BRIEF ARTICLE

Assessment of platelet activation and phagocytic activity in gastric cancer patients

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Abstract

AIM: To assess the activation of platelets and their phagocytic activity in the course of gastric cancer.

METHODS: Forty-three gastric cancer patients were recruited to the study. The patients were divided into 3 groups depending on tumor stage. Group E included 6 patients with early gastric cancer; group A 18 patients with locally advanced cancer; and group M-19 with metastatic cancer. The investigations were performed twice, prior to surgery and 12-14 d afterwards.

RESULTS: The platelet count and the level of soluble platelet selectin (sP-selectin) were found to increase with the disease progression. The level of sP-selectin was lowest in early cancer and was observed to increase after surgery in all the study patients. Irrespective of tumor stage, a statistically significant decrease was noted in the percentage of phagocytizing platelets and in the phagocytic index in gastric cancer patients as compared to healthy subjects. Despite increased platelet count and stimulation of thrombocytopoiesis, the phagocytic functions of blood platelets were markedly impaired. Tumor development seems to impair metabolic processes.

CONCLUSION: A decreasing phagocytic activity can promote both inflammatory processes and cancer growth.

INTRODUCTION

Gastric cancer is the second most common cause of deaths due to cancer worldwide[1]. Approximately 900 000 new cases of gastric cancer are diagnosed every year. The risk factors include first of all *Helicobacter pylori* (*H. pylori*) infection, which leads to chronic inflammation of the gastric mucosa and intestinal metaplasia. Irrespective of histological type, gastric cancer is characterized by high malignancy and high incidence of lymph node involvement and distant metastases. Survival prognosis depends on tumor staging and the presence of metastases. Gastric cancer is usually located in the pyloric antrum and in the pylorus, but in 25% of cases in the body and bottom of the stomach. As the clinical symptoms of gastric cancer are frequently nonspecific, it is diagnosed only at the advanced stage[2]. Therefore, there is a continuous search for alternative uses of diagnostic markers that would allow early detection of gastric cancer[3].

The proinflammatory cytokines, interleukin (IL)-6 and

IL-23, which are released during *H. pylori* infection and which strongly inhibit secretion of gastric juice, are involved in the pathogenesis and development of tumors. They facilitate tumor growth by inhibiting apoptosis of tumor cells and by inducing angiogenesis within the tumor. Platelets play an active part in cancerous diseases through secreted proinflammatory factors, chemokines and growth factors. Tumor cells exhibit prothrombotic effect by inducing platelet aggregation and by platelet activation. Upon platelet activation, platelet surface P-selectin binds to ligand CD24 present on tumor cells, causing their adherence to endothelial cells. P-selectin plays a key role both in inflammatory processes and in the pathogenesis of thrombosis in cancer patients[4].

Blood platelets also exhibit the ability to phagocytize viruses, bacteria or immune complexes. They become activated and change in shape and surface properties: this is accompanied by the formation of phagosomes containing proteolytic enzymes[5-7].

The study objective was to find out whether and how gastric cancer-related inflammation affects platelet activation and whether it changes their phagocytic functions. IL-6 was used as an inflammatory marker in gastric cancer patients.

MATERIALS AND METHODS

The study involved 43 gastric cancer patients treated at the Second Department of General and Gastroenterological Surgery, Medical University of Bialystok. The group consisted of 32 men (M) and 11 women (F), aged 31-76 years (mean age 55.4 years). The patients were divided into 3 groups by cancer (adenocarcinoma) stage[8]. Group I (E) included 6 patients (4 M, 2 F) with early stage, group II (A) had 18 patients (13 M, 5 F) with locally advanced stage and group III (M) had 19 patients (15 M, 4 F) with cancer metastases. According to the UICC classification, in group E there were 5 stage I patients (12%) and 1 stage II patient (2%), in group A - 7 stage IIIA patients (16%) and 11 stage IIIB patients (26%), and in group M - 19 stage IV patients (44%) (Table 1). All the patients were qualified for surgery. In 34 cases the surgery involved total gastrectomy and regional lymphadenectomy, omentectomy and splenectomy. In 8 of these patients, treatment was extended to pancreatic tail resection. Four patients with less advanced cancer underwent Billroth II gastrectomy with regional lymphadenectomy. Due to the stage of the cancer, in 5 patients treatment was limited to gastrointestinal anastomosis. Examinations were carried out twice, before surgery (E1, A1, M1) and 12-14 d after surgery (E2, A2, M2).

The control group (C) consisted of 40 healthy subjects (aged 20-45 years), including 22 M and 18 F. Statistical analysis of all the parameters, including age and sex, was performed within the study groups and in the control group. Results were analyzed using Statistical 8.0 program.

