

Platelet and neutrophil cross-talk-mediating cancer growth and metastasis in patients with urinary bladder cancer

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Background/Aim

The initial selectin-dependent events that mediate tumor cell tethering to platelets, leukocytes, and vascular endothelium can regulate the extravasation and colonization of metastatic cells into distant tissues. We aimed to clarify the role of selectin–selectin ligand interactions in tumor growth and progression in patients with bladder cancer.

Patients and methods

Thirty patients with bladder cancer were the participants in this study classified as follows: locally invasive group ($n=10$), urinary bladder cancer group with regional lymph node involvement ($n=10$), and urinary bladder cancer group with regional lymph nodes and distant metastasis ($n=10$). Flow cytometry was used to determine both the platelet surface expression of P-selectin (CD62P) and the neutrophil surface expression of PSGL-1 (CD162), whereas enzyme-linked immunosorbent assay was used for the assay of soluble P-selectin.

Results

Neutrophil PSGL-1 expression among the different groups studied was not statistically significant. However, there was enhanced platelet activation as evidenced by increased platelet surface expression of P-selectin together with an increase in its soluble form, which was more prominent with advancement of the disease, especially in patients with distant metastasis. Also, a strong positive correlation was found between platelet P-selectin and its soluble form with the tumor grading. In addition, stepwise multiple regression analysis showed that both P-selectin and platelet count are significant independent determinants for the stage of bladder cancer, suggesting augmentation of P-selectin–ligand interaction.

Conclusion

These data preclude that disease progression in patients with bladder cancer is dependent on the complex interaction between P-selectin and its ligand. Targeting of these molecules may represent a unique approach to tumor therapy and prevention of metastasis.

Keywords:

bladder cancer, P-selectin, P-selectin ligand

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Introduction

Most patients who die from cancer succumb to treatment-refractory advanced metastatic progression. Although the early stages of tumor metastasis result in the formation of clinically silent micrometastatic foci, its later stages primarily reflect the progressive, organ-destructive growth of already advanced metastases. Early-stage metastasis is regulated by multiple factors within tumor cells as well as by the tumor microenvironment. In contrast, the molecular determinants that control advanced metastatic progression remain essentially uncharacterized, precluding the development of therapies targeted against it [1].

Selectins, a family of mammalian lectins, are involved in adhesion reactions and expressed by leukocytes, endothelial cells, and platelets [2,3]. P-selectin and its receptor P-selectin glycoprotein ligand-1 mediate adhesion between

leukocytes, tumor cells, and platelets and play an important role in hematopoiesis, T cell interaction, and cancer growth and metastasis [4].

Upon stimulation (by histamine or thrombin), activated platelets, leukocytes, and endothelial cells degranulate, with the rapid translocation of P-selectin (CD62) to the plasma [5,6]. High expression of sP-selectin has been implicated in several inflammatory disorders, thrombotic diseases, and connective tissue disease [7].

PSGL-1 (CD162) (P-selectin glycoprotein ligand) mediates leukocyte–endothelial and leukocyte–platelet adhesion by binding to P-selectin expressed on activated endothelium and platelets and PSGL-1 mediates leukocyte–leukocyte adhesion by binding to L-selectin expressed on opposing leukocytes. PSGL-1 is unique in that it is the only selectin glycoprotein ligand that has

been shown to directly mediate cell–cell adhesion *in vitro* and *in vivo* [8].

There is accumulating evidence for the potential of selectins to contribute toward a number of pathophysiological processes, including cancer metastasis [9]. It has been known for a relatively long time that aberrant high levels of sialylated, fucosylated selectin ligands are expressed on cancer cells, especially epithelial cancer [10]. Also, cancer metastasis is associated with extravasation of tumor cells from blood into tissue. This movement is believed to follow a coordinated and sequential molecular cascade initiated, in part, by the three members of the selectin family of carbohydrate-binding proteins: E-selectin (CD62E), L-selectin (CD62L), and P-selectin (CD62P) [11].

Platelets are highly reactive components of the circulatory system, which exert not only hemostatic activity but also contribute toward the modulation of various pathological conditions including inflammation, atherosclerosis, and cancer metastasis through the release of cytokines, chemokines, and the presentation of several adhesion molecules [12]. Various experimental and clinical studies have detailed the interaction of platelets with primary tumors and circulating metastatic tumor cells. Observations have suggested that platelets not only augment the growth of primary tumors through angiogenesis but provide tumor cells with physical and mechanical support to evade the immune system and extravasate to secondary organs [13].

Although the contribution of leukocytes toward the formation of primary tumors is well known, the mechanisms by which leukocytes govern the process of metastasis remain poorly understood. Depending on the microenvironmental signals, leukocytes can either exert antitumoral activity or promote cancer progression. Tumor-associated myeloid cells including polymorphonuclear leukocytes, monocytes, and differentiated macrophages have been shown to facilitate immunosuppression, angiogenesis, tumor cell survival, and invasion and thereby contribute to metastatic dissemination [14]. Previously, it was shown that the absence of L-selectin results in the attenuation of tumor cell survival and metastasis, implicating leukocytes in the colonization process [15,16].

