Platelets increase the proliferation of ovarian cancer cells

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ABSTRACT

Platelets promote metastasis and angiogenesis but their effect on tumor cell growth is uncertain. Here we report a direct proliferative effect of platelets on cancer cells both in vitro and in vivo. Incubation of platelets with ovarian cancer cells from murine (ID8 and 2C6) or human (SKOV3 and OVCAR5) origin increased cell proliferation. The proliferative effect of platelets was not dependent on direct contact with cancer cells, and preincubation of platelets with blocking antibodies against platelet adhesion molecules did not alter their effect on cancer cells. The proliferative effect of platelets was reduced by fixing platelets with paraformaldehyde, preincubating platelets with a TGFβ1-blocking antibody, or reducing expression of TGFβR1 receptor on cancer cells with siRNA. Infusing platelets into mice with orthotopic ovarian tumors significantly increased the proliferation indices in these tumors. Ovarian cancer patients with thrombocytosis had higher tumor proliferation indices compared to patients with normal platelet counts.
INTRODUCTION

The association between malignant tumors and elevated platelet or counts raises the possibility of a pathophysiological interaction between platelets and cancer cells. Cancer cells activate and aggregate platelets \(^1\)-\(^3\). Conversely, platelets promote metastasis by protecting cancer cells against natural killer (NK) cells in the blood \(^4\), by enhancing attachment of cancer cells to the blood vessel wall \(^5\), by disrupting endothelial junctions \(^6\), and by promoting angiogenesis through selective sequestration, transporting, and releasing of several growth factors \(^7\)-\(^{11}\). In murine models of metastasis, a deficiency in platelet adhesion molecules, such as P-selectin, GP Ib\(\alpha\), or GP IIIa, reduces the number of metastatic lesions \(^{12\text{-}15}\). Recently Labelle et al. showed that TGF\(\beta\)1 secreted from platelets enhances an epithelial to mesenchymal transition in cancer cells promoting metastasis via activation of TGF\(\beta\)/Smad and NF-\(\kappa\)B pathways \(^{16}\).

We have recently shown that reducing platelet counts decreased the size and number of tumor nodules in a murine model of orthotopic ovarian cancer \(^{17}\). In the same study, we detected extravasation of platelets from tumor vasculatures and direct contact between platelets and cancer cells. In the current study, we investigated whether platelets directly affect tumor growth.

METHODS

Coincubation of platelets with ovarian cancer cells: Gel-filtered murine or human platelets were isolated from whole blood \(^{17}\). Twenty million resting or lysed platelets (prepared by three cycles of freeze-and-thaw) were incubated with cancer cells. In some experiments, platelets
were isolated from C57BL/6 mice with syngeneic tumors induced 3-4 weeks after intraperitoneal injection of 1 X 10^6 ID8 murine ovarian cancer cells \(^1\).  

Fifty thousand murine ID8 or 2C6 and human SKOV3 or OVCA5 ovarian cancer cells were incubated with serum free media overnight. In some experiments, cancer cells were transfected with TGF\(\beta\)R1 siRNA using 2 \(\mu\)g siRNA and 3 \(\mu\)l of lipofectamine reagent (Life Technologies). About 20 x 10^6 platelets were added to each well and incubated for 24 hrs at 37\(^\circ\)C. Murine cancer cells were incubated with murine platelets and human cancer cells with human platelets. In some experiments, different concentrations of blocking antibodies to GP Ib\(\alpha\) (Xia-B2, Emfret Analytics), P-selectin (RB40.34, BD Biosciences), TGF\(\beta\)1 (N1C2, Gene Tex Inc.); or Eptifibatide (0.5 \(\mu\)M, Merck); or aspirin (15 \(\mu\)g/ml, Sigma-Aldrich) were added to platelets prior to incubation with cancer cells. In control samples appropriate buffer was added to the cells instead of platelets. To differentiate between the effect of direct contact between platelets and cells from an indirect paracrine effect, we seeded platelets either on cancer cells or on porous membranes (0.4 \(\mu\)m, PET membrane BD Biosciences) separated from cells in a co-culture system. To determine cell proliferation, we measured incorporation of fluorescence-conjugated EdU (5-ethynil-2'-deoxyuridine) to newly synthesized DNA according to the manufacturer's protocol (EdU-Click-it system, Invitrogen) using flow cytometry (EPICS® XL 4-Color Cytometer, Beckman Coulter Inc. USA).  

