were administered to investigate the role of coagulation and platelet receptors, respectively. Lesional sizes were quantified by software.

Results: Laser irradiation consistently produced sub-occlusive thermal coagula. Thrombosis was triggered in all irradiated venules in a thermal coagulum-independent manner and peaked at 6.25 min post-irradiation. Heparin decreased the maximum thrombus size and caused thrombosis to reach a maximum at 1.25 min. Immunoblocking of GPIb α abated the extent of thrombosis, whereas immunoblocking of P-selectin had no effect.

Conclusions: The hemodynamic response ensues the photothermal response in a thermal coagulum-independent manner and involves primary and secondary hemostasis. Primary hemostasis is mediated by constitutively expressed GPIb α but not by activation-dependent P-selectin.

1. Introduction

Selective photothermolysis (SP) is a standard treatment modality for superficial vascular anomalies such as port wine stains (PWS) and other vessel-related dermatological disorders [1]. SP is based on the conversion of radiant energy to heat by

(de)oxyhemoglobin and the thermal denaturation of blood and vascular tissue as a result of heat diffusion, referred to as photocoagulation or the photothermal response. By employing an appropriate wavelength and irradiance, supracritical temperatures ($>70^\circ\text{C}$) can be generated in the vessel lumen and confined spatially when the pulse duration is shorter than the time required for cooling of the target structures [2], i.e., 0.5–10 ms for PWS vessels of 30–300 μm in diameter [3,4]. Normal-sized capillaries and post-capillary venules (4–26 μm inner diameter [5]) have relatively short thermal relaxation times and thus remain spared during longer pulse durations, inasmuch as heat diffusion from these vessels precludes the generation of supracritical temperatures.

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CONVENTIONAL PHOTOTHERMOLYSIS

Fig. 1. Cartoon illustrating the principles of selective photothermolysis (SP) vs. site-specific pharmaco-laser therapy (SSPLT). Irradiation of port wine stain (PWS) vessels with a yellow laser (a) is characterized by the photothermal response (b), resulting in the formation of a thermal coagulum (b, insert), and the hemodynamic response, which is characterized by thrombosis. In time the thrombus deteriorates (c), leaving a partially occluded lumen (insert: cross-sectional view: 1, thermal coagulum; 2, thrombus; 3, patent lumen). In (d), a histological section imaged by scanning electron microscopy is presented of a PWS blood vessel following 577-nm laser irradiation, exhibiting a similar damage profile as in (c) (scale bar = 10 μm). The vessel is partially occluded by a thermal coagulum (TC) composed of electron-dense, thermally denatured blood with numerous electron-lucent spherical structures (insert upper left, scale bar = 1 μm) and a thrombus (T) comprising aggregated platelets and associated fibrin. The rest of the lumen is patent (P) and contains intact erythrocytes (IE). Endothelial cells in the thermally afflicted region exhibit cytoplasmic vacuolisation and separation from the basal lamina (insert bottom right, arrow, scale bar = 1 μm). Image adapted from Ref. [10]. SSPLT is an envisaged treatment modality for refractory PWS, whereby SP is combined with the systemic administration of a prothrombotic- and/or antifibrinolytic-containing drug carrier (e). It is aspired that, following conventional SP, the drug carrier accumulates in the thrombus (f) and its contents are released by a second stimulus (e.g., heat generated by a near-infrared laser pulse) (g), resulting in local hyperthrombosis and inhibition of fibrinolysis and complete occlusion of the vascular lumen (h). Clinical evidence suggests that complete occlusion of target vasculature (h, insert: 1, thermal coagulum; 2, thrombus) is associated with well-responding lesions.

The therapeutic efficacy of SP with respect to PWS depends on the extent to which the target vasculature is afflicted by the photothermal response. Complete photocoagulation/occlusion of the vascular lumen is required for optimal lesional clearance [6], which occurs in approximately 40% of patients [7]. Contrastingly, moderately responding (20–46%) and recalcitrant (14–40%) PWS [7,8] exhibit varying degrees of incompletely photocoagulated vessels containing semi-obstructive thermal coagula (Fig. 1a–d). Consequently, alternative treatment strategies are needed to improve therapeutic outcomes in the poorly-to-non-responsive PWS patient population [9].

Recently an experimental modality, site-specific pharmaco-laser therapy (SSPLT) [10], was proposed to ameliorate PWS recalcitrance to conventional SP. The conceptual framework of SSPLT is based on the occurrence of a hemodynamic response (thrombosis) following the photothermal response [11–13]. Accordingly, SP is combined with the prior administration of a prothrombotic- and/or antifibrinolytic-containing drug delivery system to instill complete occlusion, and thus complete removal, of semi-photocoagulated target vessels by the pharmacological augmentation of the hemodynamic response (Fig. 1e–h).

