Lysis of Tumor Cells by Natural Killer Cells in Mice Is Impeded by Platelets

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ABSTRACT

Natural killer (NK) cells provide effective antitumoral activity in the blood stream of mice, leading to reduced metastasis. There are, however, tumor cells that metastasize despite the presence of an intact NK system. The capability of tumor cells to induce platelet aggregation, on the other hand, correlates with their enhanced metastatic potential. A counteractive role of platelets for the NK function in metastasis has never been conceived. Here we demonstrate for the first time that platelets directly protect tumor cells from NK lysis in vitro as well as in vivo. Using three different tumor cell lines in a mouse model of experimental metastasis, tumor seeding in the target organs was reduced when the host was platelet depleted, but only if the tumor cells were NK sensitive. Aggregation of platelets around tumor cells also inhibited in vitro NK tumorlytic activity. This protection of tumor cells by platelets was mouse strain independent and was equally observed with platelets from µg-microglobulin-deficient mice, excluding a NK inhibitory function of MHC class I on platelets. Thus, even if tumor cells are NK susceptible and cytotoxic NK cells threaten their survival in the blood, platelets are capable of protecting them from cytolysis, thereby promoting metastasis. Surface shielding by platelet aggregates seems to be the main mechanism of this protection.

INTRODUCTION

Metastasis is a complex phenomenon and involves a series of cellular interactions in which tumor cells disconnect from the primary tumor tissue, intravasate, attach to the vessel wall, and perform diapedesis to push through the endothelial layer and basement membrane. Metastasis can be regarded as a series of events in which at each step certain tumor cells with specific properties are selected (1). During the hematogenous phase of metastasis when the tumor cells are in the blood, they undergo a variety of cell-cell interactions, some of which are detrimental to their survival. This phase is particularly critical because in this hostile environment, the tumor cells are directly confronted with effectors of the host immune response. The vast majority of circulating tumor cells do not survive this stage (2, 3). NK3 cells provide the most effective antitumor cell activity in the blood stream, at least in mice. Metastasis is more pronounced in mice that have NK cells with an impaired lytic activity due to a genetic defect (4). Also, depletion of NK cells results in strongly increased metastasis (5, 6). This has been demonstrated in many different tumor models and models of experimental as well as spontaneous metastasis (5–7). The exact mechanisms by which NK cells recognize and destroy tumor cells are not completely understood, but it is clear that both processes require direct contact with the target cell (8). It is not known how some tumor cells manage to avoid contact with NK cells to escape their cytolytic action.

Despite the impressive antitumetic effect of NK cells, there is still a population of tumor cells that escapes the host immune response and completes the metastatic cascade. Apparently, the tumor cells can make use of host mechanisms for protection during the hematogenous phase of metastasis. It is still unclear which tumor cell properties determine survival in the blood. Activation of platelets by tumor cells is considered to be an important step (1). It has been reported that the capability of mouse as well as human tumor cells to induce platelet aggregation in vitro correlates positively with their metastatic potential in vivo (9). This finding led to the assumption that platelets provide support for tumor cells to adhere to and extravasate through the endothelium and the subendothelial matrix. Interference with any step of tumor cell-platelet interaction always resulted in diminished metastasis in experimental settings. The integrins gpIIb/IIIa and gpIIb on the platelet membrane have been found to be relevant interaction molecules for tumor cells to induce platelet aggregation (10). It has also been demonstrated that platelet depletion with an anti-platelet serum or inhibition of β3-integrins with competing peptides drastically decreased the number of fibrosarcoma cell colonies in the lung (11–13). Despite many in vivo investigations demonstrating the importance of platelets for metastasis and different proposed mechanisms for platelet action based on in vitro studies, the critical platelet-dependent step in the metastatic cascade has not yet been identified.

The aim of the present study was to analyze the role of NK cells and platelets in the hematogenous phase of metastasis. Here we demonstrate for the first time that platelets directly protect tumor cells from NK cell lysis in vitro as well as in vivo. In the model of experimental metastasis using NK-sensitive and NK-resistant tumor cells, platelets were only required for successful metastasis when the tumor cells were NK susceptible. No contribution of platelets to tumor cell extravasation other than preventing tumor cell destruction by NK cells was evident. Platelets inhibited NK tumor cell lysis by aggregating around the tumor cells. Because protection is dependent on the number of platelets and their aggregation but independent of MHC class I molecules, surface shielding is proposed as the main protective mechanism.

