Changes in platelet CD 62P expression and soluble P-selectin concentration in surgically treated colorectal carcinoma

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Abstract

Purpose: The aim of the present study was to assess the effect of tumor advancement and surgery treatment on P-selectin expression (CD 62P), level of sP-selectin and platelet count.

Material and methods: The study involved 27 colorectal cancer patients (CRC). They were divided into two groups: group B1 – 18 patients (T2-3N0M0) and group B2 – 9 patients (T2-4N+M0). In CRC patients the blood was collected three times: 1) before surgery (A0), 2) 3 days after surgery and 3) 12 days after surgery.

Results: The results obtained showed that CD 62P expression in CRC patients was twice higher (5.36%) than in control (2.58%) ($p<0.001$). The level of sP-selectin concentration in both groups (B1 – 74.22 ng/mL and B2 – 70.33 ng/mL) was significantly higher than in control (46.01 ng/mL) ($p<0.001$). There was no significant differences in CD 62P expression, plasma sP-selectin concentration and in PLT count between group B1 and B2. Three days after surgery in both groups of patients we observed decreased CD 62P expression and sP-selectin level compared to the results before surgery ($p<0.05$). Twelve days after surgery we found an increase in the CD 62P-positive platelets and sP-selectin in group B1 and B2. We found positive correlation between plasma sP-selectin concentration and PLT count in CRC.

Conclusions: In the current study on colorectal cancer we observed platelet hyperactivation, irrespective of tumor clinical advancement. Surgical procedure, in the early period following radical tumor resection, does not totally eliminate platelet activation *in vivo*.

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Introduction

P-selectin (CD 62P, also known as GMP-140), 140 kDa, belongs to the family of adhesion molecules. It is rich in cystein and binds in resting platelets to the membranes of α granules and Weibel-Palade bodies of endothelial cells [1]. Upon platelet activation and release from α granules, P-selectin is translocated into the platelet surface. The exposure of surface P-selectin is temporary as it is soon "shed" to the plasma and is found in a soluble form (sP-selectin) there [2]. Michelson showed that the elevated concentration of sP-selectin was accompanied by a drop in CD 62P expression [2]. The percentage of activated platelets with P-selectin expression assessed by means of flow cytometry is regarded as the "golden standard" of platelet activation [3].

P-selectin, as an adhesion molecule, plays a key role in the interaction of platelets with other cells. A small amount of PSGL-1 is also present on platelet surface and can mediate platelet-endothelium interactions *in vivo* [4]. P-selectin released from endothelial cell granules initiates leukocyte and platelet rolling on the vessel wall, which is an important process in both inflammation and hemostasis [5]. The latest studies have provided some evidence for the presence of CD 24 ligand on cancer cells, recognized by P-selectin [6,7]. Discovery of the CD 24 molecule on the cells of certain types of cancer indicates that P-selectin can mediate tumor cell interactions with platelets, leukocytes and endothelium *in vitro* [8,9]. This fact seems to be of significance in understanding the role of adhesion molecules and platelets in the formation of tumor metastasis. Cancer cell binding by platelets is an important observation – platelet aggregations formed around cancer cells play a protective role against the host immune system; in consequence, they prolong cell survival time and promote formation of metastases [10]. Moreover, platelets protect tumor cells against the environment and are the source of growth factor, such as platelet derived growth factor (PDGF), vascular endothelial growth factor (VEGF), that stimulate growth of cancer cells [11].

Quantitative thrombocytopenia or thrombocytosis, and blood platelet abnormalities found in cancers may lead to disturbances in hemostasis [12]. In neoplastic disease, platelets are capable of hyperactivation *in vivo*. An increase has been found in sP-selectin concentration or CD 62P expression in patients with thrombotic diseases, atherosclerosis and in neoplastic disease – in lung, renal, breast and colon cancer [13-17]. These abnormalities include hemorrhagic complications – a likely consequence of platelet dysfunction, vessel infiltration by cancer cells or DIC. However, the enhanced platelet activity and thrombocytosis can increase the risk of thrombosis, which is one of the most frequent causes of death due to cancer [12].

Hemostatic disorders, including platelet abnormalities in which both platelet count and function are affected are commonly found in colorectal cancer. Many studies dealing with the effect of tumor clinical advancement, presence of metastases and the influence of surgery treatment on morphological and functional parameters of platelets have yielded controversial issues. Therefore, we decided to investigate the problem in colorectal cancer patients.

The aim of our study was to assess the effect of tumor advancement on P-selectin expression (CD 62P) and plasma level of soluble P-selectin and platelet count as well as the effect of surgery on the parameters examined in colorectal cancer patients. An attempt was also made to find any correlations between these parameters in colorectal cancer patients.