The aims of the study were to determine the role and the kinetics of primary and secondary hemostasis in the hemodynamic response and to establish which key platelet surface receptors are involved in SP-induced thrombosis using a hamster dorsal skin fold model in conjunction with fluorescent labeling of platelets and intravital fluorescence microscopy. The findings comprise a basis for the continued development of SSPLT in an effort to optimize the laser treatment of aberrant cutaneous vasculature.

2. Materials and methods

1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC) were purchased from Lipoid (Ludwigshafen, Germany). Polyethylene glycol-conjugated 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine (DSPE-PEG₂₀₀₀) was provided by Genzyme Pharmaceuticals (Liestal, Switzerland). Alexa488-labeled (Molecular Probes, Eugene, OR) rat anti-mouse CD62P monoclonal antibodies (mAbs) (CD62P-Alexa488, clone RB40.34) and FITC-conjugated rat anti-mouse IgG_{1k} isotype control (IgG-FITC, clone R3-34) were obtained from Research Diagnostics (Flanders, NJ) and FITC-conjugated F(ab')₂ fragments of polyclonal rabbit anti-mouse IgG (Fab₂-FITC) from Dako Cytomation (Glostrup, Denmark). Mouse anti-human CD42b (clone 11A4) mAbs were cloned as described [14]. 5(6)-Carboxyfluorescein (CF) was acquired from Kodak Chemicals (Williston, VT), heparin Choay from Sanofi Winthrop (Gentilly, France), and fluorescent microspheres (FluoSpheres Red, Ø = 1 μm) from Molecular Probes.

Online supplemental material is indicated with a prefix 'S' throughout the manuscript.

2.1. Animals

The animal protocol was approved by the Lille University Hospital animal ethics committee. Animals were treated in compliance with NIH publication 86-23.

Sixty two male Golden Syrian hamsters (93–117 g, Dépré, Saint Doulchard, France) were anesthetized by intramuscular injection of ketamine (200 mg/kg), xylazine (10 mg/kg), and buprenorphine (0.02 mg/kg) after pre-anesthesia with diethyl ether. The dorsal chamber, which has been employed previously as a preclinical

Fig. 2. Intravital microscopy setup (a), consisting of (1) fluorescence microscope (10 $\times$ objective, NA = 0.30, Eclipse E800, Nikon); (2) Peltier-cooled CCD video camera, 25 fps, 720 $\times$ 576 pixel resolution (model L3C65-06BPV01, E2V Technologies); (3) xenon lamp (transillumination); (4) xenon lamp (epi-illumination; model 68806, Oriel); (5) analogue shutter controller; (6) mechanical shutter; (7) frequency-doubled Nd:YAG laser; (8) xyz-translator stage; (9) digital video recorder; (10) monitor; (11) laser probe; (12) reflective mirror; (13) laser beam; (14) animal platform; (15) optical fiber connected to (14); (16) dorsal skin fold chamber; (17) mirror; (18) dorsal skin fold. The animals were placed on a microscope stage-fitted platform that contained three embedded pins that interlocked with holes in the dorsal chamber frames to mechanically secure the skin fold. Insert bottom left corner: microscope optics, arrows indicate the path of light. Filter A (λ_{ex} = 480 $\pm$ 15 nm) and filter B (λ_{em} = 535 $\pm$ 20 nm) with a dichroic mirror cut-on at 505 nm (orange plane) were used for thrombus imaging (epi-illumination mode), whereas filter C (λ_{ex} = 560 $\pm$ 20 nm) and D (λ_{em} = 630 $\pm$ 25 nm) with a dichroic mirror cut-on at 595 nm were employed for thermal coagulum imaging in trans- and epi-illumination modes (C–E, 3, 15, and 17). Image analysis procedure (b). For the analysis of CF-stained thrombi (top panel), the margins of the fluorescent lesion were manually contoured by 2 independent analysts. The contoured lesions were saved as separate image files (1) and quantified for pixel area (A_{pix}) and total intensity (I_{tot}) using dedicated software. For the analysis of antibody-stained thrombi (bottom panel), the vascular lumen was roughly contoured and saved as a separate image file (2). An intensity histogram of background (2, encircled) was collected from frames of each video sequence at t = 0, 2.5, 5.0, 7.5, and 10.0 min. To characterize the thrombus (3), a threshold range was defined in the programmed macro that had an intensity cut-on value of 5 units higher than the highest recorded intensity value in the 5 background frames. The thrombus was quantified for pixel area (A_{pix} , the total number of 'thrombus pixels') and total intensity (I_{tot} , cumulative intensity of the 'thrombus pixels'). Lesional size was calculated by normalized (A_{pix}) $\cdot$ normalized (I_{tot}) and relative lesional size was calculated by [normalized (A_{pix}) $\cdot$ normalized (I_{tot})]/ $t=0$, where t represents a time point in the video sequence and $t = 0$ represents the time point immediately after the laser pulse, defined as baseline.

model for PWS vasculature [15–17], was implanted according to Bezemer et al. [15]. Reagents were infused through the subclavian vein with a 30 G needle ($\sim$ 200 μ L/30 s). The animals were sacrificed by intravenous administration of potassium chloride.