**Ki67 immunostaining.** All the studies conducted on human subjects and mice were according to the protocols approved by the Institutional Review Board and Institutional Animal Care and Use Committee of the University of Texas M.D. Anderson Cancer Center. These studies were conducted in accordance with the Declaration of Helsinki. Ovarian cancer tissue specimens collected from 10 patients with thrombocytosis (platelet counts > 450.000/\(\mu\)l) and 10 patients
with normal platelets counts (< 450,000/μl) were immunostained with polyclonal Ki67 antibody (1:200, Thermo Scientific) and counterstained with Hematoxylin.

To study the effect of platelets on proliferation of ovarian cancer cells in orthotopic murine model of ovarian cancer, we injected 1 x 10^6 SKOV3 cancer cells into the peritoneum of 20 NU/NU nude mice (Jackson Laboratory). Ten of these mice received 6 x 10^8 platelets through the tail vein once a week for four weeks starting at the day of inoculation of cancer cells. The other ten mice received buffer infusion with the same frequency. At the end of the four weeks, mice were sacrificed, their tumor resected and immunostained with Ki67 antibody as was described above.

Statistics: Data throughout the manuscript are presented as mean ± SD. Comparisons were made using Student’s t-test with p < 0.05 was considered to be statistically significant.

RESULTS and DISCUSSION

Proliferation rate of human and murine ovarian cancer cells increased significantly after coinubation with platelets (Figure 1A). This effect was platelet-specific, and red blood cells did not alter the proliferation rate (data not shown). Direct contact between platelets and cancer cells or an intact structure of platelets was not required for the proliferative response (Figure 1B). Furthermore, platelets isolated from tumor-bearing mice were similar to platelets from control mice in their proliferative effect (Figure 1B).

Blocking platelet adhesive surface proteins (GP Ibα, GPIIbβ3, and P-selectin) did not diminish proliferative effect of platelets (Figure 1C) and aspirin only partially inhibited it (Figure 1D). This effect of aspirin might be due its role in reducing platelet secretion or inhibiting COX-2 in cancer cells. Fixation of platelets with paraformaldehyde (2%) completely eliminated the
enhancing effect of platelets on proliferation of cancer cells (Figure 1D). The observation that the proliferative effect of platelets did not require direct contact with cancer cells and was not inhibited by blocking adhesion receptors led us to hypothesize that platelet effect on cancer cells mediated by their release of a growth factor.

Platelets are the main source of TGFβ1 in serum. In view of a recent study attributing much of the pro-metastatic effect of platelets to TGFβ1, we examined the effect of blocking antibody to TGFβ1, and identified a dose-dependent inhibition of the proliferative effect of platelets on ovarian cancer cells by blocking TGF β1 (Figure 1C-D). TGFβR1 is the signaling component of the TGFβ1 receptor complex and knockdown of TGFβR1 would be expected to reduce tumor cell responses to TGFβ1. Therefore, to confirm results obtained with the TGF β1-blocking antibody, we reduced expression of TGFβR1 receptors on ovarian cancer cells using siRNA prior to their incubation with platelets (Figure 1E). Consistent with results observed following treatment with the TGFβ1-blocking antibody, TGFβR1 siRNA reduced proliferation of ovarian cancer cells exposed to platelets (Figure 1F).

To study the effect of platelets in vivo, we injected syngeneic platelets to mice with orthotopic ovarian cancer on a weekly basis for four weeks, and measured the proliferation index in the resected tumors using Ki67 staining. We found that mice receiving platelet infusion had a significantly higher percentage of Ki67 positivity compared to control tumor-bearing mice (83.5% vs 66.3%, respectively; \( p<0.0005 \)) (Figure 2A). To extend these findings to human disease, we next measured proliferation indices in 20 tumor specimens collected from patients with ovarian cancer. Ten of these patients had elevated platelet counts with an average count of 635 (X 10³/µl) and a range of 480-844 (X 10³/µl), and 10 had normal platelet counts with an average of 284 (X 10³/µl) and a range of 232-391 (X 10³/µl). Thrombocytosis was associated
with a higher percentage of Ki67 positivity in tumors (68% vs. 57%, respectively; \( p=0.03 \)) (Figure 2B).

