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TUMOR CELLS MITOTIC ACTIVITY MARKER KI-67 IN ASSESSING THE NEOADJUVANT CHEMORADIOTHERAPY EFFECTIVENESS IN PATIENTS WITH RECTAL CANCER

Summary. *The aim of the study was to determine the expression patterns of protein Ki-67 in colorectal cancer tissue depending on the pathological characteristics of the tumor and to establish the possible relationship between marker level and the results of neoadjuvant chemoradiotherapy (NCRT). Ki-67 expression indexes were determined by immunohistochemistry in the biopsy and surgical material in 18 patients with adenocarcinoma of the rectum stage I-III before and after NCRT. It was found that the pre-treatment level of Ki-67 in cases of poorly differentiated tumors was significantly higher than that of highly differentiated, accounting for respectively 52,65±4,48 conv. units and 38,01±2,94 conv. units ($p<0.001$). Under the influence of NCRT the level of Ki-67 expression in the intact intestine decreased slightly. Expression of Ki-67 dramatically reduced to 32,45±1,19 conv. units ($p<0.001$) in the loci of residual tumor parenchyma compared with those before treatment (average level 46,08±3,14 conv. units) and inversely correlated with the degree of therapeutic pathomorphosis. Thus, a negative correlation between the expression of Ki-67 after NCRT and the level of the response to the applied treatment suggests the feasibility of using the Ki-67 definition to monitor the effectiveness of NCRT during combined and complex treatment of patients with rectal cancer. High expression of Ki-67 in the tumor before treatment, being characteristic of poorly differentiated tumors, corresponds to the high level of the malignant potential of the tumor.*

Key words: *rectal cancer, proliferative activity, therapeutic pathomorphosis, chemoradiotherapy.*

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MECHANISMS OF PLATELET DYSFUNCTION IN PATIENTS WITH GASTRODUODENAL ULCER BLEEDING

Summary. *Platelet reactivity was estimated in 247 patients with gastric and duodenal ulcer bleeding. Platelet aggregation was measured using aggregometry with adenosine diphosphate (5 μM), epinephrine (2.5 μM), 5-hydroxytryptamine (10 μM), collagen (1 μM) and thrombin (0.05 NIH/ml). The relationship between platelet aggregation and spatial-temporal characteristics of ulcers complicated by bleeding were shown. Adrenoreactivity of platelets was associated with terms after beginning of ulcer bleeding and hemorrhage degree. The lowest platelet response to collagen and thrombin was detected in patients with active bleeding ($p<0.001$) and unsustainable hemostasis ($p<0.01$). Additionally to gender factor, the important determinant of unsustainable hemostasis was decrease of platelet response to thrombin and adenosine diphosphate.*

Key words: *gastric and duodenal ulcer, bleeding, hemostasis, platelets.*

Introduction

Gastric and duodenal ulcer bleeding is a common cause of hospital admission and life-threatening medical emergency [Laursen et al., 2012]. Recent studies of clinical, laboratory and endoscopic features of peptic ulcers and their complications have revealed the wide list of factors which are associated with this pathology [Bratanic A. et al., 2012]. The most widely known risk-stratification tool for gastroduodenal bleeding is the Rockall scoring system [Marmo et al., 2010]. It represents an accurate and validated predictor of rebleeding and mortality [Jensen, 2012]. This approach is perfect for short-term management of GDB, but not in development of novel strategy of treatment and prevention of ulcer bleeding. Indeed, the treatment of ulcer bleeding remains only partly successful, despite the wide use of endoscopic methods, eradication of H. pylory and progress in antisecretory therapy [Holster, Kuipers, 2011]. In our view, the solution may lie in development of a new approach, directed on assessment of mechanisms of hemostasis failure rather than on detection of stigmata of bleeding. The integrative link of hemostatic system is platelets, which are the first cells activated in the place of injury, tightly related with plasma coagulation system, endothelium and connective tissue homeostasis. These blood cells have numerous receptors for different molecules, which are involved in regulation and realization of blood clotting. That is why we suggested that in vitro assessment of platelet reaction to different stimuli can be useful for understanding of hemostasis instability and establishment of pathogenetic mechanisms of unsustainable trombogenesis after bleeding.

The aim of this paper is to identify factors associated with risk of unsustainable hemostasis in patients with gastric and duodenal ulcer bleeding by in vitro assessment of platelet reactivity.