Material and methods

Patients

The study included 27 patients (10 women and 17 men; aged 52-80 years) with colorectal cancer treated surgically in II Department of General Surgery, University Hospital of Białystok. They were enrolled to the study between November 2003 and September 2004.

The diagnosis of colorectal cancer was based on clinical symptoms, and endoscopy (colonoscopy), radiology or CT. All the patients had colorectal adenocarcinoma, confirmed by histopathological examination.

Colorectal cancer patients were divided into two groups, according to TNM classification:

- Group B1 – 18 patients without metastases (7 women and 11 men; mean 67.5 years) – T2-3N0M0 (stage I and II)
- Group B2 – 9 patients with lymph node involvement (4 women and 5 men, mean 61 years) – T2-4N+M0 (stage III).

At the time of the study, there were no patients with distant metastases (M+, grade IV) of colorectal cancer.

Colorectal cancer patients awaiting surgery, who gave their written consent to the study, were recruited. The patients underwent surgical procedure of tumor resection with subsequent regional lymphadenoctomy.

Before and after surgical treatment the patients received no cytostatics nor chemo- or radiotherapy. Those who took aspirin or other platelet function affecting drugs (steroidal, anti-inflammatory) in the preceding week were excluded from the study. All the patients were given a preventive dose of heparin (LMWH) – *Enoxiparinum natricum* (Clexane) 20-40 mg once daily for 7-10 days, with the first injection administered a day before the procedure. If required, the heparin dose was increased up to 80 mg daily.

The study was approved by Bioethics Committee, Medical University of Białystok according to Guidelines for Good Clinical Practice.

Control group

Control group (C) consisted of 21 healthy subjects, men and women aged 42-74 years. They had no taken aspirin and other antiplatelet drugs during the preceding week.

Material

The blood collected from the vein to tubes in a closed system, without stasis was used for analysis. 2 ml blood samples were collected for anticoagulant EDTA-K2 to assess platelet count and 3.6 ml blood samples to evaluate P-selectin expression and level of sP-selectin using 3.2% sodium citrate as anticoagulant.

In CRC patients blood was collected three times: 1) before surgery (A0); 2) 3 days after surgery (A1), to assess the effect of surgery and the related trauma on P-selectin expression and thrombocytopoiesis, the effect of a number of factors that accompany the procedure (hypoxia, acidosis, interleukins and growth factors) and systemic hemostatic balance, 3) 12 days after surgery (A2), taking into consideration mean survival time and platelet turnover.

In control group (C) blood was collected once.

Methods

Flow cytometry Surface P-selectin expression (% of CD 62P positive platelets) was assessed using monoclonal antibodies CD 62P/RPE (DAKO, Denmark), on a flow cytometer EPICS XL Coulter according to the protocol of European Working Group on Clinical Cell Analysis [18].

Additionally, monoclonal antibodies CD 61/FITC (DAKO, Denmark) were used to identify platelets among other morphological components. IgG1/RPE (DAKO, Denmark) antibodies served as negative control antibodies.

Venous blood was taken to plastic tubes containing sodium citrate. To determine P-selectin expression on platelets *in vivo* immediately after blood collecting, a 100 μl sample was placed in a test tube containing 1 ml of 1% paraformaldehyde in PBS (without Ca^{2+} and Mg^{2+}) and incubated for 30 minutes at room temperature. Then, 20 μl alignots of the suspension were placed into two test tubes containing the following antibody sets: 1) CD 61/FITC+ IgG1/RPE, 2) CD 61/FITC+ CD 62P/RPE. This was followed by 30 min incubation in darkness and after addition of 1 ml PBS the samples were analyzed on a flow cytometer.

Signal from 10000 platelets was analyzed in each measurement. The results were presented as the percentage of CD 62P positive platelets (% CD 62P+), i.e. activated platelets with release reaction.

Assay of soluble P-selectin Blood samples were collected in citrate-containing tubes. Samples were centrifuged at 1000 x g

Table 1. **P-selectin expression and soluble P-selectin concentration and platelet count in colorectal cancer patients and in control group**

a) **P-selectin expression (CD 62P % positive)**

	n	A0	A1	A2	p
CRC group	27	5.36±1.58**	3.13±0.65	4.40±1.85**	
B1 group	18	5.69±2.09**	2.87±0.88	4.64±1.9**	A0:A1*, A1:A2**
B2 group	9	4.66±1.20**	2.45±0.50	4.41±2.55*	A0:A1*
Control group	21	2.58±1.36			

b) **sP-selectin concentration (ng/mL)**

	n	A0	A1	A2	p
CRC group	27	68.7±20.56**	55.8*±21.57	81.6±26.07 **	A0:A1*, A1:A2**
B1 group	18	74.22±28.80**	62.80±33.30	92.00±21.12**	A0:A2*, A1:A2**
B2 group	9	70.33±25.09**	52.00±21.91	63.14±13.07*	A0:A1*
Control group	21	46.01±8.09			

c) **PLT count (x 10^3/µl)**

	n	A0	A1	A2	p
CRC group	27	262.8±64.2	245.6±80.9	356.2±142.,2**	
B1 group	18	259.6±72.5	234.5±70.5	339.4±54.0	A0:A2, A1:A2*
B2 group	9	271.5±109.7	270.1±141.4	394.6±161.0**	A0:A2,A1:A2*
Control group	21	232.6±32.0			