All the patients gave their consent to the study in ac-cordance with the Guidelines for Good Clinical Practice. The study was approved by the Bioethics Committee of the Medical University of Bialystok (RI-002/154/2009). Venous blood collected for clot was used as the material for analysis.

Assessment of platelet phagocytic activity

About 1 mL of ACD (a mixture of citric acid and glucose) was added to 5 mL of venous blood collected for heparin at a concentration of 50 IU/mL blood. ACD prevents platelet aggregation by inhibiting thromboxane synthesis by platelets and reduces plasma pH, to 6.5, which decreases the physiological ability of platelets to agglutinate. Blood platelets were isolated by double centrifugation, taking advantage of the differences in the specific weight of the respective morphotic elements of blood. Whole blood was centrifuged at 250 g for 12 min, plasma was withdrawn to another test-tube and then centrifugation was repeated at 1500 g for 20 min. Some plasma (2 mL) was left over the platelet sediment and the sedimented platelets were suspended in it by gentle stirring with a plastic pipette. This platelet-rich plasma (PRP) was supplemented with 0.1 mL ACD to prevent platelet aggregation. In the PRP obtained in this way, erythrocytes and leukocytes did not exceed 1/1000 platelets[9].

Bacteria used for the phagocytic activity of blood platelets

Staphylococcus aureus ATCC 6538P strain was used for the study. The bacteria were cultured on an agar slant for 20 min, then rinsed off with 10 mL of phosphate buffer (PBS) and centrifuged at 1500 g for 20 min. The centrifuged bacterial sediment was rinsed with PBS three times and centrifuged again. The number of bacteria in the suspension was determined spectrophotometrically, by measuring optical density of the suspension. The suspension containing 120×10^6 - 140×10^6 bacteria, used to examine the phagocytic and bactericidal activity, corresponds to an optical density of T-63 at a wavelength of 540 nm. The bacteria were grown on the agar medium and counted. The suspension was diluted with PBS to obtain the platelet/bacterium ratio of 1:1.

Platelet phagocytic activity was determined by measuring the percentage phagocytizing cells and the phagocytic index; whereas bactericidal activity was determined by calculating the difference between the number of bacteria phagocytized by platelets and the number of phagocytized bacteria which survived within platelets.

Determination of the percentage of phagocytizing platelets

Bacterial suspension and PRP were incubated at 37 ℃ for 6 min, and then mixed at 75 g. After incubation, smears were made and then stained with the Pappenheim method, for 1 h using Giemsa reagent. The stained preparations were evaluated in a light microscope at × 1400 magnification. The percentage of phagocytizing platelets was described as the percentage of phagocytizing platelets per 1000 other cells in the preparation.

Table 1 Stage of gastric cancer *n* (%)

Group tested		
Tumor stage	I + II	
(TNM classification)	6 (14)	
	III	
	18 (42)	
	IV	
	19 (44)	
Depth of tumor invasion	T1	
	2 (6)	
	T2	
	4 (13)	
	T3	
	15 (47)	
	T4	
	11 (34)	
Lymph node metastases	N0	
	7 (17)	
	N1	
	4 (9)	
	N2	
	13 (31)	
	N3	
	18 (43)	
Distant metastases	M0	
	9 (47)	
	M1	
	10 (53)	

Determination of the phagocytic index

The phagocytic index was determined using 100 phagocytizing platelets. The index was calculated as the mean number of phagocytized bacteria per single platelet according to the formula: phagocytic index = number of phagocytized bacteria/number of phagocytizing platelets. Platelet count (PLT) was determined using a hematological analyzer (ADVIA 2120, Siemens). The level of soluble platelet selectin (sP-selectin) was determined by the enzyme-linked immunosorbent assay (ELISA) method using Quantikine human kit (R and D Systems, United States). The level of IL-6 was determined by the ELISA method using Quantikine human kit (R and D Systems, United States).

The results were subjected to statistical analysis using the program Statistica 8.0. The differences were considered statistically significant when the value of the test function was at the level of significance set at $P < 0.05$. The Kolgomorov consistency test was used for features consistent with normal distribution, the *t*-Student test for comparisons between the groups and the Mann-Whitney test for traits inconsistent with this distribution.

RESULTS

PLT in patients with early gastric cancer (E1 and E2) and with locally advanced cancer (A1 and A2) before and after surgery differed statistically significantly ($P < 0.05$). PLT in these groups increased statistically significantly after surgery (Table 2).

