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ORIGINAL PAPER

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Platelets promote the adhesion of human hepatoma cells with a highly metastatic potential to extracellular matrix protein: involvement of platelet P-selectin and GP IIb-IIIa

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Abstract *Purpose*: To investigate the role and possible mechanisms of platelets in liver cancer metastasis. *Methods*: The optimum conditions of hepatoma cell adhesion to the extracellular matrix (ECM) were determined. The ability of cells to adhere to the ECM was compared between human hepatoma cell lines with a highly metastatic potential (MHCC97) and human hepatoma cell lines with a lower metastatic potential (SMMC7721). By using adhesion assays and inhibition studies in vitro, the effects of platelets and their specific adhesive molecules were compared via the ability of MHCC97 and SMMC7721 to adhere to ECM protein. *Results*: The SMMC7721 cell adhesion rate to vitronectin, fibronectin, and fibrinogen, respectively, was significantly lower than that of MHCC97 cells (44.9% vs 73.6%, 47.4% vs 76.4%, and 59.3% vs 80.6%, $P < 0.05$). Both hepatoma cell adhesion to the ECM-bound platelets was unchanged whether the platelets were activated or not. SMMC7721 cell adhesion to the ECM was not affected by platelets, but MHCC97 cell adhesion to the ECM was significantly enhanced by platelets ($P < 0.01$). In addition, this effect was significantly reduced when either P-selectin or GP IIb-IIIa was blocked by monoclonal antibodies ($P < 0.05$, $P < 0.01$). In the inhibition studies, the ability of SMMC7721 to adhere to the ECM-bound activated platelets was also lower than that of MHCC97 ($P < 0.05$). However, when GP IIb was blocked by antibody, the adhesion ability of both cells was similar ($P > 0.05$). *Conclusions*: Human hepatoma cells with a highly metastatic potential proved to have a highly adhesive ability. MHCC97 cell adhesion to the ECM was significantly enhanced by platelets. The interaction of MHCC97 cells with the ECM-bound activated platelets may be mediated by platelet P-selectin and GP IIb-IIIa.

Keywords Platelet · Hepatoma cell · Metastasis · Extracellular matrix (ECM) · Adhesion · Adhesive molecule

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Introduction

Tumor metastasis is one of the main factors of malignant tumor death and the poor long-term effect of clinical treatment. Tumor cell spread is a complex, multistep process that depends on a number of cell-to-cell and cell-to-matrix interactions mediated by specific adhesion receptors or ligands between tumor cells and subendothelium. Metastasis is thought to be involved in the formation of tumor-platelet-leukocyte emboli and their interactions with the endothelium of distant organs (Kim et al. 1998). Several studies in vivo have shown inhibition of metastasis by anti-platelet agents inducing thrombocytopenia. The involvement of platelets in tumor metastasis was further demonstrated by several studies which indicated that platelets were able to enhance tumor cell adhesion to endothelial cells as well as to the subendothelial extracellular matrix (ECM) (Dardik et al. 1997).

Several adhesive molecules including selectins, integrins, and cadherins mediate cell adhesion. P-selectin belongs to the selectin family, which is located in the alpha granules of platelets and Weibel-Palade bodies of endothelial cells. Upon platelet or endothelial cell activation by thrombin, alpha granules and Weibel-Palade body membranes fuse rapidly with the plasma membrane as the granules release their contents, leading to the expression of P-selectin on the cell surface. P-selectin is known to be involved in inflammation, wound healing, immune responses, and tumor metastasis (Stone and Wagner 1993).

The platelet GP IIb-IIIa complex ($\alpha_{IIb}\beta_{IIIa}$) is a divalent cation-dependent heterodimer integrin of the glycoproteins IIb and IIIa. Ligand recognition sites reside in the N-terminal portion of both IIb and IIIa subunits. High affinity ligand recognition requires both subunits and consequently may involve multiple ligand contact points. The inactive GP IIb-IIIa complex is present on the surface of resting platelets. After activated with thrombin or ADP, the GP IIb-IIIa complex becomes an active form that can bind to matrix macromolecules, such as fibrinogen, fibronectin, vitronectin, and Von Willebrand factor (Liu et al. 1997; Akiyama et al. 1990; Loftus et al. 1994).