2.2. Liposome preparation

Liposomes ($\emptyset$ = 132 $\pm$ 11 nm, section-S3) composed of DPPC:DSPC:DSPE-PEG₂₀₀₀ (10:85:5 mole ratio) were loaded with a self-quenching concentration CF (100 mmol/L, section-S3) as described previously [18]. Unencapsulated CF was removed by dialysis (Spectra/Por 7, MWCO-100,000, Spectrum Laboratories, Rancho Dominguez, CA) in phosphate buffered saline (PBS) at room temperature (RT) until a residual solvent concentration of 40 μ mol/L CF (measured spectrophotometrically, OD₄₅₀ = 0.59) was obtained during gentle stirring.

Raising the temperature above the phase transition temperature of the lipid bilayer (55.5 $^{\circ}$ C, Fig. S1) is associated with a gel-to-liquid-crystalline phase transition of component phospholipids, leading to rapid release of CF and a reduction in fluorescence quenching. Hyperfluorescence during intravital microscopy following the laser pulse therefore served as an indicator for thermal denaturation of plasma components ($>$ 45 $^{\circ}$ C [19]) and erythrocytes ($<$ 51 $^{\circ}$ C [20]), i.e., precursor events in thermal coagulum formation.

2.3. Intravital microscopy, laser-induced thrombosis

The microscopy and thrombus induction setup is explained in Fig. 2a. To verify that non-obstructive thermal coagula (Fig. 1c and d) were formed at the laser parameters employed for thrombus induction, the microscope optics were configured as described

previously [15]. Vascular patency was determined by infusion of fluorescent microspheres (2 $\times$ 10⁹ microspheres/kg, 100- μ L injection volume) [15]. Thermal coagula (n = 15) were visualized using a filter set whose excitation wavelength range (λ_{ex} = 540–580 nm) matches a high absorption range of thermally denatured blood [21] and whose emission range (λ_{em} = 605–655 nm) is broad enough to generate brightfield images in transillumination mode. In this configuration, simultaneous visualization of the laser-induced thermal coagulum (appearing dark against a lighter background), skin fold anatomy, and fluorescent microspheres (section 'Shear Rates') was possible. For thrombus imaging, mAb- and CF-labeled platelets were visualized at λ_{ex} = 480 $\pm$ 15 nm and λ_{em} = 535 $\pm$ 20 nm. In some antibody experiments, transillumination was applied for contrast enhancement. Endovascular events were recorded for a period of 8 min in the thermal coagulum experiments and for 15 min in the thrombosis experiments.

2.4. Intravital platelet labeling

After implantation of the dorsal skin fold chamber, platelets were stained cytochemically by infusion of 180 μ L of the liposome suspension (n = 17) to study the involvement of primary hemostasis. Resting and activated platelets endocytose free CF [22], infused in unencapsulated form and released from the liposomes, resulting in the formation of a fluorescent thrombus following laser-induced endovascular damage. High molecular weight heparin was administered after liposome infusion at a concentration of 2000 IU/kg (n = 11) to study the involvement of secondary hemostasis.

Platelets were fluorescently immunolabeled by infusion of mouse anti-human CD42b mAbs (350 μ g/kg, n = 10) secondarily labeled with Fab₂-FITC (250 μ g/kg) during 15 min before infusion to assess the role of glycoprotein (GP)Ib α in the hemodynamic

Fig. 3. Laser-induced semi-obstructive thermal coagulum formation in hamster dorsal skin fold venules visualized in brightfield and fluorescence mode. Fluorescent microspheres were injected intravenously to monitor blood flow. Thermal coagula either remained attached (a–d) or detached immediately after the laser pulse (e–h) without causing luminal occlusion due to the limited penetration depth of 532-nm light in blood. Translucent aggregates resembling a thrombus appeared at the site of laser irradiation (arrowheads), even in the absence of a thermal coagulum (h, arrowhead). A = arteriole, V = venule, semi-opaque ellipses correspond to sites of laser impact, arrows indicate direction of flow, the time relative to the laser pulse is indicated in the upper right corner (min:sec).

response. Fab₂-FITC was used as negative control (250 µg/kg, *n* = 5). Fractional immunoblocking of GPIbα (~3% of platelets, section-S5) with fluorescent CD42b mAbs allowed simultaneous visualization and quantification of platelet aggregation under mild inhibitory conditions. The anti-CD42b mAbs exhibited strong cross-reactivity with resting and activated hamster platelets (section-S4), reaching a level of saturation at ~2.5 µg/10⁶ platelets (section-S5), and induced a mild reduction in platelet count (section-S6).