The selectin family of adhesion molecules binds to glycoconjugate ligands expressed on opposing cells. Selectin ligands such as PSGL-1, ESL-1, and CD24 are expressed mainly by leukocytes. A small amount of PSGL-1 is also present on the platelet surface and can mediate platelet–endothelium interaction *in vivo* [17]. Carlow *et al.* [18] have reported that PSGL-1 is the high-affinity counter-receptor for P-selectin. Also, Xu *et al.* [19] showed a critical role for PSGL-1 in regulating lymphocyte homing and leukocyte trafficking during inflammation. The interaction of the PSGL-1 cytoplasmic domain with the actin cytoskeleton is essential for rolling on P-selectin, and thereby suggests a novel paradigm for adhesion receptors that mediate leukocyte rolling under flow.

Recently, Läubil and Borsig [20] discussed the current evidence for selectins as potential facilitators of metastasis.

They reported that cell interactions with selectins are possible because of a frequent presence of carbohydrate determinants–selectin ligands on the cell surface of tumor cells from various types of cancer. The degree of selectin ligand expression by cancer cells is well correlated with metastasis and a poor prognosis for cancer patients. Initial adhesion events of cancer cells facilitated by selectins result in the activation of integrins and the release of chemokines, and are possibly associated with the formation of a permissive metastatic microenvironment. In addition, Schneider *et al.* [21] have reported that integrin expression and signaling are perturbed in cancer cells, allowing them to ‘escape’ from cell–cell and cell–matrix tethers, and invade, migrate, and colonize within new tissues and matrices. Integrin signaling through $\alpha\text{v}\beta 3$ and VLA-4 on tumor cells can promote tumor metastasis to and proliferation in the bone microenvironment.

The aim of the present study was to assess the platelet surface expression of P-selectin (CD62P) and the neutrophil expression of PSGL-1 (CD162) and sP-selectin in the plasma of patients with bladder cancer, as selectin–selectin ligand-mediated interactions between cells in the microenvironment of the tumor are an important axis and molecules associated with this axis may serve as candidates for targeted tumor therapy and prevention of metastasis.

Patients and methods

This study was carried out on 30 patients (24 men and six women) with urinary bladder cancer (UBC) admitted to the Urology Department, Theodor Bilharz Research Institute, Giza, Egypt, in addition to 10 healthy individuals (eight men and two women) serving as a control group (Table 1).

The study protocol was approved by the institutional ethical committee for the protection of human participants and conformed to the guidelines of the 1975 Declaration of Helsinki.

All the patients studied were subjected to detailed history taking, thorough clinical examination, abdominal and pelvic ultrasonography, chest radiograph, computed tomography scan, urine cytology, and histopathological diagnosis of urinary bladder biopsies obtained by cystoscopy.

Table 1 Demographic data of all groups studied

Groups	Age (years)	M/F
Control group		
Range	49–74	4/1
Mean $\pm$ SD	58.8 $\pm$ 7.8	
Group I		
Range	53–80	9/1
Mean $\pm$ SD	66.2 $\pm$ 9.12	
Group II		
Range	57–78	4/1
Mean $\pm$ SD	64.8 $\pm$ 6.2	
Group III		
Range	47–75	7/3
Mean $\pm$ SD	61.3 $\pm$ 9.2	

F, female; M, male.

On the basis of the above-mentioned investigations, the patients were classified into three groups as follows (Table 1):

Group I: This group included 10 patients (nine men and one woman, mean age 66.2 ± 9.12 years) with locally invasive UBC (*de novo*).

Group II: This group included 10 patients (eight men and two women, mean age 64.8 ± 6.2 years) with UBC accompanied by regional lymph node involvement.

Group III: This group included 10 patients (seven men and three women, mean age 61.3 ± 9.2 years) with UBC accompanied by regional lymph node involvement as well as distant metastasis.

The histopathological picture of cystoscopic bladder biopsies taken from the 30 patients included in the study indicated that 11 patients (36.66%) had transitional cell carcinoma, six patients (20.0%) had squamous-cell carcinoma, five patients (16.66%) had adenocarcinoma, two patients (6.66%) had adenoid squamous-cell carcinoma, and six patients (20%) were undifferentiated.

For all the patients studied, 4 ml blood samples were collected under complete aseptic conditions and distributed into the following tubes.

Two milliliters of blood was collected in an EDTA-containing tube for a complete blood picture and flow cytometric assay of platelet CD62 P-selectin and neutrophil expression of CD162.

Two milliliters of blood was left to stand for clot formation. Sera were separated and used for the assay of soluble P-selectin using the enzyme-linked immunosorbent assay technique.

Preparation of resting platelets

The EDTA blood sample was centrifuged at 600 rpm for 10 min to obtain platelet-rich plasma. One milliliter of platelet-rich plasma was incubated with an equal volume of 2% paraformaldehyde for 10 min at room temperature. Washing was carried out twice by the addition of 10 ml of the washing solution (PBS + 1% FBS), followed by centrifugation at 2000 rpm for 10 min to obtain the platelet pellet sediment. Finally, the platelet pellet was resuspended in 6–8 ml of washing solution.