In this study, we focus on the molecular basis of the ability of human hepatoma cell lines – with various metastatic potentials – to adhere to ECM proteins through platelets and the corresponding adhesive molecules. It was found that in a human hepatoma cell line with a highly metastatic potential (MHCC97) adhesion to the ECM was significantly enhanced by platelets only or the ECM-bound activated platelets through platelet P-selectin and GP IIb-IIIa.

Materials and methods

Antibodies

Mouse anti-human P-selectin MAb (CD62P, clone AK4) was purchased from Sigma Chemical. Mouse monoclonal antibody against human platelet glycoprotein IIb (CD41, clone 5B12) and IIIa (CD61, clone Y2/51) was from Dako Chemical.

Cell cultures

A human hepatoma cell line with a lower metastatic potential (SMMC7721) was obtained from Secondary Military Medical University, Shanghai, China. Cells were grown in RPMI-1640 (Gibco, pH 7.2) medium supplemented with newborn calf serum, 1.2 g/l sodium bicarbonate, 5.7 g/l HEPES, 0.11 g/l sodium pyruvic acid, and 0.3 g/l L-glutamine.

A human hepatoma cell line with a highly metastatic potential (MHCC97) was established in our institute (Tian et al. 1999). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco, pH 7.2) supplemented with human AB type serum and 3.7 g/l sodium bicarbonate.

Labeling of tumor cells

Tumor cells ($2–3 \pm 10^6$) were washed with PBS and suspended in 1 ml Ca^{2+}, Mg^{2+} free PBS. Cells were labeled with 5.0 μM Calcein AM (Molecular Probes, USA), at 37 °C for 1 h (MacCoubrey et al. 1990), washed with PBS three times, and resuspended in PBS at a concentration of 1 ± 10^6/ml. Analysis of cell fluorescence intensity was performed on an fmax96 fluorescence microplate reader (Molecular Devices, USA) with Ex 485 nm and Em 538 nm.

Platelet preparation

Platelet-rich plasma (PRP) was prepared according to the reported method (Dardik et al. 1997). The concentration of platelets in PRP was $210–262 \pm 10^9$/l. Activation of platelets in PRP were performed by addition of 0.15 U thrombin/ml (Sigma Chemical) and incubated at 22 °C for 30 min without stirring (Stone and Wagner 1993). The platelets were used in cell adhesion assays within 30 min after preparation.

Preparation of ECM plates

Vitronectin (Takara, Japan), fibronectin and fibrinogen (Sigma Chemical) were diluted with PBS as required, and coated 96-well high-adhesive culture plate in triplicate with 50 μl per well, respectively. Fifty microliters of poly-p-lysine (100 μg/ml) and 1% BSA was coated in triplicate as the maximum or the minimum adhesive control, respectively. Coated plates were incubated at 4 °C for 16 h, and then blocked with 1% BSA at room temperature for 2 h. ECM-coated plates were washed with PBS and stored at 4 °C for further use and the optimum concentrations of ECM proteins were determined.

Cells adhesion to ECM proteins

In time-course experiments, labeled hepatoma cells (diluted with PBS to yield a suspension of 5 ± 10^4 cells in 50 μl) were incubated on ECM plates for varying durations at 37 °C with 5% CO_2. Non-adhesive cells were removed by washing with HEPES solution (10 mM HEPES, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 140 mM NaCl, 1 mM $MnCl_2$, pH 7.5). Adhesive cells were dissolved by 50 μl 0.1% Triton-X100 per well, and cell fluorescence intensity was determined. According to adhesion time curves, the optimum adhesion incubation times were determined.