The level of sP-selectin was lowest in group E1 and only this value was found to differ statistically significantly compared to the control group ($P < 0.05$). In the other groups, the increase was not statistically significant in comparison with the control group. A statistically significant difference in the level of sP-selectin was observed between the groups A1 and A2 (Table 2).

The level of IL-6 was found to be markedly higher in the groups prior to surgery (E1, A1 and M1) as compared to the control, the differences being statistically significant (Table 2).

Statistically significant differences were noted in the percentage of phagocytizing platelets between groups E and A, before and after surgery. In the study groups E1, A1 and M1 the percentage values of phagocytizing platelets were statistically significantly lower than those obtained in the control group ($P < 0.001$) (Table 3).

The phagocytic index was statistically significantly lower in the groups before surgery (E1, A1 and M1) as compared to the control (Table 3).

DISCUSSION

Gastric cancer is accompanied by inflammation and at the same time by impairment of immune mechanisms. *H. pylori* is responsible for gastric mucosa inflammation, resulting in the stimulation of neutrophils and macrophages, and in an increased production of reactive oxygen species. Chronic inflammation and reduced secretion of hydrochloric acid lead to stomach ulceration or, by causing intestinal metaplasia of the stomach, turn into dysplasia, which is considered a precancerous condition[10]. Platelets take an active part in the initiation and development of the inflammatory process by adhering to the cells of the vascular walls and by the release of chemokines, cytokines, proteases and procoagulants[11]. Cancer cells undergo proliferation under the effect of epidermal growth factor, platelet-derived growth factor (PDGF) and insulin-like growth factor-1 released from blood platelets. Vascular endothelial growth factor (VEGF) and angiopoietin exert a pro-angiogenic effect[12-14]. Fibrinogen, fibronectin, vitronectin and vWf enhance platelet-endothelium adhesion by the formation of connections between GP II b-III a and integrin $\alpha v \beta 3$ or intercellular adhesion molecule-1. Adhesion molecules belonging to the P-selectin group have a special function during aggregation of platelets and cancer cells. P-selectin translocated from α-granules onto the platelet surface during platelet activation facilitates their interaction with endothelial cells, monocytes, neutrophils and lymphocytes. Cancerous diseases are usually accompanied by overproduction of platelets. Thrombocytosis, which can be the cause of increased risk of metastases, has been observed in the cancer of the stomach, colon, lungs, kidneys, prostate and reproductive organs[15,16].

IL-6 is a mediator of platelet production and a direct stimulator of megakaryocytes[15]. Ikeguchi *et al*[17] showed the usefulness of IL-6 determination in the diagnosis of gastric cancer. They found a statistically significant cor-

Table 2 Mean values of platelet counts, levels of soluble platelet-selectin and interleukin-6 in cancer patients-early carcinoma-E advanced carcinoma with lymph node involvement-A, metastatic cancer-M and in control group (mean ± SD)

	PLT	sP-selectin	IL-6
Study group E1	247.33 ± 42.92	67.93 ± 74.49	20.68 ± 6.31
	E1 *vs* C	E1 *vs* C	E1 *vs* C
	0.8 < P < 0.9	P < 0.05	P < 0.01
Study group E2	662.83 ± 335.23	153.37 ± 174.59	10.90 ± 8.43
	E1 *vs* E2	E1 *vs* E2	E1 *vs* E2
	P < 0.05	0.5 < P < 0.6	0.1 < P < 0.2
	E2 *vs* C	E2 *vs* C	E2 *vs* C
	P < 0.05	0.6 < P < 0.7	0.05 < P < 0.1
Study group A1	344.28 ± 266.40	193.23 ± 143.70	11.51 ± 8.44
	A1 *vs* C	A1 *vs* C	A1 *vs* C
	0.05 < P < 0.1	0.05 < P < 0.1	P < 0.001
Study group A2	584.25 ± 237.54	278.54 ± 158.64	20.42 ± 12.88
	A1 *vs* A2	A1 *vs* A2	A1 *vs* A2
	P < 0.05	P < 0.001	0.2 < P < 0.3
	A2 *vs* C	A2 *vs* C	A2 *vs* C
	P < 0.01	0.2 < P < 0.3	P < 0.05
Study group M1	335.84 ± 154.08	173.71 ± 116.79	8.44 ± 5.63
	M1 *vs* C	M1 *vs* C	M1 *vs* C
	P < 0.01	0.1 < P < 0.2	P < 0.001
Study group M2	451.55 ± 210.74	178.96 ± 84.56	15.26 ± 12.73
	M1 *vs* M2	M1 *vs* M2	M1 *vs* M2
	0.2 < P < 0.3	0.1 < P < 0.2	0.1 < P < 0.2
	M2 *vs* C	M2 *vs* C	M2 *vs* C
	P < 0.01	0.5 < P < 0.6	P < 0.05
Control group C	247.78 ± 44.74	130.38 ± 48.35	2.45 ± 1.44

PLT: Platelet count; sP-selectin: Soluble platelet selectin; IL: Interleukin.