MATERIALS AND METHODS

Animals. Specific pathogen-free female C3H/HeN, DBA/2, and C57Bl/6 mice 5–7 weeks of age were obtained from Charles River (Sulzfeld, Germany). The β2-microglobulin-deficient mice (14) were kindly provided by I. Autenrieth (LMU, Pettenkofer-Institut, Munich, Germany) and B. Kyewski (Deutsches Krebsforschungszentrum, Heidelberg, Germany). Tumor Cells. CFS1/LacZ is a lacZ gene-transduced methylcholanthrene-induced fibrosarcoma cell line of C3H/HeN mouse origin (15). ESb-L.NA-10s, a lacZ gene-transduced ESb cell line derived from the highly metastatic subline of the methylcholanthrene-induced DBA/2 lymphoma L5178YE (Eb; 11), was kindly provided by V. Schirrmacher (Deutsches Krebsforschungszentrum, Heidelberg, Germany). B16F10 melanoma cells derived from C57Bl/6 mice were kindly provided by A. Bosserhoff (Institut für Pathologie, Universität Regensburg, Regensburg, Germany). As target cells for the in vitro NK cytotoxicity assay, YAC1 Moloney virus-induced lymphoma cells were used. All cells were cultured in RPMI 1640 supplemented with 10% heat-inactivated FCS, 100 IU/ml penicillin, and 0.1 mg/ml streptomycin (all from Life Technologies, Inc., Eggenstein, Germany) in a humidified atmosphere containing 5% CO2 at 37°C. YAC1 cell culture medium also contained 50 µM β-mercaptoethanol (Merck, Darmstadt, Germany).

Reagents. The monoclonal rat-anti-mouse interleukin 2 receptor β chain IgG2b-secreting hybridoma (TM-β1) was a generous gift of T. Tanaka (Bio-
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Medical Research Center, Osaka University Medical School, Japan). The antibodies were used as described (16). Polyclonal rat IgG (Sigma, Deisenhofen, Germany) was used as control antibody. Polyclonal rabbit anti-mouse platelet serum was generated as described (12), and 100 μl were injected i.p. per mouse for induction of thoracotomy. The anti-mouse platelet MAb MWR30 (rat IgG1) was generated by fusion of mouse myeloma cells (X63.Ag8.653) with spleen cells from a rat immunized with purified mouse platelets (11). The antigen recognized by MWR30 is the fibrinogen receptor (gpIIb/IIIa, CD41) as shown by sequencing of the precipitated material. Affinity-purified MWR30 was labeled with FITC (Sigma) at a FITC:protein ratio of 3:1 or with phycoerythrin (Sigma) at a phycoerythrin:protein ratio of 2:1 by standard methods and separated by gel filtration on a PD-10 column (Pharmacia). Hirudin (Serva, Heidelberg, Germany), apyrase grade III (Sigma), high molecular weight heparin (Sigma), and antibodies to P-selectin (Pharmingen, Hamburg, Germany) were purchased.

Experimental Metastasis. CFS1/lacZ tumor cells after detachment with a trypsin (0.05%)-EDTA (0.02%) solution (Life Technologies, Inc.) and ESb-L.NA-10s tumor cells were washed twice. CFS1/lacZ (1 × 10^6) or ESb-L.NA-10s cells (2 × 10^6) in 0.2 ml medium were injected into the lateral tail vein of mice. B16F10 cells (1 × 10^6) were injected in 0.2 ml medium culture medium retroorbitally. Because the number of lung colonies formed in NK cell-depleted mice, the number of CFS1/lacZ tumor cells was reduced as indicated in the figure legends to obtain evaluable numbers of metastases. Animals were killed by cervical dislocation 3 days after CFS1/lacZ or ESb-L.NA-10s tumor cell inoculation. To quantitate micrometastases, curesections of the lungs from mice injected with CFS1/lacZ cells or of the livers from mice injected with ESb-L.NA-10s cells were fixed in formaldehyde (2%)/glutaralde- dehyde (0.2%); Merck) in PBS for 10 min at 4°C. After washing with PBS, the sections were incubated in X-gal staining solution consisting of X-gal (1 mg/ml; AppliChem, Darmstadt, Germany; stock solution of 40 mg of X-gal dissolved in 1 ml of N,N-dimethylformamide (Merck)) diluted in 5 mM K4Fe(CN)6, 5 mM K3Fe(CN)6, 3.5% CH3OH, 2 mM MgCl2, 0.01% (v/v) sodium deoxycholate, and 0.02% (v/v) NP40 (all from Sigma) in PBS for 3–12 h at 37°C. The organs were rinsed with PBS and stained with eosin. Tumor colonies were determined by counting the metastatic foci microscopically using a measuring grid. Tumor colonies in at least six different sections per organ were counted. The results are given as mean ± SD colonies/mm^2. To determine the number of surface metastases after B16F10 injection, the mice were killed on day 10, lungs and livers were removed and fixed in 4% PBS-buffered formaldehyde, and surface metastases were counted.