Material and methods

A cohort study was conducted in 140 patients with a diagnosis of gastroduodenal ulcer bleeding. Patients

considered eligible for enrollment had to be over 18 years of age, suffer from typical symptoms of acute bleeding from gastric and duodenal ulcers, confirmed by positive upper gastrointestinal endoscopy. Exclusion criteria were age younger than 35 years or over 75 years, any allergy to established medications, coagulopathy, infarction of myocardium and ischemic stroke in the last 6 months, pregnancy, cirrhosis or use of a proton pump inhibitor or H2-receptor antagonist in the 2 weeks prior to enrollment in the study. The patients with malignant ulcers or trauma were also excluded. Patients were divided into two groups according to initial endoscopic data: group 1 with sustained hemostasis; group 2 with failure of initial hemostasis corresponding to Forrest classification. It is commonly used when stratifying patients with upper gastrointestinal hemorrhage into high and low risk categories for mortality. It is also a significant method of prediction of the risk of rebleeding. High risk category (with un-sustained hemostasis) includes Forrest grade I and IIa-b [Chiu, 2010]. Patients who had these endoscopic stigmata were included into the 2nd group. Low risk lesions (with sustained hemostasis) correspond to Forrest grade IIc and III. Patients with these endoscopic features belonged to the 1st group. Whole blood for the in vitro study was sampled from patients with peptic ulcer bleeding at the moment of hospital admission before therapy. Blood was collected from the antecubital vein into plastic syringes containing sodium citrate at a final concentration of 0.38% with proportion 9:1 and centrifuged at 200 x g for 20 minutes at 25°C to prepare platelet-rich plasma (PRP). We evaluated the platelet aggregation in the presence of different proaggregants as followed: adenosine diphosphate (ADP; 5 μM), epinephrine (2.5 μM), 5-hydroxytryptamine (10 μM), collagen (1 μM) and thrombin (0,05 NIH Unit/ml). The reaction tubes were pre-incubated for 1 min at 37°C, and then 20 μL of each agonist in EC50 concentrations were added [Баринов и др., 2011; Barinov at al., 2013]. Measurement of platelet aggregation with aggregometer was carried out according to the method previously described [Баринов и др., 2006; Lombardi, 2012]. The aggregometer was 490-2D (Chrono-log, USA). Collagen, ADP, Epinephrine, Thrombin and 5-hydroxytryptamine were from Sigma (USA).

Data were collected and analyzed using the statistical package MedCalc version 12.3 (MedCalc Software Inc., 1993-2012). Descriptive statistics were used to analyze and report the data. For presentation of nominal data the % and standard error (m) were calculated; for presentation of numerical data the median (Me) and standard error (m) were calculated [Petrie, Sabin, 2005]. The chi-squared and the rank Kruskal-Wallis and Dunn's tests were used to determine differences between patients with sustained and unsustainable haemostasis. The significance threshold was set at $P<0.05$.

Results. Discussion

Acute peptic ulcer bleeding occurred in 185 men (74,9±2,8%), average age 54±1,4 years old and in 62 women (25,1±2,8%), average age 70,2±1,9 years old. One hundred and six patients had a bleeding from gastric ulceration, 128 had duodenal ulcers and thirteen patients had both gastric and duodenal ulcers. In 130 cases ulcer bleeding occurred in patients with comorbidities including the pathology of cardiovascular system, digestive system diseases and acute inflammatory processes. There were no gender differences in frequency of cardiovascular pathology in patients with ulcer bleeding. Disorders of thrombogenesis were more often in patients with such comorbidities as cancer, portal hypertension and acute inflammatory diseases. According to endoscopic characteristics the most often location of ulcers complicated by bleeding was duodenum (128 patients; 51,8±2,6%), rather than gastric body and pylorus ulcers (22,2±1,5% and 20,6±1,3% of patients respectively). In 13 patients (5,3±1,3%) several ulcer were found. However, location and size of ulcers were not related with the efficacy of hemostasis. Endoscopic study revealed active bleeding (F1) in 21 patients (8,5±1,8%); 121 cases (48,9±3,2%) of F2a and F2b class; Forrest class 2c in 83 (33,7±3,1%) and F3 in 22 (8,9±1,8%) patients.

There were no significant differences in coagulation system indexes ($p>0,05$) and platelet count in patients of the 1st and the 2nd groups. Nevertheless, analysis of platelet aggregation has shown the difference in platelet response to all agonists. Despite the absence of significant links between collagen- and thrombin-induced platelet aggregation and ulcer size or location, association of platelet reactivity with endoscopic characteristics of hemostasis was found. The lowest platelet response to collagen and thrombin was detected in patients with F1 ($p<0,001$) or F2a-b Forrest classes ($p<0,01$). The decrease of collagen- and thrombin-induced aggregation was associated with reversible pattern of the curve. This fact can be explained by the defect of platelet degranulation or impairment of secondary agonists effects. To verify these points we analyzed the platelet response to ADP and ATP, which are considered to be the main paracrine factors magnifying the effect of tissue (collagen) and coagulation system (thrombin) stimuli to platelets. Interestingly, characteristics of ADP-induced aggregation in patients with gastroduodenal ulcer bleeding were similar to thrombin and collagen-induced aggregation - higher in patients with sustainable hemostasis and low or absent in cases with recent or active bleeding respectively. There were significant differences in platelet aggregation induced by ADP ($p<0,01$), collagen ($p<0,01$) and thrombin ($p<0,001$) in patients with different state of hemostatis according to the endoscopic features.