Activated platelets were stained by infusion of CD62P-Alexa488 mAbs (500 µg/kg, *n* = 10) to assess the role of P-selectin in the hemodynamic response. IgG-FITC (500 µg/kg, *n* = 4) was used as isotype control to determine the extent of non-specific binding. The anti-CD62P mAbs exhibited cross-reactivity with activated but not resting hamster platelets (section-S4), although some nonspecific binding to resting hamster platelets was observed (Fig. S2). The anti-CD62P mAbs induced negligible reduction in platelet count (section-S6).

Fig. 4. Aggregation of 5(6)-carboxyfluorescein (CF)-labeled platelets at the site of laser-induced damage in the absence (a–f) and presence (g–l) of heparin (Hep). The bright ellipse in (a) is the laser spot. A = arteriole, V = venule, arrows indicate direction of flow, and the time relative to the laser pulse is indicated in the upper right corner (min:sec). The arrowhead in (b) indicates a region of residual hyperfluorescence as a result of heat-mediated CF release from thermosensitive liposomes. The arrowhead in (g) points to a remnant thermal coagulum. The lesion sizes (hemodynamic contribution only) with minima (Min) and maxima (Max) are plotted vs. time for CF-labeled platelets (m, *n* = 14) and for CF-labeled platelets in the presence of heparin (n, *n* = 11). The Min and Max values represent the minimum and maximum values of all lesion sizes calculated for all animals in the experimental group. In (o), the relative lesion size is depicted vs. time. The vertical lines indicate peak sizes for CF (dark) and CF + Hep (light). In (m) and (n), lesion sizes (normalized (A_{pix}) · normalized (I_{tot})) were normalized to baseline ($t = 0$, lesion size immediately after the laser pulse) for each experiment and are presented as mean ± SEM per experimental group.

All mAbs were diluted with 0.9% sodium chloride solution to a final injection volume of 200 μ L.

2.5. Laser irradiation

The target venules had a mean $\pm$ SD diameter of 157 ± 35 μ m (range = 86–252 μ m). Lesions were induced with a frequency-doubled Nd:YAG laser (532 nm, Entertainer, Laser Quantum, Cheshire, UK) at a power of 224 mW, a mean $\pm$ SD incident radiant exposure of 289 ± 38 J/cm², a 2.3×10^{-3} -mm² spot size, and a 30-ms pulse duration [15]. The pulse duration for this wavelength falls within the clinically employed range [23].

2.6. Shear rates

Blood flow measurements were performed using fluorescent microspheres. Shear rates were determined ($n = 95$) based on the measured blood flow velocity and vessel radius (section-S7). At a mean $\pm$ SD flow of 0.48 ± 0.21 mm/s and a mean $\pm$ SD venular diameter of 0.15 ± 0.03 mm, the mean $\pm$ SD shear rate was 7.0 ± 3.7 s⁻¹ (range = 1.8–19.6 s⁻¹).

2.7. Lesion quantification

The quantification of laser-induced lesions was performed differently for cytochemically (CF-) vs. immunolabeled platelets (Fig. 2b, section-S8). For the former, isolated video frames of laser-induced lesions were manually contoured and quantified for pixel area (A_{pix}) and total intensity (I_{tot}) in SigmaScan Pro (Systat Software, Point Richmond, CA). Contrastingly, immunolabeled thrombi were demarcated in SigmaScan Pro using a thresholding algorithm, whereby 'thrombus pixels' were defined as pixels with an intensity of 5 grayscale units above the highest background intensity, and quantified for A_{pix} and I_{tot} . For both quantification techniques, A_{pix} was normalized to baseline ($t = 0$, lesional area immediately after the laser pulse) and I_{tot} was normalized to the maximum total intensity.

Lesional size was defined by normalized (A_{pix})-normalized (I_{tot}) in both quantification methods and expressed comprehensively (thermal coagulum + thrombus) or individually (thrombus). Normalization of A_{pix} and I_{tot} and lesional size calculations were performed per experiment/animal. Lesional sizes were averaged per experimental group, normalized to baseline, and plotted as a function of time. The relative lesional size was calculated by dividing the lesional size at each time interval by the baseline value.