Results are expressed as mean ±SD; **p<0.001 vs C, *p<0,05 vs C; A0 – before surgery; A1 – 3 days after; A2 – 12 days after surgery; CRC – colorectal cancer patients; B1 – tumor without metastases; B2 – tumor with lymph node metastases

within 30 minutes. Then plasma were kept frozen at -80°C, and thawed before determination of sP-selectin. sP-selectin levels were determined using the Quantikine human sP-selectin immunoassay kit (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions.

Platelet count PLT count was determined on a hematological analyzer ADVIA 120 (Bayer). Platelet count in healthy subjects is 150-350 x 10^3/µl.

Statistical analysis

The results were subjected to statistical analysis using non-parametric U-Mann-Whitney tests to compare data between the groups B1 and B2 CRC patients and with control findings (group C) before surgery. Wilcoxon's test was applied to compare differences in the parameters in time (before and 3 and 12 days after surgery). And Pearson correlation coefficient was calculated.

Results were presented as mean ±SD. Differences were considered significant for p<0.05 and highly statistically significant for p<0.001. Statistical package SPSS 8.0 PL was used for calculations.

Results

Platelet CD 62P expression in colorectal cancer patients before surgery (A0) was over twice higher than in healthy subjects (p<0.001) (*Tab. 1a*). The percentage of CD 62P-positive platelets was higher in metastasis-free patients – group B1 as compared to patients with lymph node involvement – group B2, but the differences were not statistically significant. However, highly statistically significant were the differences in the expression of CD 62P in the study groups in comparison to controls (p<0.001) (*Tab. 1a*). The level of soluble P-selectin was slightly higher in study patients than in control group (68.7 ng/mL vs 46.01 ng/mL; p<0.001) (*Tab. 1b*). It was higher in group B1 than in group B2, although the difference was not statistically significant (p<0.067) (*Tab. 1b*). In patients with colorectal cancer (A0), PLT count was higher than in control group, although the differences were statistically insignificant. No differences in PLT count were also found with regard to tumor advancement – group B1 and group B2 (*Tab. 1c*).

Three days after surgery (A1) colorectal cancer patients showed significantly decreased platelet CD 62P expression, which approached control values. In both study groups the percentage of CD 62-positive platelets was significantly lower as compared to the expression before surgery (A0:A1, p<0.05) (*Tab. 1a*). The level of sP-selectin in both groups was decreased as compared to the level before surgery, but it was still higher than in controls (*Tab. 1b*). At the same time, in both study groups, platelet count was slightly reduced in comparison to that noted prior to surgery (*Tab. 1c*).

Twelve days after surgery (A2), we found an increase in the percentage of CD 62P-positive platelets both in the group of colorectal cancer patients and in clinical advancement subgroups (B1 and B2) (*Tab. 1a*). The percentage values of CD 62P in the group B1 were lower as compared to that group before surgery (*Tab. 1a*). At the same time we observed increased concentration of sP-selectin in CRC patients, especially in group B1. Differences in sP-selectin between the groups were statistically significant (*Tab. 1b*). Moreover, a statistically significant increase was observed in PLT count in the study groups as compared to the values obtained in the patients 3 days after (A1) and before the procedure (A0; $p<0.05$) (*Tab. 1b*). A significant increase in platelet count – thrombocytosis ($394.6x10^3/\mu l$) was noted in the group B2, i.e. in patients with metastases to the lymph nodes; it was statistically significant both in comparison to PLT before surgery (A0) and 3 days after the surgery (A1) (A0:A2, A1:A2; $p<0.05$) (*Tab. 1c*).

The relationship between plasma sP-selectin and CD 62P expression and PLT count in CRC patients was also evaluated. We found no statistical correlation between sP-selectin and CD 62P. However, we observed positive correlations between sP-selectin and PLT count in group B1 ($r=0.6027$, $p<0.023$) and in group B2 ($r=0.8242$, $p<0.006$).

Discussion

Numerous studies point at the occurrence of hyperactive platelets in cancer patients [12,14-16,20]. Upon platelet activation, tumor growth-inducing substances TXB2 and VEGF are released [21], and P-selectin involved in neoplastic spread can be found on platelet surface. On the other hand, a number of factors released by tumors such as cancer procoagulant, thrombin, ADP, tissue factor (TF) are capable of direct platelet activation [22].