Analysis of purine signaling in platelets has shown the decrease of ADP-induced platelet aggregation, the degree of which was associated with different endoscopic characteristics ($p<0,01$) and correlated with platelet response to thrombin ($r=0,714$; $p<0,001$) and collagen ($r=0,584$; $p<0,01$). Another association of platelet reactivity with clinical and instrumental data were shown during analysis of epinephrine and 5-HT effects. In vitro measurement of platelet aggregation induced by epinephrine has shown its relation

with temporal and chronological characteristics. During the first 6 hours after bleeding manifestation, high epinephrine-induced platelet aggregationn was observed; later, however, the decrease of platelet adrenoreactivity was detected. This fact can reflect the phases of compensatory reaction of sympatho-adrenal system and time-dependent changes of platelet sensitivity to systemic regulators after 12 hours after the manifestation of the ulcer bleeding. There was no significant relation between size, Forrest class, severity of bleeding and 5-HT-induced platelet aggregation. Nevertheless, the relation between 5-HT effect and location of ulcer was shown: the minimal 5-HT-induced platelet aggregation was measured in patient with gastric ulcers, and the highest values ($p<0,01$) were found in cases of duodenal ulcer bleeding which were associated with associated pathology of gastrointestinal tract ($p<0,01$).

Thus, analysis of functional state of platelet in patients with gastroduodenal ulcer bleeding allows to establish the following: 1) variability of platelet response to different agonists used in EC50. 2) relationship exist between platelet aggregation and spatial-temporal characteristics of ulcers complicated by bleeding; 3) platelet reactivity was associated with different parameters of hemostasis and outcome.

In this work we analyzed in vitro the mechanisms of hemostasis failure in patients with gastroduodenal ulcer bleeding. In order to study the mechanisms of unsustainable hemostasis platelets were chosen as a model. The choice is explained by the following facts: 1) platelets are the first and obligatory participants of thrombogenesis [Michelson, 2013]; 2) platelet surface is the place for reactions of coagulation cascade realization, reacts with thrombin and tissue factor through PAR-1 and PAR-4 [Schlagenhauf et al., 2014]; 3) platelets are the targets for different systemic factors, involved in reaction of organism to bleeding, including epinephrine, norepinephrine, 5-HT and so on [Stalker et al., 2012]. Molecular and functional characteristics of platelets can be informative in assessment of the individual reactivity of an organism and the key mechanisms that lead to the defects in thrombogenesis. According to this concept, we assessed the efficacy of platelet model for investigation of mechanisms of platelet dysfunction in patients with ulcer bleeding by in vitro analysis of platelet aggregation.

Despite the classical postulate that there are four major risk factors for bleeding peptic ulcers namely Helicobacter pylori infection, non-steroidal anti-inflammatory drugs (NSAIDs), stress and gastric acid and that the reduction or elimination of these risk factors lessen ulcer recurrence and rebleeding rates, we support another hypothesis. According to the current theories of ulcerogenesis we suggest that development of ulcer bleeding and alteration of thrombogenesis mechanisms, which might determine the spontaneous resolution of bleeding, are related to the factors involved both in regulation of haemostasis and inflammation [Petaja, 2011]. And assessment of platelet aggregation induced by different agonists indirectly confirmed this thought.

In this work we found out that the changes in agonist-induced platelet aggregation and hemostasis failure in patients with ulcer bleeding can reflect the role of systemic, tissue, paracrine and coagulation factors in platelet dysfunction and failure of thrombogenesis. The specific relations between platelet response to different regulators and special-temporal characteristics of ulcers and hemostasis characteristics were shown. The results of platelet aggregation analysis had shown the significant differences in thrombin induced aggregation in patients with spontaneously stopped bleeding (1st group) and active or recent bleeding (2nd group). Really thrombin is considered to be a potent inductor of platelet aggregation and in control group it induced high irreversible aggregation. So reduction of platelet reactivity on this agonist was a big surprise. Thrombin effect on platelet is realized through PAR-1 and PAR-4 which are the most numerous type of receptors on platelet (1500-2000 per platelet), and additional;y through GP Ib [Баринов, Сулаева, 2012].