Assay of platelet CD62 by flow cytometry

Platelet suspension (100 μ l) was incubated with 10 μ l PE mouse anti-human CD62p monoclonal antibody (Monoclonal anti CD62P; Beckman Coulter, Brea, California, USA) for 20–30 min at room temperature in the dark. After incubation, washing was carried out twice by adding 2 ml of washing solution (PBS + 1% FBS), followed by centrifugation at 2000 rpm for 5 min. Then the supernatant was removed by aspiration. Finally, 500 μ l of wash buffer was added and the tube was shaken gently. For each sample, a matching control tube (without CD62P) was used for the correction of nonspecific binding. The samples were analyzed by flow cytometry.

Assay of neutrophil CD162 by flow cytometry

EDTA blood (100 μ l) was incubated with 10 μ l PE mouse anti-human CD162 monoclonal antibody (Monoclonal anti CD162; BD Biosciences, New Jersey, USA) for 15 min at 4°C in the dark. Immediately after incubation, 2 ml of freshly prepared diluted (1 + 19 with deionized water) lysing solution (EasyLyse) (Leinco technologies, Inc., St. Louis, Missouri, USA) was added and left to stand for 10–15 min at room temperature. For each sample, the matched control tube (without CD162) was used to correct for nonspecific binding. Then the samples were analyzed by flow cytometry.

Statistical analysis

All statistical calculations were carried out using computer programs Microsoft Excel 2003 (Microsoft Corporation, New York, USA) and SPSS (Statistical Package for the Social Science; SPSS Inc., Chicago, Illinois, USA) version 15 for Microsoft Windows. Data were statistically described in terms of range, mean $\pm$ SD. Comparison of quantitative variables between the study groups was carried out using the Mann–Whitney *U*-test for independent samples when comparing two groups and the Kruskal–Wallis analysis of variance test when comparing more than two groups. For comparison of categorical data, the χ^2 -test was performed. The correlation between various variables was assessed using the Spearman rank correlation equation for non-normal variables. Regression analysis was performed to evaluate any association between platelet count and platelet activation marker (CD62) with the growth and spread of the tumors. A *P* value of less than 0.05 was considered statistically significant.

Results

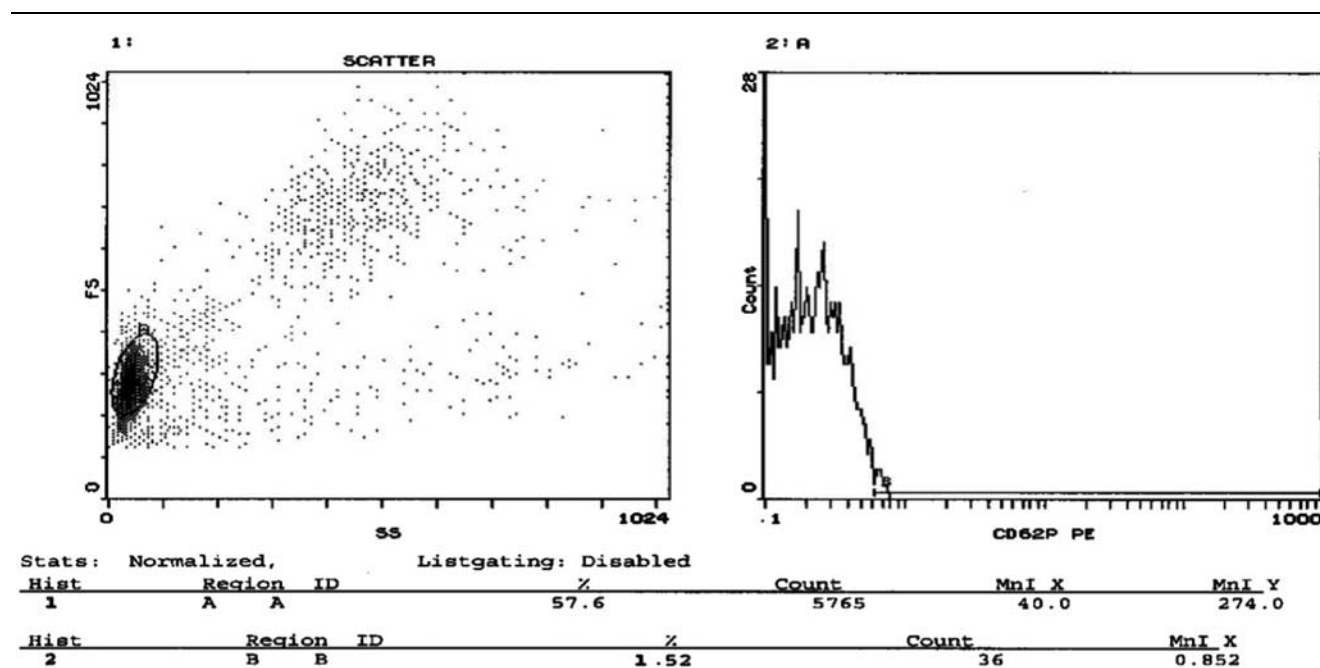
The results of all the parameters studied are presented in Table 2. Hemograms showed that the platelet count was significantly elevated in group III as compared with the control group and groups I and II with *P* values of 0.002, 0.015, and 0.004, respectively, although there was no significant difference on comparing both group I and group II with the control group with *P* values of 0.571 and 0.449, respectively. Also, a nonsignificant difference was observed on comparing groups I and II (*P* = 0.796). However, a comparative statistical study showed a nonsignificant difference in the absolute neutrophil count among the different groups studied (*P* = 0.440).