Various labeled cells (5 ± 10^4 cells/50 μl) were incubated on ECM-coated plates (six wells each) at designated concentrations for a given time at 37 °C with 5% CO_2. Cell-to-ECM adhesion experiments were performed as mentioned above. Adhesive abilities were presented as adhesive rate (%).

Adhesive rate (%) = (Sample fluorescence – minimum fluorescence) / (maximum fluorescence – minimum fluorescence) ± 100%.

Cell adhesion to ECM-bound platelets

Platelet-coated ECM plates were prepared by addition of 50 μl of activated or resting PRP at 22 °C for 45 min, followed by washing with PBS. To test the blockage effects of anti-platelet antibodies, ECM-bound activated platelets were incubated with MAbs (20 μg/ml) at 22 °C for 45 min and washed with PBS before the addition of various tumor cells (Dardik et al. 1997; Stone and Wagner 1993).

Labeled MHCC97 and SMMC7721 cells were incubated in BSA-coated wells, ECM-coated wells, resting platelet-coated wells, activated platelet-coated wells, and MAbs-acting wells, respectively. Adhesive abilities were presented as adhesive fluorescence intensity (AFI).

Statistical analysis

Data are presented as mean ± SD and analyzed with SPSS 9.0 for Windows. Statistical analysis of differences was performed using the paired one-tailed Student's *t*-test.

Results

Labeling of tumor cells

Hepatoma cells were vitally stained with fluorescent dye, Calcein AM. Fluorescent intensity was linearly positively correlated with the tumor cell count, and correlative experiments were performed in linear range.

Tumor cell adhesion to ECM proteins

MHCC97 and SMMC7721 cells adhered to the ECM in a time-dependent and protein-concentration-dependent manner, respectively. At the following concentration of vitronectin (10 μg/ml) and fibronectin (15 μg/ml), the submaximal adhesion of these two cell lines occurred at 2 h. For fibrinogen (15 μg/ml), the submaximal adhesion occurred at 1.5 h. Therefore, further experiments testing the effects of platelets and antibodies on tumor cell adhesion were performed in similar conditions.

The adhesive rate of SMMC7721 cells to vitronectin, fibronectin, and fibrinogen, respectively, was significantly lower than that of MHCC97 cells (44.9% vs 73.6%, 47.4% vs 76.4%, and 59.3% vs 80.6%, $P < 0.05$, Table 1). The results suggest that human hepatoma cells with a highly metastatic potential prove to have highly adhesive ability, which may come from the differential behaviour of cell adhesion molecules (mainly integrins).

Effect of platelets on tumor cell adhesion to the ECM

The AFI of tumor cell adhesion to ECM-bound activated platelets was similar to that of tumor cell adhesion to ECM-bound resting platelets ($P > 0.05$) (Table 2). The AFI of SMMC7721 cell adhesion to the ECM-bound platelets was similar to that of cell adhesion to the ECM ($P > 0.05$). However, the AFI of MHCC97 cell adhesion to the ECM-bound platelets was much stronger than that of simple cell adhesion ($P < 0.01$). These results indicated that SMMC7721 cell adhesion to the ECM was not affected by platelets, but MHCC97 cell adhesion to the ECM was significantly enhanced by platelets.

Table 1. Fluorescent intensity of tumor cell adhesion to ECM-coated surface

Groups	SMMC7721	MHCC97
BSA	0.347 ± 0.106	0.244 ± 0.023
Poly-p-lysine	1.799 ± 0.189	1.985 ± 0.224
Vitronectin	1.00 ± 0.119	1.526 ± 0.277
Adhesive rate (%)*	44.9	73.6
Fibronectin	1.036 ± 0.248	1.575 ± 0.219
Adhesive rate (%)*	47.4	76.4
BSA	0.395 ± 0.114	0.309 ± 0.031
Poly-p-lysine	1.645 ± 0.219	1.964 ± 0.414
Fibrinogen	1.136 ± 0.139	1.644 ± 0.285
Adhesive rate (%)*	59.3	80.6