According to our results the risk of unsustainable hemostasis significantly increased with combined failure of platelet response to thrombin and ADP. It is well known that ADP is a weak agonist for platelet aggregation that is explained by low number of purine receptors on platelet surface (near 150 per platelet) [Баринов и др., 2014]. However, ADP release from dense granules during primary induction and activation of two types of receptors ($P2Y_1$ and $P2Y_{12}$) induce potent stimulation of Gq- and Gi-protein-associated signaling. The latter includes activation of phospholipase C, inositol-3-phosphate, protein kinase C, calcium release, inhibition of adenylyl cyclase with additional stimulation of phosphatidylinositol-3-kinaseγ, Akt and Rap 1b, that provide the potent amplification of primary agonists (collagen, thrombin et al) effect [Stalker et al., 2012]. The results of ADP effect are recruitment of new platelets into aggregation with progression of clot formation and activation of GPIIb-IIIa for fibrinogen links necessary for clot stabilization. Alteration of platelet response to purines can explain the failure of clot stabilization (according to endoscopic characteristics of ulcers) under the high level of fibrinogen on one side and reversible character of platelet aggregation curve after potent agonist stimulation on the other side.

Conclusion and perspectives of the further investigation

Thus, estimation of platelet reactivity in vitro allows indicating key mechanisms of hemostasis failure in patients with ulcer bleeding. Additionally to gender-associated factor, the important determinants of unsustainable hemostasis were a decrease of platelet response to thrombin and ADP.

Further investigation of molecular mechanisms of abnormal platelet response among patients with ulcer bleeding could be important for new therapeutic strategy development.

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Сулаева О.Н.

МЕХАНИЗМЫ ДИСФУНКЦИИ ТРОМБОЦИТОВ У ПАЦИЕНТОВ С КРОВОТЕЧЕНИЯМИ ИЗ ЯЗВ ГАСТРОДУОДЕНАЛЬНОЙ ЗОНЫ

Резюме. *В работе проведена оценка реактивности тромбоцитов у 247 пациентов с кровотечениями из язв желудка и двенадцатиперстной кишки. Агрегацию тромбоцитов оценивали при индукции АДФ (5 мкМ), эпинефрином (2,5 мкМ), 5-гидрокситриптамином (10 мкМ), коллаген (1 мкМ) и тромбина (0,5 NIH/мл). Показаны взаимосвязи между агрегацией тромбоцитов и пространственно-временными характеристиками язв, осложненных кровотечением. Адренореактивность тромбоцитов была ассоциирована со сроками после начала кровотечения язвы и тяжестью кровотечения. Наиболее низкий ответ тромбоцитов на коллаген и тромбин был зарегистрирован у пациентов с активным кровотечением ($p<0,001$) и при нестабильном гемостазе ($p<0,01$). Важными детерминантами неустойчивого гемостаза, помимо пола, является снижение реакция тромбоцитов на тромбин и аденозиндифосфат.*

Ключевые слова: *язва желудка и двенадцатиперстной кишки, кровотечение, гемостаз, тромбоциты.*

Сулаєва О.Н.

МЕХАНІЗМИ ДИСФУНКЦІЇ ТРОМБОЦИТІВ У ПАЦІЄНТІВ З КРОВОТЕЧЕЮ З ВИРАЗОК ГАСТРОДУОДЕНАЛЬНОЇ ЗОНИ

Резюме. *У роботі проведена оцінка реактивності тромбоцитів у 247 пацієнтів з кровотечами з виразок шлунка та дванадцятипалої кишки. Агрегацію тромбоцитів оцінювали при індукції АДФ (5 мкМ), епінефрином (2,5 мкМ), 5-гідрроксиприптаміном (10 мкМ), колаген (1 мкМ) і тромбіном (0,5 NIH/мл). Показано наявність взаємозв'язку між агрегацією тромбоцитів і просторово-хронологічними характеристиками виразкового процесу, ускладненого кровотечею. Адренореактівність тромбоцитів динамічно змінювалася відповідно до термінів від маніфестації виразкової кровотечі й корелювала з тяжкістю кровотечі. Найбільш низька відповідь тромбоцитів на колаген і тромбін була зареєстрована у пацієнтів з активним кровотечею ($p<0,001$) і при нестабільному гемостазі ($p<0,01$). Важливими детермінантами нестійкого гемостазу, крім статі, є зниження реакція тромбоцитів на тромбін і аденозиндифосфат.*

Ключові слова: *виразка шлунка та дванадцятипалої кишки, кровотеча, гемостаз, тромбоцити.*

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МОДЕЛЮВАННЯ НАЛЕЖНИХ ПОКАЗНИКІВ ЦЕНТРАЛЬНОЇ ГЕМОДИНАМІКИ У ВОЛЕЙБОЛІСТОК РІЗНОГО АМПЛУА У ЗАЛЕЖНОСТІ ВІД АНТРОПОМЕТРИЧНИХ І СОМАТОТИПОЛОГІЧНИХ ОСОБЛИВОСТЕЙ ОРГАНІЗМУ