Table 3 Assessment of phagocytic activity of blood platelets in gastric cancer patients-E, -A, -M and in control group (mean ± SD)

	Percentage of phagocytizing platelets	Phagocytic index
Study group early carcinoma E1	1.08 ± 0.08	1.02 ± 0.04
	E1 *vs* C	E1 *vs* C
	P < 0.001	P < 0.001
Study group early carcinoma E2	1.27 ± 0.10	1.07 ± 0.05
	E1 *vs* E2	E1 *vs* E2
	P < 0.05	0.05 < P < 0.1
	E2 *vs* C	E2 *vs* C
	P < 0.01	P < 0.01
Study group advanced carcinoma with lymph node involvement A1	1.10 ± 0.11	1.02 ± 0.11
	A1 *vs* C	A1 *vs* C
	P < 0.001	P < 0.001
Study group advanced carcinoma with lymph node involvement A2	1.23 ± 0.08	1.03 ± 0.06
	A1 *vs* A2	A1 *vs* A2
	P < 0.05	0.7 < P < 0.8
	A2 *vs* C	A2 *vs* C
	P < 0.001	P < 0.001
Study group metastatic cancer M1	1.13 ± 0.10	1.0 ± 0.07
	M1 *vs* C	M1 *vs* C
	P < 0.001	P < 0.001
Study group metastatic cancer M2	1.20 ± 0.12	1.05 ± 0.08
	M1 *vs* M2	M1 *vs* M2
	0.2 < P < 0.3	0.7 < P < 0.8
	M2 *vs* C	M2 *vs* C
	P < 0.001	P < 0.001
Control group C	2.26 ± 0.57	1.83 ± 0.37

relation between IL-6 and tumor stage. An elevated level of IL-6 indicated disease progression and higher malignancy, and was thus an unfavorable prognostic factor. The level of IL-6 was found to correlate with the disease stage and increased in gastric cancer relapse[18]. Ashizawa *et al*[19] described the importance of IL-6 measurement in gastric cancer patients with local lymph node involvement and distant metastases. Due to its pyrogenic action, IL-6 is responsible for cachexia, fever, body mass reduction and other devastating symptoms of tumor progression. Determination of IL-6 can be a prognostic factor of survival in gastric cancer patients[19-21]. In our study, the level of IL-6 was found to increase in all gastric cancer patients. Its values were the highest in patients with early cancer (over an 8-fold increase), whereas the lowest in those with metastases (over a 3-fold increase), as compared to the control group. This seems to confirm that inflammation can be the primary stage of the neoplastic process. When the inflammatory process begins to extinguish, neoformation develops intensively. PLT was found to increase along with disease progression.

Platelets are a rich source of pro- and anti-angiogenic factors that regulate the process of tumor growth. The angiogenesis-stimulating factors include tumor necrosis factor-α, PDGF, granulocyte-macrophage colony-stimulating factor, IL-6 and metalloproteinases. After angiogenesis initiation, platelets adhere to vascular endothelium and aggregate. It has been shown that VEGF released from platelets stimulates the development of megakaryocytes and platelets, activates endothelial cells to release vWF, and thus facilitates platelet adhesion to

the vascular wall[22,23].

According to Park *et al*[24], the statistically significantly lower values of MPC in gastric cancer patients are caused by degranulation and release of granular contents following platelet activation. Osada *et al*[25] found no statistically significant differences in the platelet count between gastric cancer patients and control subjects. The number of CD62P antigens on the platelet surface after TRAP activation in gastric cancer patients increased from 6 to 12 times as compared to a 3-fold increase in healthy subjects. The large number of glycoproteins on the platelet surface observed in gastric cancer after platelet activation *in vitro* may indicate prothrombotic tendencies. Platelets exhibited far greater activity, with their number remaining unchanged.

During platelet activation, the membranes of platelet α-granules combine with the platelet cell membrane, whereas P-selectin is expressed on the surface as CD62P receptor. Part of the extracellular domain is rinsed off to the blood and occurs as sP-selectin, which has a long half-life and is a good plasma marker to assess platelet activation. An increase in the level of sP-selectin is accompanied by a decrease in P-selectin expression on the platelet surface. Soluble P-selectin is a marker of platelet hyperactivity, endothelial dysfunction and inflammation, and may serve as a biomarker associated with venous thrombosis in the course of cancer. At the same time it plays a major role linking inflammation with thrombosis[26].