Additionally, a computational analysis method was developed for validating the manual image analysis. Good agreement was found between manual and automated analysis (section-S9).

2.8. Statistical analysis

Statistical analysis (means, standard deviations, and independent hetero- and homoscedastic Student's *t*-tests) was performed with SPSS (SPSS, Chicago, IL). Shapiro-Wilk tests confirmed the normal distribution of continuous data. The type of *t*-test used was based on Levene's test of equality of variances. A *p*-value of ≤ 0.05 , designated by (*) throughout the text, was considered statistically significant. A *p*-value of ≤ 0.01 is designated by (**).

3. Results

3.1. Induction of subocclusive thermal coagula

The laser-induced formation of subocclusive thermal coagula (photothermal response) was investigated first to ensure that our model emulated the endovascular damage profile associated with SP-treated refractory PWS vasculature (Fig. 1d). Laser irradiation consistently resulted in the formation of subocclusive thermal coagula in all irradiated vessels as evidenced by the uninterrupted flow of fluorescent microspheres (Video-S1/S2). Thermal coagula remained attached to the vessel wall (Fig. 3a–d) or detached within a few seconds after the laser pulse (Fig. 3e–h).

3.2. The photothermal response triggers primary and secondary hemostasis

Next, the manifestation of primary hemostasis (platelet aggregation) following photothermolysis was studied. Platelets were labeled by the systemic infusion of CF and liposomes containing a self-quenching concentration of CF were co-infused to serve as a thermal damage indicator. Laser irradiation was associated with transient hyperfluorescence as a result of a heat-induced liposomal membrane transition and release of CF (Fig. 4b, arrowhead, Video-S3), confirming local denaturation of plasma proteins and erythrolysis (thermal coagulum formation).

Platelet adhesion and the development of a nidus occurred in all irradiated venules within seconds after thermal coagulum formation (Fig. 4a–f), which was characterized by a rapid growth phase during the first 1.25 min and a slow growth phase in the subsequent 5.0 min. At 6.25 min the comprehensive lesional size reached a maximum with a 9.5-fold greater volume with respect to baseline (**, Fig. 4m and o). The slow growth phase encompassed extensive thromboembolic activity in which clot build-up exceeded breakdown, given the zigzag pattern of the maxima and the upward trend of the lesional size curve, respectively (Fig. 4m). The slow growth phase was ensued by clot breakdown as evidenced by the rapid decline in lesional size after 6.25 min (Fig. 4m and o) and extensive embolization of large platelet

Fig. 5. Laser-induced thrombus formation in the absence (a–f) and presence (g–l) of heparin (Hep) in venules (V) where the thermal coagulum had dislodged. Platelets were fluorescently labeled by the systemic administration of free and liposome-encapsulated 5(6)-carboxyfluorescein (CF). A = arteriole, white arrows indicate direction of flow, black arrows indicate the site of laser irradiation, the time relative to the laser pulse is indicated in the upper right corner (min:sec).

aggregates (Video-S3). At 15.0 min, the lesions had gradually sloughed off to 58% of their maximum (**). Platelet adhesion and aggregation at the site of laser irradiation occurred in the presence and absence of a thermal coagulum (Fig. 5a–f).

Heparin, an antagonist of factor (f)Xa and thrombin formation [24], was infused to determine the involvement of coagulation (secondary hemostasis) (Fig. 4g–l). Heparin reduced the maximum lesional size to 49% of the heparin-untreated group ($p = 0.07$, Fig. 4n vs. m) and caused the peak of thrombosis to occur at 1.25 min (Fig. 4n and o). At this time point the comprehensive lesional size had increased 2.6-fold (Fig. 4o), whereby the maximum relative lesional size was 27% of that in the heparin-untreated group (*).

In contrast to the heparin-untreated group, clot breakdown started after 1.25 min (Fig. 4n) and encompassed extensive embolization of platelet aggregates and, in some instances, portions of the thermal coagulum. The breakdown phase plateaued at 6.25 min at a lesional size that did not differ from baseline ($p = 0.08$), suggesting that the presence of residual platelet aggregates was minimal and/or that the thermal coagulum had partly or entirely dissociated from the vascular wall. As reported for the heparin-untreated group, the presence of a thermal coagulum was not required for platelet adhesion and aggregation to occur at the site of laser irradiation (Fig. 5g–l).

The accumulation of CF-labeled platelets at the site of endovascular damage and the inhibitory effect of heparin demonstrate that the photothermal response triggers primary as well as secondary hemostasis, respectively, in a thermal-coagulum adhesion/embolization-independent manner. To examine which key platelet receptors are involved in cell adhesion/aggregation during the hemodynamic response, the hemodynamic response was studied following immunoblocking of GPIIb/IIIa (CD42b) and P-selectin (CD62P).

