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STRESS PHYSIOLOGY: CLINICAL IMPLICATIONS

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Stress is ever-present. Living organisms are continuously challenged by stressors which disturb their state of equilibrium or homeostasis. To maintain homeostasis, the stress response evolved as an adaptive mechanism involving multiple systems, working in concert to extinguish the stressors and then to extinguish the stress response itself. With adaptive regulation of the stress response, the stressor is extinguished, equilibrium is maintained, adaptive coping is established, and the effects are beneficial. With maladaptation, or dysregulation of the stress response, equilibrium is not maintained and often, the consequences may be pathological and/or harmful.

For instance, inability to dampen or hyper-reactivity the stress response, has been implicated in the pathophysiology of melancholic depression, anxiety, diabetes, gastrointestinal disorders, obsessive-compulsive disorders, substance abuse, obesity, inflammatory disorders, cardiovascular disease, osteoporosis, impairments in learning and memory, and immunosuppression. Inability to initiate or hypo-reactivity of the stress response, has been associated with such illnesses as chronic fatigue syndrome, hypothyroidism, rheumatoid arthritis and some subsets of post traumatic stress disorder.

Both psychological and physical stressors have been found to have a negative association with stress response dysregulation and illness. In addition, improved psychological and physical coping have been correlated with improved stress response regulation and health outcomes. Mind body intervention, including cognitive-behavior therapy, relaxation training, lifestyle modifications addressing nutrition and exercise, and coping skills training has been applied successfully in the treatment of a wide variety of clinical conditions including heart disease and hypertension, chronic pain, insomnia, a various women's health conditions.

MECHANISMS IN HEMOSTASIS AND THROMBOSIS – I

MONITORING HEMOSTASIS UNDER FLOW CONDITIONS (I)

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Thrombus formation has been studied through a parallel-plate perfusion device. Blood from healthy individuals on surfaces coated with adhesive protein, and blood from patients suffering from various bleeding disorders have been considered. Special attention has been dedicated to von Willebrand factor. The parallel flow arrangement allows physiological flow rates, with representative arterial blood velocities. In addition, the perfusion chambers mimics the stress distribution of an actual blood vessel, in a simplified geometry that facilitates quantitative speculations. Carefully controlling shearing stresses is particularly interesting to investigate their role in both platelet activation and radial cells segregation. Most of the efforts concentrate in the investigation of the earlier steps of the hemostasis process, beginning with platelet-substrate interaction, possibly leading to firm adhesion, a prerequisite for thrombus growth. Specific features of the investigation method include continuous recording of the surface exposed to flowing blood, with several type of microscopic techniques (inverted microscope with epifluorescent illumination and intensified CCD, reflection interference contrast microscopy, confocal microscopy). Systematic and automated analysis of the recording is an original contribution. The experimental technique together with automated analysis allows counting of platelets interacting with the surface, already a measure of adhesion effectiveness. In addition, platelets can be identified from frame to frame, allowing to track their surface behavior, possibly alternating rolling and stable adhesion. From pause time analysis the bond strength can be estimated, in terms of detachment rate constant. A parallel investigation on the significance of the sampling has been carried out, leading to the surprising result that poorly representative experimentation, both in terms of sample size (number of interacting cells observed) and rate (speed of the camera) can provide strongly misleading results. Cell behavior under flow is also modeled by means of computational fluid dynamics techniques (CFD), trying to predict mutual cell interactions, distribution in space and vessel geometry effects.

MONITORING HEMOSTASIS UNDER FLOW CONDITIONS (II)

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Recent *in vitro* and *in vivo* studies have emphasized the dynamic and complex nature of platelet thrombus growth, and the requirement for multiple and sequential adhesive receptor-ligand interaction in this process. von Willebrand factor (VWF) plays a key role in promoting both primary adhesion and aggregation under high shear condition. The membrane glycoprotein (GP) Ibalpha can tether platelets to immobilized VWF A1 domain opposing elevated shear forces, but alone cannot support permanent attachment, owing to a high dissociation rates. The efficiency with which platelets adhere and aggregate at sites of vascular injury depends on the synergistic and/or sequential action of various adhesive proteins, soluble agonists and the contribution of each of the individual receptors modulated by flow-dynamic conditions. In our review, we will focus on the contribution of VWF and its platelets receptors GPIb, alphaIIbbeta3 and of acid soluble collagen and its receptors GPVI and integrin alpha2beta1 on platelet adhesion and aggregate formation, under high and low shear rates. We will also discuss the signaling mechanism used by the above described platelet receptors to induce platelet activation by studying intracellular Ca++ oscillations and Ca++ fluxes in regulating the dynamics of platelet adhesion. We will also focus on the roll of receptors interplay and of soluble agonist in promoting initial thrombus formation and we will present models explaining the synergistic requirement for adhesive and soluble stimuli for an efficient platelet adhesion and aggregation.