Ikeda *et al*[27] reported a slight, statistically insignificant difference in the level of sP-selectin determined in gastric cancer patients before and after surgery. However, in our study the level of sP-selectin was statistically significantly lower only in patients with early cancer (group E) as compared to the control, and it was accompanied by the lowest platelet count. In this group, the level of IL-6 was the highest, which suggested acute inflammation. The decrease in the platelet count may be due to their consumption at the site of the inflammatory and then neoplastic process. The lowest level of sP-selectin seems to confirm earlier activation of platelets and disintegration of sP-selectin. In the other groups of patients, an increase in platelet count was accompanied by a rise in sP-selectin, although the differences were not statistically significant. The increased level of sP-selectin reflects intravascular platelet activation.

P-selectin takes part in the process of platelet aggregation and in interactions with cancer cells. Platelets bind to cancer cells and form aggregates around them, thus protecting them against the host immune system and in consequence allowing survival and formation of metastases. Factors released from tumor cells, *e.g.*, cancer procoagulants, thrombin, adenosine diphosphate, tissue factor show the capacity of direct platelet activation. Therefore, a correlation can be found between the level of sP-selectin, platelet count and their activation. Adhesion molecules, such as integrins and glycoproteins are involved in the formation of the platelet-tumor cell aggregates and metastases. The increased expression of

P-selectin on the platelet surface is strongly related to the occurrence of metastases and therefore the blockade of P-selectin can be used to inhibit the formation of cancer metastases[13].

Blood platelets exhibit the capacity of phagocytizing and digesting bacteria independently. The process of phagocytosis lasts only 6 min, *i.e.*, it is 5-times faster than in granulocytes. It also seems to be more efficient as the platelet count is 40-times higher and platelets remain in the circulation after digesting bacteria[6]. Platelet activation involves release of defensins, as well as Platelet factor 4 and CXC chemokine ligand 4 belonging to the family of chemokines, which show bactericidal properties. These actions are aided by H_2O_2 and reactive oxygen species that have a toxic effect on bacteria[28]. However, White[29] questions the ability of platelets to phagocytize bacteria, claiming that platelets can absorb but not kill them, which is due to the fact that platelet lysosomes do not contain myeloperoxidase. He believes that the mechanism of bacterial absorption is in their sequestration by the open canalicular system, through which P-selectins are transported to the platelet surface.

We noted a statistically significant decrease in the percentage of phagocytizing platelets and the phagocytic index in gastric cancer patients irrespective of staging, as compared to healthy subjects. Despite increased platelet count and thrombocytopoiesis stimulation, the phagocytic functions of blood platelets are markedly impaired. It seems that cancer development reduces the effectiveness of the ongoing metabolic processes. The decreasing phagocytic activity of platelets can promote both inflammatory processes and neoplastic metastases.

A decreased phagocytic activity of blood platelets indicates directly that the mechanisms of nonspecific immunity are impaired. On the other hand, an increased level of sP-selectin suggests platelet stimulation and their continuous activation in the course of gastric cancer. A growing tumor promotes platelet stimulation and activation. The lowest platelet count and the lowest level of sP-selectin were observed in early cancer, which seems to confirm the involvement of platelets in the formation of inflammatory foci and their intravascular activation.

COMMENTS

Background
Helicobacter pylori (*H. pylori*) is responsible for gastric mucosa inflammation, resulting in the stimulation of neutrophils and macrophages, and in an increased production of reactive oxygen species. The proinflammatory cytokines, interleukin (IL)-6 and IL-23, which are released during *H. pylori* infection and which strongly inhibit secretion of gastric juice, are involved in the pathogenesis and development of tumors.

Research frontiers
Platelets play an active part in cancerous diseases through the secreted proinflammatory factors, chemokines and growth factors. Tumor cells exhibit prothrombotic effect by inducing platelet aggregation and by platelet activation.

Innovations and breakthroughs
The authors noted a statistically significant decrease in the percentage of phagocytizing platelets and the phagocytic index in gastric cancer patients irrespective of staging, as compared to healthy subjects. Despite increased platelet

count and thrombocytopoiesis stimulation, the phagocytic functions of blood platelets are markedly impaired.

Applications

The lowest platelet count and the lowest level of soluble platelet selectin were observed in early cancer, which seems to confirm the involvement of platelets in the formation of inflammatory foci and their intravascular activation.

Peer review

In this study, the level of IL-6 was found to increase in all gastric cancer patients. Its values were the highest in patients with early cancer, whereas the lowest in those with metastases, as compared to the control group. This seems to confirm that inflammation can be the primary stage of the neoplastic process.

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