We found increased platelet activation *in vivo* in colorectal cancer patients, which was manifested by over twice as high CD 62P expression on platelet surface and elevated level of soluble P-selectin as compared to healthy subjects. Slightly higher expression of CD 62P and higher sP-selectin level were observed in patients with stage I and II carcinoma, in comparison to stage III, the differences being statistically insignificant. Lack of differences between these markers of platelet activation and clinical staging may suggest that tumor causes platelet activation independent of metastasis and could be related to the inflammatory response which is associated with CRC. Some authors obtained different results, perhaps due to differences in the number of study patients – our group was small, while groups of other investigators were large. Findings similar to ours have been reported by Mantur et al. for renal cancer [15]. The author observed an increase in both surface and soluble P-selectin, but found no effect of lymph node involvement on platelet activation in renal cancer patients [15]. However, Ferroni, who studied colorectal cancer, revealed higher sP-selectin level in patients with more advanced carcinoma and distant metastases [17]. Similarly, in lung cancer Roselli found a correlation between the level of sP-selectin and the presence of distant metastases, and as we know many investigators have suggested platelet involvement and direct contribution of P-selectin to the formation of metastases [14,23,24].

We found a correlation between sP-selectin and PLT count but not between the examined markers of platelet activation. It may indicate that sP-selectin is rather derived from blood platelets. This hypothesis could be in agreement with the findings of Ferroni et al., who observed no correlation between sE-selectin and sP-selectin, and suggested that the increased level of sP-selectin originated from platelets rather than endothelial cells due to platelet activation [17]. The same positive correlation between sP-selectin and PLT count was also noted by Fijnheer et al. [25]. According to some authors, sP-selectin is a better and more useful marker of platelet activation, especially in patients with thromboembolic disorders, as compared to cytometric analysis of CD 62P [2,25]. It is likely that P-selectin expression is temporary, and the receptor is then thrown into the circulation and labeled as soluble P-selectin, while the percentage of CD 62P+ returns to baseline level. This hypothesis is based on the observation that an increase in plasma sP-selectin is accompanied by a decrease in CD 62P expression [2].

Data on the effect of surgery on platelet activation in cancer patients are scarce. Our patients underwent resection of colorectal tumor with regional lymphadenectomy. Three days after surgery we noted a significant decrease in CD 62P expression, nearly to the values observed in healthy subjects. Also sP-selectin level was reduced, although not so much as expression of CD 62P, indicating that in the early postoperative period in colorectal cancer patients platelets still show a considerable activation potential. This can be associated with the inflammatory process accompanying the surgical procedure, wound healing and cytokine production (e.g. IL-6 involved in acute phase reaction and stimulating thrombocytopoiesis) [26]. At the same time we observed a decrease in platelet count, most likely caused by the loss of more active platelets due to bleeding or shortened survival time in cancer patients, or caused by heparin administration in the perioperative period. According to some authors, heparin, which is a P- and L-selectin inhibitor, suppresses tumor growth and formation of metastases [27,28]. Experimental studies have demonstrated that even a single heparin dose inhibits platelet interaction with tumor cells, which is P-selectin-mediated [27]. This could explain a considerable drop in CD 62P expression compared to sP-selectin in our patients.

Twelve days after surgery we found enhanced stimulation of thrombocytopoiesis and platelet activation in patients with colorectal cancer. We noted an increase in CD 62P expression and sP-selectin level in both study groups of patients as compared to day 3 and interestingly, we observed statistically significant differences in sP-selectin level between the groups. This may indicate that the procedure had a certain effect on platelet activation, but did not totally eliminate the platelet activating factors. Lower platelet activation indicated by much lower sP-selectin level in stage III patients may be caused by shorter survival time or lower potential of PLT, considering that high-activity platelets have been utilized in the formation of metastases in these patients.

Stimulation of thrombocytopoiesis after surgery has been observed by Folman et al. [29,30]. The authors noted the maximum increase in platelet count between day 7 and day 20 after surgery, associating it with the level of thrombopoietin, major regulator of thrombocytopoiesis [29]. Platelet turnover and production activation due to their intra-surgical exploitation result in the appearance of young platelets in the circulation 12 days after surgery and hence raised platelet count. The young platelets show higher metabolic activity, are more sensitive to the action of activating factors and have more surface receptors [15].

Based on the expression of surface P-selectin and the level of soluble P-selectin, the current study has revealed that colorectal carcinoma induces intravascular platelet hyperactivation, irrespective of clinical advancement. Positive correlation between sP-selectin and PLT count seems to indicate that sP-selectin may be derived from blood platelets. Surgical procedure exerts a significant effect on the stimulation and platelet count, but in the early period following radical tumor resection it does not eliminate platelet activation *in vivo*.

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