To calculate the total number of CFS1/lacZ micrometastases per lung, the lung volume was determined by water displacement. The area of 1 mm^2 of a section of 50 μm thickness represents a volume of 0.05 mm^3. On the basis of this assumption, a calculation of tumor foci for the whole organ was performed because CFS1/lacZ cells colonize uniformly in all regions of the lung.

NK Cytotoxicity Assay. Cytotoxicity activity was tested in a standard 4-h ^51Cr-release assay. Briefly, splenic effector cells were cultured with YAC-1 target cells labeled with ^51Cr (NEN DuPont, Bad Homburg, Germany) at E:T ratios of 100:1, 50:1, and 25:1 for 90 min in quadruplicates for 4 h at 37°C. Label release into the supernatant was used to calculate the cytotoxicity in % = (a – b)/(c - b) × 100, where a is cpm released in the test well, b is spontaneous release from target cells without addition of effector cells, and c is total release. For supernatant transfer experiments, 2-h supernatants from platelets or platelets cocultured with YAC1 cells at a ratio of 10,000 platelets per YAC1 cell were transferred to the NK cytotoxicity assay without any further dilution. Spontaneous release was always <10%.

Platelet Preparation. Mice were bled retroorbitally under ether anesthesia, and blood of 10 mice was pooled into a tube containing 0.5 ml 0.1 M sodium citrate. Platelet-rich plasma was obtained by centrifugation at 300 × g for 10 min at room temperature. The platelets were packed by centrifugation at 1300 × g for 10 min, washed three times with culture medium, and used immediately. Isolated platelets did not show signs of activation or degranulation as tested by fluorescence-activated cell sorter analysis. To determine platelet numbers in mice, blood (20 μl) was obtained from the retroorbital plexus using siliconized microcapillaries and immediately diluted 1:100 in Unopette kits (Becton Dickinson, Heidelberg, Germany). The diluted blood sample was allowed to settle for 20 min in an improved Neubauer hemocytometer, and platelets were counted under phase contrast at ×400.

Flow Cytometry. Tumor cells were harvested as described above, counted, resuspended in culture medium to a final concentration of 10^6 cells/ml, and coincubated with platelets in different ratios for 30 min at 37°C. The cells were subsequently stained with MAb MWR30-FITC or control rat IgG-FITC at a final concentration of 5 μg/ml for 20 min on ice and analyzed on a FACSscan (Becton Dickinson; 11). Cells were gated by forward/side scatter characteristics.

Statistics. If not indicated otherwise in the figure legends, results are expressed as mean values ± SD for five mice per group.

RESULTS

Effect of NK Cell Depletion on Metastasis. The role of NK cells was analyzed in a model of experimental metastasis by injecting syngeneic tumor cells i.v. and quantitating tumor colonies in the target organ. NK cell-depletion was achieved by injection of MAb to the interleukin 2 receptor β chain (TM-β1; 16). This treatment has been shown to selectively and completely eliminate splenic NK functions of mice without affecting T-cell functions. Depletion of NK cells, as evidenced by the absence of any NK lytic activity in the spleen of antibody-treated mice (data not shown), resulted in strongly increased numbers of CFS1 lung colonies compared with control animals (Fig. 1). This effect was so strong that to distinguish between individual metastatic foci in the NK-depleted mice, the number of inoculated CFS1 tumor cells was reduced from 1 × 10^6 to 1 × 10^5 per animal. Similar results were obtained when lung colonization by B16 melanoma cells was compared in normal versus NK-depleted mice (Fig. 1). Not only increased numbers of B16 colonies were detected in the lungs of NK-depleted mice, but the livers were also filled with metastases. This is in clear contrast to the metastatic behavior of B16 cells in normal mice, where no liver metastasis is observed. Colony formation of the NK-resistant ESb thymoma cells in the liver was not affected by NK cell depletion (Fig. 1).