3.3. Partial inhibition of CD42b reduces the extent of laser-induced thrombosis

CD42b is a transmembrane subunit of the constitutively expressed GPIIb-IX-V receptor complex with heterotypic binding sites for von Willebrand factor (vWF), Mac-1, CD62P, α -thrombin, FXI/XIIa, and high-molecular-weight kininogen [25]. Although the major physiological function of CD42b is the adhesion of circulating platelets to vWF in the subendothelial matrix at high shear, which leads to activation of integrin $\alpha_{IIb}\beta_{III}$ (GPIIb/IIIa) and subsequent aggregation [26], it has also been shown to mediate platelet adhesion under low shear conditions, i.e., in venules [27,28]. Inhibition of CD42b has further been correlated to significantly reduced platelet microparticle formation [29]. At a shear rate of $7.0 \pm 3.7 \text{ s}^{-1}$ in hamster venules, CD42b may therefore constitute an important receptor during the hemodynamic response, inasmuch as both platelet aggregation and coagulation prevail.

Anti-CD42b mAbs were infused to investigate the role of GPIIb/IIIa in the hemodynamic response (Fig. 6a–f). Fractional immunoblocking of CD42b imposed no deleterious effect on thrombus formation during the first 3.5 min compared to CF-stained lesions, but significantly reduced clot size during the subsequent 8.0 min (*, Fig. 6m). Thrombosis peaked at 2.75 min (Fig. 6m and o) followed by a steep deterioration phase, characterized by embolization of platelet aggregates and, sparsely, portions of the thermal coagulum, that stabilized at 4.25 min. Thrombi remained enveloped around the thermal coagulum up to 15.0 min (** vs. baseline, $p = 0.09$ vs. CF), suggesting that, at an estimated 3% GPIIb/IIIa inhibition, clot fortification by cross-polymerized fibrin imposed greater resistance to deterioration than a clot composed of predominantly platelets (Fig. 6m, CD42b vs. CF + Hep).

Fig. 6. Aggregation of anti-CD42b- (a–f) and anti-CD62P-labeled platelets (g–l) at the site of laser-induced damage. A = arteriole, V = venule, arrows indicate direction of flow, and the time relative to the laser pulse is indicated in the upper right corner (min:sec). The arrowheads in (c) and (i) point to a residual thermal coagulum. The lesional sizes (hemodynamic contribution only) with corresponding minima (Min) and maxima (Max) are depicted vs. time for CD42b-labeled platelets (m, $n = 10$) and CD62P-labeled platelets (n, $n = 10$). Lesional sizes (normalized (A_{pix}) · normalized (I_{tot})) were normalized to baseline ($t = 0$, lesional size immediately after the laser pulse) for each experiment and are presented as mean $\pm$ SEM per experimental group. The Min and Max values represent the minimum and maximum values of all lesional sizes calculated for all animals in an experimental group. In (o), the thrombus dynamics expressed as normalized relative lesional size are depicted for CF-labeled platelets in the absence and presence of heparin (Hep) and anti-CD42b- and anti-CD62P-labeled platelets. Mean values were normalized to maximum.

3.4. Inhibition of CD62P does not affect the extent of laser-induced thrombosis

CD62P is a cell adhesion molecule constitutively expressed in platelet α -granules [30] and endothelial cell Weibel–Palade bodies [31] that, upon cell activation, is translocated to the outer membrane to mediate platelet–platelet [32], platelet–leukocyte [33], and platelet–endothelial cell interactions [34]. In addition to cell recruitment, the expression of CD62P potentiates a procoagulant state by enhancing fibrin deposition [35] through the incorporation of P-selectin glycoprotein ligand (PSGL-1)-expressing, tissue factor-bearing microparticles derived from the above-mentioned cells [36]. As CD42b, CD62P may therefore play an instrumental role in the laser-induced hemodynamic response.

Anti-CD62P mAbs were infused to assess the role of P-selectin in the hemodynamic response (Fig. 6g–i). With the known inhibitory properties of the RB40.34 clone (section-S4), it was expected that the anti-CD62P mAbs would reduce the extent of thrombosis. Although a slight reduction in thrombus size manifested itself in the rapid growth phase, no inhibitory effect was observed in the slow growth and breakdown phases vs. CF-stained thrombi (Fig. 6n, black vs. dotted line, respectively). The maximum thrombus size was reached at 5.75 min and both lesional size curves (CD42b- and CF-stained thrombi) exhibited a similar progression up to 8.0 min. The 8.0-min time point marked a deflection in the downward trend in lesional size in the CD62P group, which may have been a result of increased CD62P expression (as evidenced by an increase in fluorescence intensity and not the lesional area, data not shown) and/or the nonspecific binding of the mAb (section-S4).