*$P < 0.05$

As shown in Table 2, platelet P-selectin and GP IIb-IIIa may not be involved in mediating the interaction of SMMC7721 cells with the ECM-bound activated platelets, while the enhancing effect of MHCC97 cell adhesion to the ECM by activated platelets was significantly reduced when P-selectin or GP IIb-IIIa was blocked by monoclonal antibodies ($P < 0.05$, $P < 0.01$). Meanwhile, the adhesive ability of SMMC7721 cells to the ECM or to the ECM-bound platelets was significantly lower than that of MHCC97 cells ($P < 0.05$, $P < 0.01$). When P-selectin or GP IIIa was blocked by monoclonal antibodies, the adhesive ability of SMMC7721 cells to the ECM-bound activated platelets was also lower than that of MHCC97 cells ($P < 0.05$). However, when GP IIb was blocked by antibodies, the adhesion ability of both cells was similar ($P > 0.05$). This suggests that platelet GP IIb may play a more important role in the enhancing effect of activated platelets mediating the interaction of human hepatoma cell lines – with a highly metastatic potential – with the ECM.

Discussion

Several ECM protein receptors belong to a large family of integrins that are involved in cell-cell interaction and/or cell-matrix attachment, performing cell recognition and cell adhesion tasks (Liu et al. 1997). In the process of the malignant transformation of tumor cells, the cell's adhesive ability is commonly changed in relation to two aspects: first, the adhesion of tumor cells to cells and tumor cells to host cells; and second, the adhesion of tumor cells to the ECM. We found that hepatoma cell lines with higher or lower metastatic potentials adhered well to ECM proteins; the adhesive ability of MHCC97 cells to the ECM was significantly more than that of SMMC7721 ($P < 0.05$). However, various adhesive glycoproteins differ in their ability to support tumor cell adhesion, adhesive capacity being: vitronectin < fibronectin < fibrinogen. This is the reason why we chose fibrinogen for the adhesion experiment with platelets.

The arrest of metastasizing tumor cells within the blood stream is a prerequisite for their extravasations. This step is rate-limiting during hematogenous metastasis. The ability of platelets to support tumor cell arrest during blood flow may contribute to the role of platelets in hematogenous metastasis (Felding-Habermann et al.

Table 2. Adhesive fluorescence intensity (AFI) of tumor cell adhesion concerning platelets*. *P* value of interblock: compared between SMMC7721 group and MHCC97 group

Groups	SMMC7721**	MHCC97***	*P* value of interblock
Cell adhesion simply[a]	0.469 ± 0.028	0.578 ± 0.045	< 0.05
Coated resting platelets[b]	0.454 ± 0.062	1.079 ± 0.090	< 0.01
Coated activated platelets[c]	0.511 ± 0.027	1.109 ± 0.109	< 0.01
Blocked with P-selectin MAb[d]	0.558 ± 0.075	0.762 ± 0.127	< 0.05
Blocked with GP IIb MAb[e]	0.606 ± 0.102	0.685 ± 0.221	> 0.05
Blocked with GP IIIa MAb[f]	0.473 ± 0.119	0.746 ± 0.123	< 0.05

*AFI of BSA-coated well SMMC7721: 0.338 ± 0.053, MHHCC97: 0.268 ± 0.010
**Compared in SMMC7721 group: [b] vs [c] $P > 0.05$, [a] vs [b/c] $P > 0.05$, [c] vs [d/e/f] $P > 0.05$
***Compared in MHCC97 group: [b] vs [c] $P > 0.05$, [a] vs [b/c] $P < 0.01$, [c] vs [d/e] $P < 0.05$, [c] vs [f] $P < 0.01$