Interaction of Platelets with Tumor Cells in Vitro. The role of platelets in metastasis was investigated to find out how some tumor cells avoid being killed by NK cells. CFS1, B16, and ESb tumor cells were tested in vitro for their potential to interact with platelets. Tumor cells were coincubated with freshly isolated platelets, and adhesion of platelets to the tumor cell membrane was determined by flow cytometry. Bound platelets were stained with MWR30, a fluoresceinated mouse platelet/megagaryocyte-specific rat MAb directed against the fibrinogen receptor (gpIIb/IIIa, CD41; 11). Single cells were gated by
forward/side scatter characteristics, which were not altered significantly by bound platelets. CFS1 and B16 cells in contrast to ESb cells strongly induced platelet aggregation, leading to deposition of platelet aggregates on their surface (Fig. 2A). The intensity of the platelet-specific marker signal associated with the tumor cells was dependent on the number of added platelets (data not shown). Microscopic inspection revealed individual tumor cells covered with platelets (Fig. 2B) and, in addition, large aggregates of tumor cells and platelets that had been excluded in the flow cytometric analysis. These data showed that CFS1 and B16 cells strongly activated platelets and induced their aggregation, whereas ESb cells did not.

Effect of Platelet Depletion on Metastasis. To investigate the significance of such platelet aggregation on metastasis in vivo, mice were rendered thrombocytopenic 24 h before tumor cell inoculation by injection of rabbit anti-mouse platelet serum. This treatment decreased the number of circulating platelets to <10% of the normal value (from $1.0 \times 10^6$ to $1.0 \times 10^5$ /µl) and kept it at this level for at least 48 h (data not shown). Such platelet depletion resulted in a reduction of metastasis of CFS1 and B16 cells by >95% compared to metastasis in animals with normal platelet numbers (Fig. 3). Liver colonization with ESb cells, however, was not affected by platelet depletion.

Effect of NK Cell Depletion and Platelet Depletion on Metastasis. To investigate whether NK cells and platelets play counteracting roles in metastasis, mice were depleted of NK cells by TM-b1 treatment, and the role of platelets in this NK cell-depleted system was evaluated. Metastasis of CFS1 and B16 cells in NK cell-depleted mice was high, and no difference in the number of metastatic foci between platelet-depleted and control group was measurable (Fig. 4). Metastasis of NK-resistant ESb cells was not affected by either treatment (Fig. 4).

The efficacy of this platelet-mediated protection was estimated with CFS1 tumor cells by calculating the percentage of inoculated tumor cells that reached the lung and formed micrometastases. On the basis of a mean lung volume of $350 \pm 50$ µl, it was found that: (a) in NK cell-depleted mice, virtually all inoculated tumor cells (93%) had formed metastatic foci; and (b) thrombocytopenia did not alter this

![Fig. 2. Platelet-specific staining of tumor cells after coculture with platelets. Tumor cells were stained with the fluoresceinated monoclonal rat anti-mouse CD41 (fibrinogen receptor, gpIIb/IIa) antibody (MWReg30, 5 µg/10^5 tumor cells/ml). A, in the cytofluorometric analysis, the shaded area represents nonspecific background staining of the CFS1, B16, or ESb tumor cells with MWReg30. Positive staining with MWReg30 was found when platelets (1000 platelets/tumor cell) were added to CFS1 and B16 tumor cells. B, microscopic analysis of a coculture of platelets with CFS1 tumor cells (1000 platelets/tumor cell; left, phase contrast; right, fluorescence microscopy; x400).](image-url)
To test a possible involvement of platelet MHC class I molecules for inhibition of NK cytotoxicity, we used platelets from different allogeneic mouse strains and, in addition, platelets from β₂-microglobulin-deficient mice, which express little if any functional MHC class I molecules (14). All platelets inhibited lysis of YAC1 NK target cells to a comparable extent (Table 2), indicating that the observed metastasis-enhancing effect after NK depletion was not restricted to the lung. These data confirm the notion that NK cells provide an effective antitumoral defense in the blood of mice. Addressing the question how some tumor cells manage to escape NK cell cytolysis in the blood, we found that metastasis of two NK-sensitive tumor cell lines, CFS1 and B16, was platelet-dependent, whereas the NK-resistant ESb tumor cells metastasized platelet independently.