In terms of the special researches, the platelet surface expression of P-selectin by flow cytometry increased in different patient groups compared with the control group (*P* = 0.000). Also, the expression was higher in group III as compared with both group I (*P* = 0.000) and group II (*P* = 0.000). Moreover, the expression was increased in group II compared with group I (*P* = 0.000) (Figs 1–3).

Statistical analysis indicated a significantly high sP-selectin level in different patient groups compared with the control group (*P* = 0.000). In addition, a significantly high level was detected in group III compared with both group I (*P* = 0.000) and group II (*P* = 0.001). Moreover,

Table 2 Results of studied parameters in different groups of patients with bladder cancer

	Control group	Group I	Group II	Group III
Platelet count ($\times 10^3/\mu\text{l}$)				
Mean $\pm$ SD	274 $\pm$ 69	274 $\pm$ 86	259 $\pm$ 51	376 $\pm$ 81 ^{a,b,c}
Range	249–398	157–400	190–322	229–521
Absolute neutrophil count ($\times 10^3/\mu\text{l}$)				
Mean $\pm$ SD	3.72 $\pm$ 1.27	3.73 $\pm$ 1.03	4.30 $\pm$ 1.47	4.40 $\pm$ 1.23
Range	2.1–6.1	2.6–6.2	2.1–6.2	2.8–6.5
Platelet CD62 (%)				
Mean $\pm$ SD	0.59 $\pm$ 0.38	3.15 $\pm$ 1.27 ^a	8.34 $\pm$ 1.71 ^{a,b}	20.32 $\pm$ 7.23 ^{a,b,c}
Range	0.24–1.47	1.52–4.98	5.58–11.30	11.70–31.00
Neutrophil PSGL-1 (%)				
Mean $\pm$ SD	99.6 $\pm$ 0.4	99.7 $\pm$ 0.2	98.6 $\pm$ 1.8	98.1 $\pm$ 1.8
Range	98.7–100	99.3–100	94.7–100	95.0–100
sP-selectin				
Mean $\pm$ SD	13.9 $\pm$ 4.04	19.3 $\pm$ 4.52 ^a	27.9 $\pm$ 3.78 ^{a,b}	35.0 $\pm$ 4.05 ^{a,b,c}
Range	8–20	13–27	21–34	29–42

^aStatistically significant from control group.^bStatistically significant from the locally invasive tumor group.^cStatistically significant from invasive tumor with the distant metastasis group.**Figure 1**

Flow chart showing the platelet expression of CD62P (%) from a patient in group I.

a significantly high level of sP-selectin was found in group II in comparison with group I ($P = 0.001$).

However, enhanced platelet surface P-selectin expression, together with increased sP-selectin, on flow cytometric assay of neutrophil PSGL-1 (CD162) expression among the different groups studied was statistically no significant (Figs 4–6).

Correlation analysis

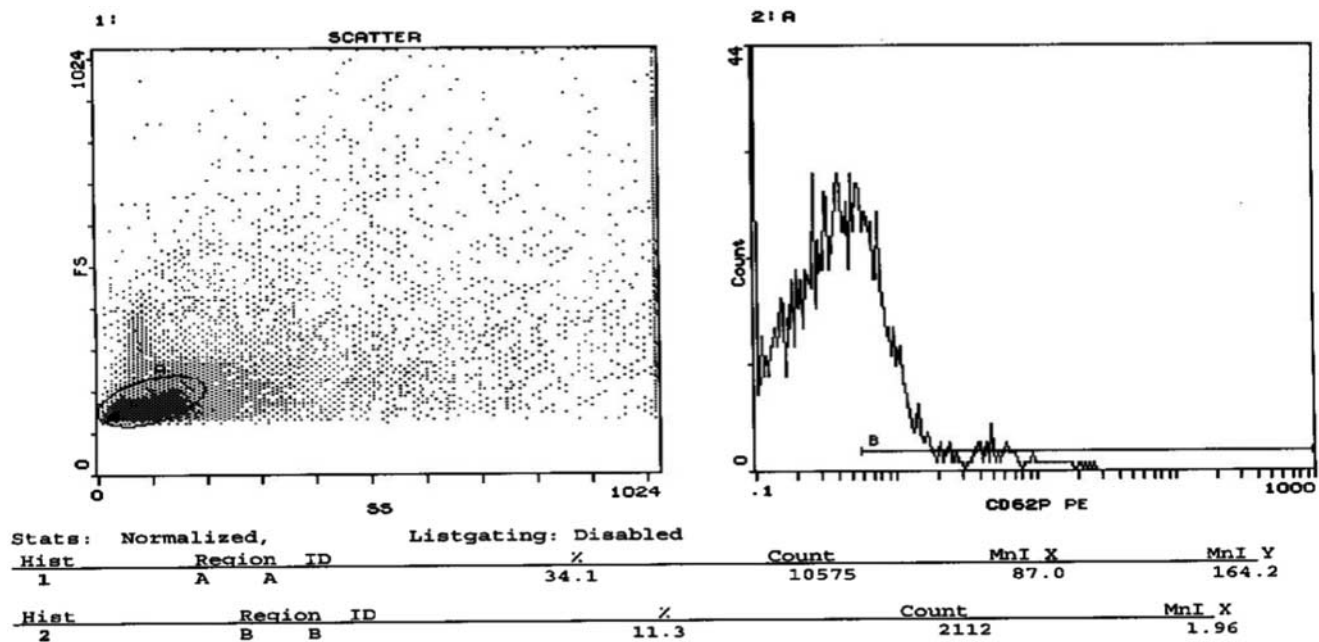
Platelet surface expression of CD62P by flow cytometry showed a significant direct correlation with soluble P-selectin in the control group ($r = 0.979$, $P = 0.00$), group I ($r = 0.952$, $P = 0.00$), group II ($r = 0.994$, $P = 0.00$), and group III ($r = 0.853$, $P = 0.002$).