4. Discussion

A laser-mediated vascular injury model was employed with which the photothermal and hemodynamic response could be concomitantly studied in refractory PWS vessel analogues by intravital fluorescence microscopy. With this model we corroborated that primary hemostasis (platelet aggregation) comprises an integral component of endovascular laser–tissue interactions in semi-photocoagulated blood vessels [11–13]. Additionally, we demonstrated that (1) secondary hemostasis (blood coagulation) is triggered by SP, (2) primary and secondary hemostasis occur in the absence of a thermal coagulum, (3) CD42b is involved in the adhesion of platelets at the site of laser irradiation under low flow conditions, (4) the adhesion of platelets at the site of laser irradiation is accompanied by platelet activation, and (5) CD62P does not mediate platelet adhesion during the hemodynamic response. Moreover, the kinetics of laser-induced thrombosis in low-flow refractory PWS vessel analogues were established. Knowledge about the involved platelet receptor and thrombosis kinetics is particularly important in the continued development of SSPLT as a clinical modality.

As this study demonstrated, an intricate and causal relationship exists between the photothermal and the hemodynamic response. This relationship appears to be manifested at different levels: the nucleation centers (erythrocytes), the endothelium, and the thermal coagulum. Firstly, rapid heat build-up in erythrocytes causes the cells to swell and rupture [11,37]. Erythrocytes contain adenosine diphosphate [38] and phosphatidyl serine [39] that, when liberated and exposed, trigger platelet adhesion [40] and activation [41] and initiation of coagulation [42], respectively. Erythrocyte membrane disruption and complete disintegration have been reported to occur at 47–49 °C and 50 °C, respectively [20,37]. These temperatures were exceeded in the *in vivo* experiments as corroborated by the CF-encapsulating thermosensitive liposomes and by the consistent formation of thermal

coagula at the employed laser settings. Furthermore, it has been shown that ruptured erythrocytes remain attached to the thermal coagulum [43], causing the biochemical template for thrombosis to become stationary following laser irradiation. Image analysis of laser-induced lesions confirmed that thrombo-embolic activity always occurred around the thermal coagulum, even when the thermal coagulum had attached to or was overlaying a non-irradiated portion of the vessel downstream of the irradiation site. Consequently, erythrolysis constitutes an important trigger of the hemodynamic response following the photothermal response. This is underscored by the finding that laser-induced thrombosis *in vivo* occurs in irradiated blood vessels perfused with washed erythrocytes, erythrocyte ghosts, or hemolysate but not with platelet-rich plasma, PBS, or a non-absorbing exogenous chromophore [12].

Secondly, the heat-afflicted endothelium evidently plays a role in the hemodynamic response. High-fluence laser irradiation of microvessels causes ultrastructural perturbation of the endothelial cell membrane and denudation of the endothelial monolayer [13,44,45], which triggers primary [46,47] and secondary hemostasis [42]. It should be noted that the volumetric heat production, $\mu_a \phi$, where μ_a is the absorption coefficient (cm^{-1}) and ϕ is the fluence rate (J/cm^2), that led to such endothelial damage [13] was very high ($\sim 17,656 \text{ kJ}/\text{cm}^3$, section-S10.1) and not representative for either the clinical setting or our model. However, Tan et al. [44,45], who employed a substantially shorter pulse duration and much larger spot size for treating PWS, showed that endothelial damage can be achieved at significantly lower $\mu_a \phi$. Contrastingly, at a thousand-fold lower $\mu_a \phi$ of $\sim 16 \text{ kJ}/\text{cm}^3$ (section-S10.2), thermal coagulum formation and endothelial denudation were absent and only one component of the hemodynamic response (platelet adhesion and aggregation) prevailed, albeit transiently [18]. Inasmuch as our model produced a thermal coagulum at a $\mu_a \phi$ of $\sim 66 \text{ kJ}/\text{cm}^3$ at the blood–endothelium interface (section-S10.3), a contribution of thermally afflicted endothelium to the hemodynamic response is warranted. This was experimentally corroborated by the fact that thrombosis occurred in laser-irradiated vessel segments where the thermal coagulum had dislodged (e.g., Fig. 3h, Video-S2) and is in agreement with previous reports [12].