Many different mechanisms have been proposed by which platelets may promote metastasis. These include: (a) stabilizing tumor cell arrest in the vasculature; (b) stimulation of tumor cell proliferation; (c) promoting tumor cell extravasation by potentiating tumor cell-induced endothelial cell retraction; and (d) enhancing tumor cell interaction with the extracellular matrix as reviewed by Honn et al. (1). In the absence of NK cells with all three tumor cell lines, equally strong metastasis was observed in both thrombocytopenic and control mice. Under these conditions, virtually all inoculated fibrosarcoma cells metastasized, irrespective of the presence or absence of platelets, suggesting that, at least in our model of experimental metastasis,

**Table 1** Effect of NK cell depletion and platelet depletion on lung metastasis

Numbers of micrometastases and percentage of inoculated tumor cells were assessed based on counted tumor lung colonies, thickness of tissue sections, and lung volume.

<table>
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<tr>
<th>NK cells</th>
<th>Platelets</th>
<th>Inoculated tumor cells</th>
<th>Micrometastases per Lung</th>
<th>% of inoculated tumor cells</th>
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</thead>
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<tr>
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<td>Present</td>
<td>$1 \times 10^6$</td>
<td>8</td>
<td>$5.5 \times 10^4$</td>
</tr>
<tr>
<td>Present</td>
<td>Absent</td>
<td>$3 \times 10^4$</td>
<td>4</td>
<td>$2.8 \times 10^4$</td>
</tr>
<tr>
<td>Absent</td>
<td>Present</td>
<td>$1 \times 10^6$</td>
<td>0.4</td>
<td>$2.8 \times 10^4$</td>
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<tr>
<td>Absent</td>
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<td>$3 \times 10^4$</td>
<td>4</td>
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DISCUSSION

Our studies show that depletion of NK cells increased the number of CFS1 and B16 tumor colonies in a model of experimental metastasis, demonstrating the NK sensitivity of these tumor cells in vivo. In the case of CFS1 fibrosarcoma cells, virtually all inoculated tumor cells colonized the lungs after NK cell depletion, whereas in NK-bearing mice, <10% developed into metastases. Comparable results were obtained with B16 melanoma cells, which besides metastasizing more strongly to the lungs also metastasized into the liver after NK cell depletion, indicating that the observed metastasis-enhancing effect after NK depletion was not restricted to the lung. These data confirm the notion that NK cells provide an effective antitumoral defense in the blood of mice. Addressing the question how some tumor cells manage to escape NK cell cytolysis in the blood, we found that metastasis of two NK-sensitive tumor cell lines, CFS1 and B16, was platelet-dependent, whereas the NK-resistant ESb tumor cells metastasized platelet independently.

![Fig. 3. Metastasis in thrombocytopenic mice.](image)

Mice had received either TM-β1 or control rat immunoglobulin (1 mg in 100 μl PBS) i.p. 1 week before and either rabbit anti-mouse platelet serum or control rabbit serum (100 μl) i.p. 24 h before the i.v. injection of CFS1 (1 × 10⁶), B16 (1 × 10⁴), or ESb (2 × 10⁵) tumor cells. The number of micrometastases per mm² was quantified on stained lung sections for CFS1 or on liver sections for ESb tumor cells 4 days after tumor cell injection. Surface metastases were counted on lungs on day 10 after B16 tumor cell injection. The results are given as means; bars, SD.