In patients of group II, a significant direct correlation between the platelet count and both platelet surface expression of CD62P ($r = 0.701$, $P = 0.024$) and soluble P-selectin ($r = 0.764$, $P = 0.010$) was observed. However, in patients of group III, a significant direct correlation was found between platelet surface expression of CD62P and soluble P-selectin with the tumor grading ($r = -0.870$, $P = 0.001$, and $r = -0.881$, $P = 0.001$), respectively. These results provide evidence for the role of platelets in tumor growth and distant dissemination of malignant tumors.

Regression analysis

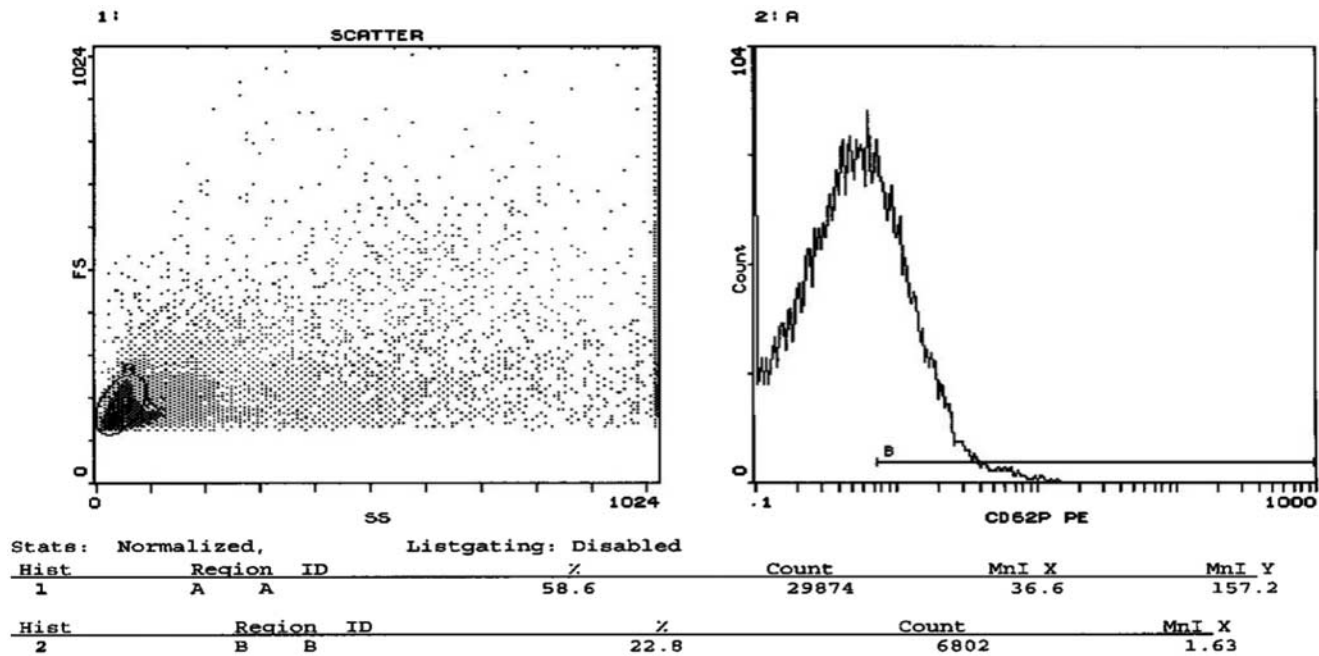
Stepwise multiple linear regression analysis indicated that platelet count and sP-selectin are independent

Figure 2



Flow chart showing the platelet expression of CD62P (%) from a patient in group II.

Figure 3



Flow chart showing the platelet expression of CD62P (%) from a patient in group III.

determinants for the stage of bladder cancer as shown in Table 3. These data provide strong evidence for the impact of enhanced platelet activation on tumor growth and spread in patients with bladder cancer.