1996; Yu et al. 2000). Platelets directly promote all the midway steps of hematogenous tumor cell metastasis (Honn et al. 1992). In cancer patients, especially in the advanced stage, the expression of the adhesion molecules on platelets and their released products increase (Zhang et al. 1998). It was reported that melanoma cell adhesion to the ECM was enhanced by platelets, regardless of their activation state (Dardik et al. 1997). In this study, we observed that both SMMC7721 and MHCC97 cell adhesion to the ECM-bound platelets was unchanged whether platelets were activated or not. This fact may be partly explained by the activation of several adhesion molecules such as GP IIb-IIIa accomplished by the ligands themselves (Hynes 1992) or by the ECM protein.

In the melanoma cell adhesion to vascular endothelial cells (ECs) experiment, the interaction between activated platelets and ECs involved both P-selectin and GP IIb-IIIa (Dardik et al. 1998). P-selectin-deficient mice showed a significantly slower growth of subcutaneously implanted human colon carcinoma cells and generated fewer lung metastases from intravenously injected cells. It was demonstrated that intravenously injected tumor cells home into the lungs of P-selectin-deficient mice at a lower rate. Tumor cells lodged in lung vasculature after intravenous injection often are decorated with platelet clumps, and these are markedly diminished in P-selectin-deficient animals (Kim et al. 1998). Similarly, monoclonal antibodies against P-selectin strongly inhibited tumor metastasis in an animal model of human stomach carcinoma cells by generated in ascites, and the expression of plasma P-selectin in metastatic nude mouse was significantly higher than that of non-metastatic mouse (Chen et al. 1998).

It was reported that the interaction of melanoma cells and KB carcinoma cells with ECM-bound platelets is mediated by platelet GP IIb-IIIa and by tumor cellα_v integrins independently of the nature of the β subunit. Platelets of a Glanzmann thrombastenia (GT) patient lacking GP IIb-IIIa were unable to support tumor cell adhesion to the ECM (Dardik et al. 1997). A study has showed that thrombin, an activating factor of platelets, is capable of enhancing in vitro human colon adenocarcinoma cell metastatic potential in terms of adhesive properties and migratory response. The effect of thrombin resulted in an up-regulated cell-surface expression of β_3 integrins. Antibodies against β_3 integrins effectively blocked both the enhanced adhesion and migration (Chiang et al. 1996). Other researchers found that acquired β_3 integrin expression emerged during melanoma progression (Danen et al. 1994). Further research has shown that melanoma cell interaction with the matrix or blood cells and platelets associated with adherent microthrombi, and this resulted in stable melanoma cell arrest. Platelet-mediated melanoma cell arrest largely depended on the presence of plasma proteins, which were required for the formation of large thrombi. Analyzing the positions of arrested melanoma cells revealed that 93% or more of the attached tumor cells were associated with platelet-containing thrombi. Platelet-dependent arrest of tumor cells was blocked by platelet inhibition through treatment with PGE1 or anti-$\alpha_{IIb}\beta_3$ MAb. These results indicated that β_3 integrin expression by tumor cells supported their interaction with platelets and tumor cell arrest (Felding-Habermann et al. 1996).

In this study, we found that platelets enhanced the ability of a hepatoma cell line with a highly metastatic potential to adhere to the ECM, but not a hepatoma cell line with a lower metastatic potential, and this effect can be reduced by a decreased content of activated platelets (date not shown). Our results may imply that the change in the adhesive abilities of various hepatoma cells was really related to the biological characteristics of the tumor itself, and also related to platelets. For tumor cells with high metastatic speciality, platelets may be capable of promoting the adhesive properties of tumor cells and migratory response by tumor cells passive arrested and the enhanced effect of tumor cell adhesion to the ECM. Platelet P-selectin and GP IIb-IIIa may play an important role in mediating adhesion. The present study provides a new look at the mechanism of liver cancer emboli formation. It may be a new anti-tumor metastasis treatment target.

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