Thirdly, photocoagulation is associated with protein denaturation [48,49] that embodies conformational rearrangements and cross-linking/aggregation of unfolded/misfolded proteins [50] and corollary amyloid fibril formation [51]. There is increasing evidence that misfolded proteins/amyloids activate platelets via CD42b [52] and initiate the contact activation pathway through the auto-activation of fXII by anionic surfaces [53]. Inasmuch as thermal coagula are in part comprised of thermally denatured (unfolded/misfolded) proteins and anionic moieties exposed on the surface of ruptured erythrocytes, these laser-induced lesions may constitute the basis for the initiation of primary and secondary hemostasis that persisted around the thermal coagula. These postulations are, however, hypothetical and are currently being investigated in a separate study.

Although several specific pathways underlying endovascular laser–tissue interactions require further elucidation, it is unequivocal that SP affects both primary and secondary hemostasis in semi-photocoagulated vasculature. Primary hemostasis involves platelet *adhesion*, *activation*, and *aggregation*. The immunostaining experiments with anti-CD42b and anti-CD62P mAbs revealed that platelet adhesion to the thermal coagulum and/or to the thermally afflicted vascular wall is mediated by GPIIb α , but not CD62P, and that the platelets become activated upon adhesion, respectively. For platelet aggregation, integrin $\alpha_{\text{IIb}}\beta_{\text{III}}$ (CD41) activation is required [54], which could not be studied directly because none of the assayed antibodies cross-reacted with hamster $\alpha_{\text{IIb}}\beta_{\text{III}}$. Nevertheless, experimental data confirms the activation of integrin

$\alpha_{IIb}\beta_{III}$, and thus aggregation, in the hemodynamic response. Strong evidence is provided by the facts that a >9-fold expansion of the thrombus following laser irradiation cannot be accounted for by solely platelet adhesion and that embolization of large platelet aggregates was observed in the breakdown phase. The involvement of GPIIb α in the adhesion process further implies activation of integrin $\alpha_{IIb}\beta_{III}$ (concur with the binding of ligands to the GPIIb-IX-V complex during platelet adhesion [55]), which was supported by the reduction in thrombus growth following partial immunoneutralization of GPIIb α . Also, $\alpha_{IIb}\beta_{III}$ activation was corroborated by the positive immunolabeling of CD62P that is co-expressed when $\alpha_{IIb}\beta_{III}$ is activated [56]. Finally, we performed the same experiments in the dorsal vasculature of 3 surplus BALB/c mice (where anti-CD41 antibodies do cross-react with the respective platelet epitope), and confirmed $\alpha_{IIb}\beta_{III}$ activation during laser-induced thrombosis (Video-S4).

During secondary hemostasis, prothrombin is converted to thrombin by the prothrombinase complex, which exerts pleiotropic effects on both platelets and coagulation. The binding of thrombin to the GPIIb-IX-V complex induces platelet adhesion and spreading, dense granule secretion, and $\alpha_{IIb}\beta_{III}$ activation and subsequent aggregation [57]. It also accelerates the hydrolysis of protease-activated receptor (PAR)-1 (a thrombin receptor) [58] that further contributes to platelet activation. With respect to coagulation, thrombin mediates the cross-polymerization of fibrin that acts as a support matrix in thrombus fortification. The complete inhibition of thrombin generation at high heparin concentrations did not completely abrogate thrombus formation but substantially reduced the extent and duration of thrombosis. These observations confirm the involvement of the previously mentioned primary hemostasis mechanisms, which do not require thrombin, and attest to a role of coagulation in the hemodynamic response.

Inasmuch as thrombin generation is antagonized by heparin at the level of fXa in the common pathway, it is impossible to deduce whether coagulation was initiated through the tissue factor pathway or the contact activation pathway. The activation of coagulation in our model is, however, in contrast with previous studies [11,13,59], where no fibrin cross-polymerization (secondary hemostasis) was found following laser-induced thrombosis. It should be stressed, however, that the influence of secondary hemostasis was considerably inferior to that of primary hemostasis given that very mild inhibition of CD42b and complete inhibition of thrombin formation generated comparable thrombus kinetics curves. This is quite surprising given the fact that venous thrombosis predominantly entails activation of coagulation rather than platelet aggregation [60]. Unfortunately, the lack of mouse anti-human fibrin mAb cross-reactivity with hamster fibrin (section-S4) precluded the intravital qualification and quantification of fibrin cross-polymerization in the thrombi.

In conclusion, the hemodynamic response ensues the photo-thermal response and involves the activation of primary and, to a lesser extent, secondary hemostasis. Laser-induced venular thrombi reach a maximum size at 6.25 min after laser irradiation under very low flow conditions. Primary hemostasis, encompassing platelet adhesion, activation, and aggregation, is mediated by constitutively expressed GPIIb α but not by activation-dependent P-selectin.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jdermsci.2011.04.015.

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