Discussion

It has been shown that inflammation is a critical component of tumor progression. It is becoming clear

Figure 4

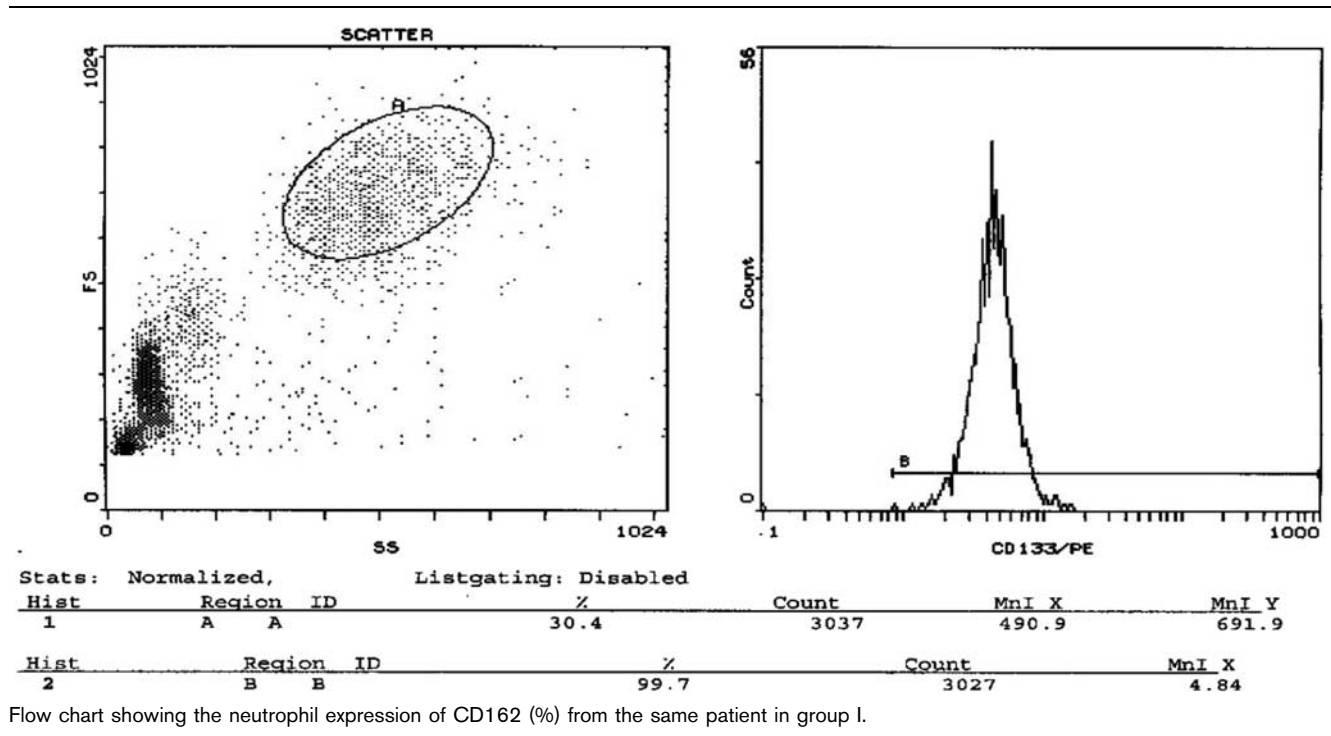
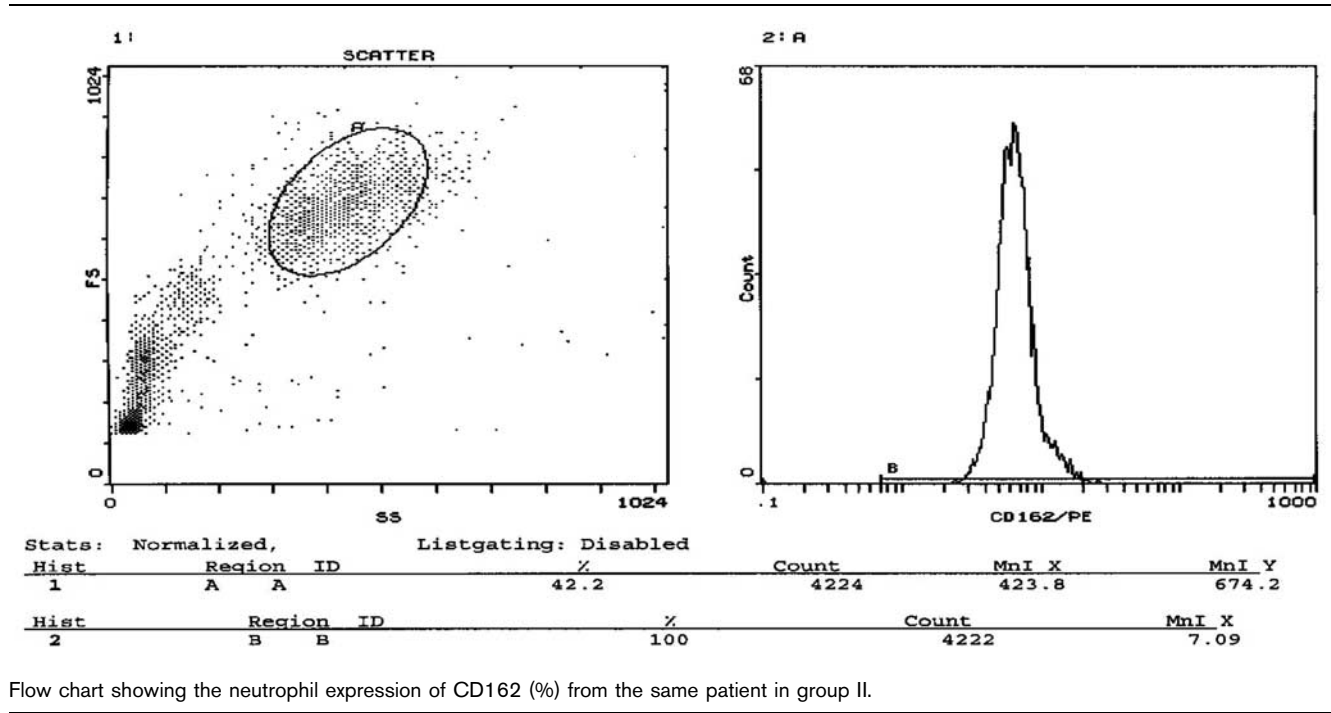