MECHANISMS OF PLATELET ACTIVATION BY AGONISTS

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Human platelets are activated by several agonists that trigger signalling upon binding to their membrane receptor(s), as in the cases of collagen- and ADP-induced activation, or upon cleavage of their membrane receptor(s), as in the case of thrombin-induced activation. Collagen, the most thrombogenic component of extracellular matrix, elicits a platelet response through distinct receptors, including integrin $\alpha 2$-$\beta 1$ and glycoprotein VI. ADP, a weak platelet agonist that amplifies the platelet responses induced by other agonists, interacts with two specific P2 receptors: P2Y1 mediates a transient increase in the concentration of intracellular calcium, shape change and aggregation, P2Y12 mediates inhibition of adenylyl cyclase. P2Y1 initiates the platelet activation, whereas P2Y12 is essential for a sustained aggregation response to ADP. A third purinergic receptor, P2X1, has a controversial role. The serine protease thrombin is a potent activator of platelets and elicits signalling, at least in part, through G-protein-linked PAR1 and PAR4. By cleaving the amino terminal exodomain of PARs, thrombin unmasks a new N terminus that, as a tethered ligand, binds to the body of the receptor resulting in self-activation. PAR1 is the most important thrombin receptor and mediates platelet responses induced by low thrombin concentrations, whereas PAR4 seems to mediate platelet activation only to high thrombin concentrations. In addition to PARs, thrombin also interacts with at least two glycoproteins on human platelets, GPIb α and GpV, which are both part of the multimeric complex GpIb-IX-V. GpIb α seems necessary for the complete platelet activation and for the optimal PAR1 cleavage by thrombin. GPV, which is cleaved by thrombin, seems to negatively modulate the platelet response to thrombin, through undefined mechanism(s). For these agonists at least two or more receptors responsible for signal transduction have been described. This *receptors redundancy* may be necessary to modulate platelet stimulation *in vivo*.

INVESTIGATION OF PLATELET DEFECTS

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Defects of platelet function should be suspected when a patient has a bleeding diathesis that is characterized by mucocutaneous hemorrhage despite normal platelet count. The utility of performing global tests of primary hemostasis, such as the bleeding time and the PFA-100 system device is uncertain. The diagnostic approach to disorders of platelet function is based on a two-step strategy. The first step include simple, rapid and cost-effective tests, which are sensitive to the most common platelet function disorders. Since light transmission aggregometry is not sufficiently sensitive to defects of platelet secretion, which are the most common platelet function diroders, platelet function should be screened by lumiaggregometry, which allows the parallel monitoring of platelet aggregation and secretion. Several platelet agonists, each exploring individual pathways of platelet activation, should be employed. Additional tests include inspection of the peripheral blood smear and clot retraction. The combined analysis of the results of these tests will allow to raise a diagnostic hypothesis, which will then be verified in the second step of the diagnostic strategy, which include specific confirmatory tests. Due to the many pre-analytical and analytical variables that affect the results of platelet functions tests, both steps of the diagnostic strategy for platelet disorders should be done in specialized centres.

PAI-1 DEFICIENCY AND BLEEDING

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Background. Less than half of the patients referred for evaluation of bleeding tendency will have a hemostatic defect identified. Abnormalities of the fibrinolytic system are rarely diagnosed. Only case reports of deficiency of plasminogen activator inhibitor type 1 (PAI-1) have been published.

Aim. An improved method for analysis of PAI-1 with increased precision at the low end was used to better identify patients with very low activity of PAI-1. Using this assay, we are attempting to define the importance of low PAI-1 activity in various settings and to quantify the problem.

Methods. PAI-1 activity was analyzed with a commercially available chromogenic kit, but more measuring points were added at the lower end, the curve was read using a point-to-point method and a low in-house control added. Patients referred for investigation of bleeding tendency (group A, n=586), patients with congenital hemophilia (group B, n=100) and patients going for prostatic surgery (group C, n=62) as well as two groups of controls - blood

donors (control A, n=100) and age&sex-matched healthy persons (control B, n=100) were enrolled. The severity of bleeding was quantified in different ways - clinically significant or not for group A, according to bleeding frequency, factor requirement and joint score for group B and with exact measurement of intraoperative blood loss plus estimation of postoperative bleeding for group C. The 4G-5G polymorphism was analyzed in a subset. All studies are conducted at Karolinska University Hospital.