![Fig. 4. Metastasis in thrombocytopenic mice after NK cell depletion.](image)

Mice had received either TM-β1 or control rat immunoglobulin (1 mg in 100 μl PBS) i.p. 24 h before the i.v. injection of CFS1 (1 × 10⁴) or ESb (2 × 10⁵) tumor cells. The number of micrometastases per mm² was quantified on stained lung sections for CFS1 or on liver sections for ESb tumor cells 4 days after tumor cell injection. Surface metastases were counted on lungs on day 10 after B16 tumor cell injection. The results are given as means; bars, SD.

rate of colonization. In normal NK cell-bearing mice, 5.5% of inoculated tumor cells colonized the lungs. Platelet depletion reduced the rate of tumor cell colonization to 0.2%, demonstrating the potential of platelets to prevent NK cell lysis of CFS1 tumor cells (Table 1).

**Effect of Platelets on NK Cell Cytotoxicity in Vitro.** Addition of increasing numbers of platelets into the standard NK assay system resulted in a platelet number-dependent inhibition of YAC1 killing (Fig. 5). To test a possible involvement of platelet MHC class I molecules for inhibition of NK cytotoxicity, we used platelets from
stabilization of the tumor cells in the vasculature and enhancement of tumor cell interactions with the subendothelial matrix are not essential but may represent platelet effects of secondary importance.

On the other hand, in the presence of NK cells, lack of protecting platelets is detrimental to NK-sensitive tumor cells, as shown in different metastasis models where platelet depletion or inhibition of platelet aggregation clearly reduced metastasis (11, 12, 15, 17). Also, an inverse correlation of the antimetastatic thrombomodulin expression and intrahepatic metastasis in human hepatocellular carcinoma has been reported (18). Conversely, measures leading to enhanced platelet aggregation, e.g., provided by inflammatory stimuli, correlated with enhanced metastasis (1, 13). In both human and rat studies, organs that are preferred sites for metastasis have been shown to have significantly higher procoagulant activity than nonpreferred organs (19). Our flow cytometric data show that not all tumor cells bind platelets to the same extent. The NK-resistant ESb tumor cells did not induce significant platelet aggregation on their surface. Also, in the case of the CPS1 and B16 tumor cells, a subpopulation of the cells bound platelets much more efficiently than the rest, as indicated by higher fluorescence intensity after staining with the FITC-labeled, platelet-specific antibody MWRég30. This subpopulation with higher platelet-aggregation activity may represent those tumor cells that are most efficiently protected against NK cell lysis.

Different adhesion molecules have been suggested to be involved in this tumor cell-platelet aggregation phenomenon. The finding that blocking antibodies to P-selectin inhibited platelet aggregation around tumor cells and partially reversed the platelet-mediated inhibition of NK lysis in vitro supports the hypothesis that this molecule plays a role in platelet-tumor cell adhesion (20–24). Thrombospondin (25) as well as the fibrinogen receptor (gpIIb/IIIa, CD41; 26) and tumor cell av integrins (27) may be involved in tumor cell attachment to platelet thrombi. Ultrastructural studies with the human carcinosarcoma Colo 526 revealed a two-step process in which a first event of adhesion and degranulation of individual platelets in contact with a glycoprotein or glycolipid on the tumor cell membrane was followed by a wave of aggregation involving residual platelets (28).

Besides providing a mere physical barrier and steric hindrance between tumor cells and NK cells, platelets may also interfere with recognition or killing of tumor cells by NK cells. In covering the tumor cells, platelets bring MHC class I antigen into the vicinity of the tumor cell surface, which could down-regulate NK cytotoxic activity (29). However, because platelet-caused NK inhibition was independent of the platelet donor haplotype and was equally observed with platelets from β2-microglobulin-deficient mice, a MHC class I-dependent inhibitory mechanism is unlikely. An alternative interpretation is that platelets do not (only) shield tumor cells but inhibit cytolytic functions of NK cells by either release of suppressive or tumor cell protective factors (30, 31). The two theories are not mutually exclusive. Our supernatant transfer experiments indicate that platelet-derived soluble inhibitory factors might, indeed, be involved.

The role of the NK system for tumor defense in humans is still a matter of debate. This study shows that the main contribution of platelets to metastasis seems to consist in protection of tumor cells from NK lysis. Clinical studies have shown that anticoagulant therapy improves tumor response rates and survival of small cell lung cancer patients (32). It remains to be carefully analyzed in further clinical studies whether therapies with anticoagulants could be particularly useful in reducing the metastatic spread in cancer patients.

### REFERENCES

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