Figure 5

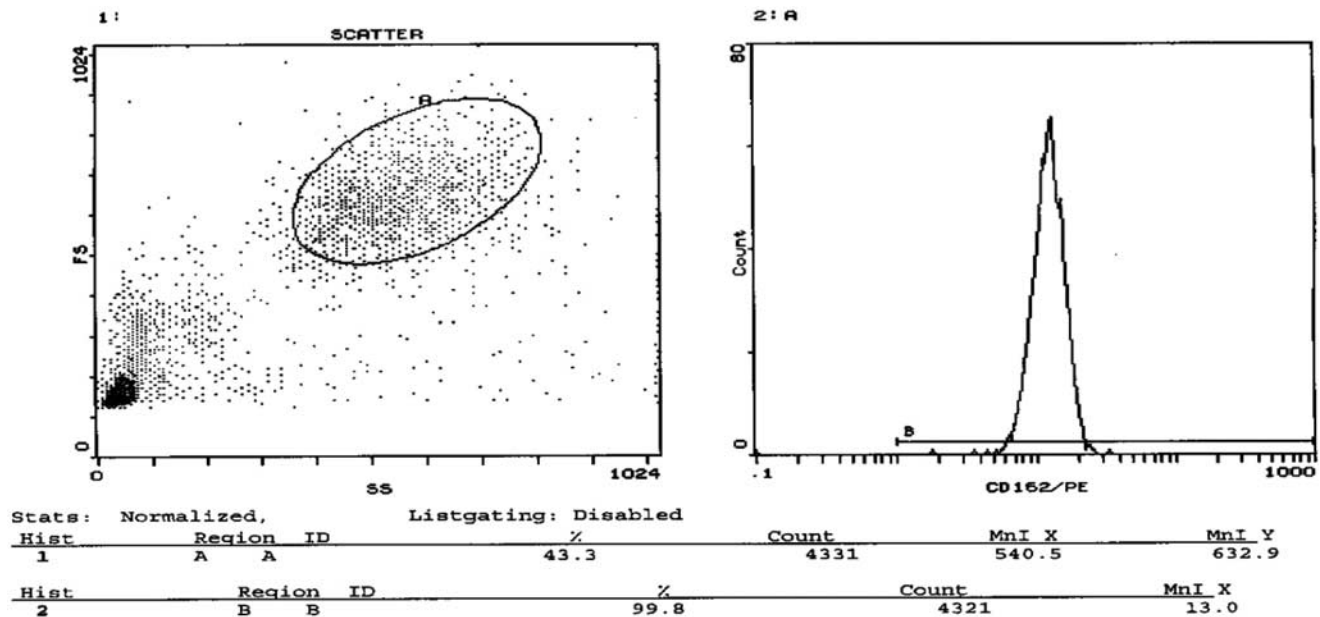


that the tumor microenvironment, which is largely governed by inflammatory cells, is a major participant in neoplastic progression, promoting proliferation, survival, and migration [22]. The selectin–selectin ligand axis is actively involved in tumor progression and drives this process. The involvement of selectins and their ligands in tumor progression takes place at three levels: interaction of tumor cells with platelets and leukocytes, resulting in the

formation of circulating emboli; interaction of tumor cells with endothelial cells leading to extravasation of the tumor cells; and utilization of reciprocal promalignancy signals delivered by the selectins or by their ligands to interacting cells that express the corresponding coreceptor [3].

In the present study, the platelet count was high in patients with bladder cancer as compared with controls.

Figure 6



Flow chart showing the neutrophil expression of CD162 (%) from the same patient in group III.

Table 3 Stepwise multiple linear regression analysis

Variables	β	95% CI	T	P
Platelet count	0.253	0.001–0.020	2.373	0.025 ^a
sP-selectin	0.494	0.031–0.468	2.350	0.027 ^a

CI, confidence interval.

^a $R=0.868$; $R^2=0.753$; $SE=2.031$.

Moreover, the platelet count was significantly higher in patients with invasive tumors with distant metastasis as compared with those with a locally invasive tumor and invasive tumors with regional lymph node involvement.