In summary, we observed that platelets increase the proliferation of ovarian cancer cells in vitro and in vivo. The in vitro effect was not mediated by platelet GP Ib\( \alpha \), GP IIbIIIa, or P-selectin; was partially dependent on platelet signaling through COX-1; and was dependent on released TGF\( \beta 1 \) binding to tumor cell TGF\( \beta 1 \)R. These results corroborate that platelet-derived TGF\( \beta 1 \) is important for platelet-tumor cell cross-talk \(^{16}\) and extend our understanding of mechanisms by which platelets contribute to tumor progression.

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AUTHORSHIP AND CONFLICT OF INTEREST

M.S.C. designed and performed experiments, analyzed and interpreted data; J. B-M. designed and performed experiments, analyzed and interpret data; H.G.V. performed experiments; R.S. performed experiments; B.Z. performed experiments; M.K. interpreted data, A.K.S. designed experiments and interpreted data; V.A-K. designed experiments, interpreted data, wrote the paper.

Authors declare no conflict of interest.
REFERENCES


FIGURE LEGEND

Figure 1. Proliferative effect of platelet on ovarian cancer cells in vitro. (A) After incubation with platelets, the proliferation rate of murine (ID8 and 2C6) and human (SKOV3 and OVCAR5) ovarian cancer cells was assessed by measuring Edu incorporation. Results are normalized to Edu positivity among buffer-exposed cells. (B) Effect of manipulating platelets on proliferative response of cancer cells was investigated by either directly incubating platelets on ID8 cells (direct) or separating them using a porous membrane (indirect). Lysed platelets were prepared by repeated applications of freeze and thaw and sonication. Platelets from tumor-bearing mice were isolated from moribund C57BL/6 female mice (n=3) by IVC venipuncture 3-4 weeks after inoculation of ID8 cells. (C) Blocking antibodies against GP Ibα (5 μg/ml), P-selectin (4 μg/ml), TGFβ1 (5 μg/ml), or GPIIb-IIIa blocking agent Eptifibatide (0.5 μM) or aspirin (15 μg/ml) was added to platelets before incubation with cancer cells, and after 24 hrs Edu incorporation was measured. Results were normalized to cancer cells incubated with buffer alone. (D) Increasing concentrations of TGFβ1 blocking antibody and aspirin were used for blocking platelet-induced cancer cell proliferation. All results are compared to the second bar. (E) Knockdown of TGFβR1 gene expression after transfection of SKOV3 human ovarian cancer cells with siRNA/liposome mixture (2 μg siRNA and 3 μl lipofectamine) was investigated by measuring protein and mRNA products, using Western-blotting with anti-TGFβR1 antibody (above) and real-time RT-PCR (below), respectively. (F) ID8 cells were transfected with either TGFβR1 siRNA or scrambled siRNA before incubation with platelets. All results are compared to the second bar. The reduction in the proliferative response induced by scrambled siRNA was not statistically significant (p>0.09).

Each experiment was repeated at least 3 times in triplicates. The cumulative results are summarized as bargraphs. For all statistical analyses t-test was used (* p<0.05 and ** p<0.01).
Figure 2. Proliferative effect of platelet on ovarian cancer cells in vivo. The proliferation index was measured in (A) tumors resected from tumor-bearing mice infused with buffer (control) (n=10) or platelets (n=10) and (B) surgical specimens from ovarian cancer patients with normal platelet counts (n=10) or thrombocytosis (n=10), by Ki67 immunostaining. From each tissue block five sections were prepared, and from each section five fields were counted at X 200 magnification of a light microscope by 2 different observers as independent blinded assessments. The ratio of positive Ki67 nuclear staining to total number of nuclei was measured for each field. The cumulative results are summarized as bargraphs and a representative Ki67 staining is shown above each column.
Figure 1.

A

![Graph showing relative EdU positivity](image)

B

![Graph showing relative EdU positivity](image)

C

![Graph showing relative EdU positivity](image)

D

![Graph showing relative EdU positivity](image)

E

![Image of Western blot](image)

F

![Graph showing relative EdU positivity](image)
Figure 2.

A

![Image of histological sections showing Ki67 positivity in Control vs. Platelet Infusion samples.]

**Graph:**

- **Y-axis:** Ki67 positivity (%)
- **X-axis:** Control vs. Platelet Infusion
- **Legend:** starred *p = 0.005

B

![Image of histological sections showing Ki67 positivity in Normal Count vs. Thrombocytosis samples.]

**Graph:**

- **Y-axis:** Ki67 positivity (%)
- **X-axis:** Normal Count vs. Thrombocytosis
- **Legend:** starred *p = 0.004

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