Results. Study of group A and control A and B: We defined low PAI-1 activity as <1 U/mL, since values of absorbance of less than 1 U/mL did not differ significantly from the 0-line. The PAI-1 activity increases with body mass index (r-square=0.2, $p<0.001$), as previously described. The proportion of individuals with low PAI-1 activity is 2-3 times higher among females than males in the different groups. Younger patients have slightly lower PAI-1 activity, but that can be ascribed to the effect of exogenous estrogens on the subset of young women. The adjusted odds ratio for low PAI-1 activity among patients compared to matched healthy controls was 3.23 (95% CI, 1.22±8.56, $p=0.019$). There was no interaction with 4G-5G genotypes. No typical pattern of bleeding was seen among patients with PAI-1 deficiency. Study of group B and C: Analysis is hampered by the lower prevalence of low PAI-1 activity among men. Among the patients with transurethral prostatic resection low PAI-1 activity was found in 4 of 62 patients. Three of these four patients had bleeding complications compared to 16 of 58 patients with normal PAI-1 ($p=0.082$) and after adjustment for resection time, resected mass and systolic blood pressure this becomes borderline significant. In none of the populations could we identify a quantitative effect of the PAI-1 levels above 1.0 U/mL on the risk of bleeding. Analysis of group B is still going on.

Conclusions. There appears to be a qualitative effect on the risk of bleeding from PAI-1 activity of less than 1 U/mL. This effect may become more pronounced in certain situations, such as surgery in areas with a high fibrinolytic activity. However, further studies will be needed to define the clinical relavance of this hemostatic defect.

PLATELET PATHOPHYSIOLOGY

BIOLOGICAL AND CLINICAL CONSEQUENCES OF MYH9 MUTATIONS

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Eighteen distinct classes of myosin have been established based on phylogenetic analysis of the motor domain. Class II comprises filament-forming (also named conventional) myosins that are found in muscle and non-muscle cells. Humans express three isoforms of non-muscle class II myosins, termed A, B, and C. Like all conventional myosins, these are hexameric proteins composed of two heavy chains and two pairs of light chains. MYH9 is the gene for the heavy chain of non-muscle myosin IIA (NMMHC-IIA). Myosin IIA is ubiquitously expressed in cells and tissues, where it is thought to play several roles, including cytokinesis, cell motility, and maintenance of cell shape. Heterozygous MYH9 mutations originate a complex disorder named MYH9-related disease (MYH9-RD). At birth, patients presents only macrothrombocytopenia and leukocyte inclusion bodies, but many of them subsequently develop deafness, cataracts and/or a glomerulonephritis leading to end-stage renal failure. Several questions on MYH9-RD are still unanswered: which are the cellular and molecular consequences of MYH9 mutations? Why several cell/tissues expressing NMMHC-IIA are not affected? Why mutations of one gene produce different clinical pictures? Recently, analysis of large case series of patients with different MYH9 mutations and development of cell-animal models for MYH9-RD allowed hypothesizing some answers to these questions. First, it has been suggested that processing of mutant NMMHC-IIA varies in different cell types, in that a dominant negative effect of the mutant allele has been observed in leukocytes, while haploinsufficiency has been demonstrated in megakaryocytes and platelets. Moreover, the study of tissue distribution of myosin II isoforms suggested that cells expressing only II-A manifest the congenital defects, while tissues expressing additional myosin II isoforms show either late onset of abnormalities or no pathological signs. Finally, genotype/phenotype correlation studies suggested that the main determinant of MYH9-RD clinical features is the type of MYH9 mutation. Advances in understanding MYH9-RD pathogenesis are going to improve our clinical approach to patients with MYH9 mutations.

ANIMAL MODELS IN PLATELET PATHOPHYSIOLOGY

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It is commonly recognized that blood platelets play a role in hemostasis and thrombosis but over the last few years increasing evidence has accumulated on a central role of platelets in inflammation and atherogenesis too. Studies on human platelets have several obvious limitations, thus crucial informations on the pathophysiologic role of platelets in disease have been obtained from animal models, and in particular from the manipulation of mice genome.

Recent examples of studies on *in vivo* platelet pathophysiology evaluated by our group are the role of matrix metalloproteinase type 2 (MMP2) in primary hemostasis and thrombosis and the role of platelet P-selectin in lung eosinophil infiltration in allergic asthma. Platelets contain and release, upon activation, MMP2 that in turn potentiates platelet response to