This is in agreement with Sierko and Wojtukiewicz [23], who reported that thrombocytosis is frequently observed in cancer patients, although the mechanisms underlying thrombocytosis have not been fully elucidated as yet. It has been reported that tumor-derived factors with thrombopoietin (TPO)-like activity, growth factors, platelet-derived microparticles, factors released from bone marrow endothelial cells, and growth factors secreted by megakaryocytes play a role in this process and the consequent activation of platelets [12]. Although TPO, through signaling through its cognate receptor, is a key cytokine involved in the regulation of megakaryocyte differentiation leading to platelet production [24–26], the authors have reported that a high platelet count, observed in cancer patients, was found not to be correlated with TPO levels.

We showed enhanced platelet activation in patients with bladder cancer as evidenced by elevated platelet P-selectin expression. Enhanced platelet P-selectin expression was more pronounced in patients in groups II and III. This finding is in agreement with Polek *et al.* [6], who

suggested that elevated P-selectin expression may be a good marker of some types of carcinoma, such as neoplastic pulmonary diseases, breast, renal, colon cancer, and blood cancer. Recently, Starlinger *et al.* [27] have reported that in comparison with standard platelet markers, thrombospondin-1 is a sensitive and stable parameter suitable for monitoring in-vitro platelet activation. Using a specific equation, they proved that cancer patients show increased in-vivo activation of platelets as evidenced by a significantly decreased ratio of thrombospondin-1 variants in comparison with healthy volunteers.

In addition, it was reported that surgical procedures in the treatment of cancer did not completely eliminate the factors responsible for platelet activation and did not normalize it [28]. Moreover, the rapid expression of P-selectin on the surface of activated platelets has been strongly implicated in the progression and metastatic spread of malignancies [29,30]. Accordingly, the authors concluded that this could provide evidence for the role of P-selectin in cancer progression and distant metastasis.

During platelet activation, P-selectin is translocated onto the external platelet membrane. Surface exposure of P-selectin is temporary and the molecule undergoes endocytosis or shedding to the circulation, where it appears as soluble P-selectin [25]. The present study showed a significantly high sP-selectin level in different patient groups, especially in patients with lymph node involvement and distant metastasis. Abnormal platelet activation, together with elevated platelet activation markers including sP-selectin, is frequent in patients with bladder cancer and has been reported by many authors [12,31,32].

Elevated serum level of sP-selectin may occur in patients with non-Hodgkin's lymphoma and Hodgkin's disease [33]. The authors reported that alterations in the expression and function of this adhesion molecule may play an important role in the progression of lymphomas. The abnormally high plasma level of sP-selectin may be related to metastasis, which might be used as a therapeutic indicator in patients with nasopharyngeal carcinoma [34]. It was suggested that patients with cancer have a higher level of sP-selectin compared with normal individuals. This clearly indicates that P-selectin can mediate tumor cell interaction with platelets, leukocytes, and endothelium [35]. However, this was in contrast to the results obtained by other authors [32]. Although they found that the serum levels of sP-selectin were significantly higher in all patients with bladder cancer in comparison with the controls, their levels did not differ in terms of tumor stage and grade. However, this disagreement may be attributed to the difference in geographical locations, which may affect the risk and predisposing factors for bladder cancer such as bilharziasis in Egypt.

The current study showed that the neutrophil surface expression of PSGL-1 was neither increased nor decreased in the patients studied as compared with the control group. There was no statistically significant difference in its expression between all the patient groups studied. PSGL-1 (CD162) is a counter-receptor for P-selectin and possibly for the other selectins [36], as normal mature neutrophil granulocytes express 26500 ± 4500 copies of PSGL-1 on their surface [37]. As we have shown, the increase in the platelet count (although not significant in all groups), together with the increase in the platelet surface expression of P-selectin, and also the increase in its soluble form in the sera of bladder cancer patients, especially those with distant metastasis, would eventually augment the interaction of this adhesive molecule and its ligand PSGL-1 on neutrophils.

Recently, it has been reported that circulating platelets and leukocytes interact productively through P-selectin and its ligand PSGL-1, and the formation of heterotypic aggregates is a feature of systemic inflammatory, thrombotic, and neoplastic diseases [38]. Hence, the association between thrombosis and cancer has been well established in metastatic patients. In addition, tumor progression is associated with the activation of coagulation and fibrin formation, both of which are implicated in cancer proliferation and metastasis dissemination [39].

Stepwise multiple regression analysis showed that both sP-selectin and platelet count are significant independent determinants for the stage of bladder cancer. Accordingly, sP-selectin and platelet count are independent risk factors for the prognosis of patients with bladder cancer and could be useful tools to predict and control the progression of bladder tumors.

Conclusion

It can be concluded that disease progression in patients with bladder cancer is dependent on the complex interaction between the tumor and the host inflammatory

response through platelet–neutrophil interaction through these adhesion molecules. Migration of cancer cells to the vasculature and the formation of tumor–platelet aggregates facilitate immune evasion. The molecules involved in this interaction would exclusively serve as a unique approach for targeted tumour therapy and metastasis prevention.

Acknowledgements

Conflicts of interest

There are no conflicts of interest.

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