Human Platelets Exert Cytotoxic Effects on Tumor Cells

By Gretchen M. Ibele, Neil E. Kay, Gerhard J. Johnson, and Harry S. Jacob

Monocytes are thought to play a role in host resistance to tumor cell growth in animals and humans. In addition, platelets are known to be involved in tumor metastases. To investigate the interaction of these two cell types and their effect on tumor cells, human monocytes and platelets were examined using an in vitro monocyte–tumor cell cytotoxicity assay. Monocytes alone resulted in 32% ± 1.5 (mean ± SEM) tumor cell kill. When platelets were added to monocytes in a 1:1 ratio, an increase in cytotoxicity to 61% ± 3.2 was observed. The cytotoxicity noted when platelets were added to a fixed number of monocytes and tumor cells was dependent on the number of platelets added. A decrease in cytotoxicity from 32% ± 1.5 to 12% ± 2.3 was observed when contaminating platelets were removed from monocyte preparations. Platelets added to tumor cells in the absence of any monocytes were also toxic, resulting in a maximum kill of 95% at a 4:1 platelet/tumor cell ratio. Secreted products of freshly isolated platelets may be responsible for much of the observed cytotoxicity, since supernatants from the platelets were toxic for tumor cells. Platelets pretreated with a cyclooxygenase inhibitor (ASA) or a lipoxygenase inhibitor had decreased cytotoxicity compared with untreated platelets. Our results indicate that products of platelet arachidonate metabolism are toxic for tumor cell lines. They also suggest that the role of the platelet must be considered when studying monocyte–tumor cell cytotoxicity.

MONOCYTES are known to be present in tumor infiltrates, and in animal models the degree of monocyte infiltration has been shown to correlate inversely with the extent of disease.1 Unstimulated monocytes are capable of inhibiting the in vitro growth of malignant cell lines, and activated macrophages have been shown to have enhanced cytotoxicity for malignant, but not for normal cells.2 Monocytes are thus felt to play an important role in host resistance to tumor cell growth.

Platelets have been convincingly demonstrated to be involved in the development of some tumor metastatic lesions.3 Small clusters of malignant cells coated with adherent platelets break free from the primary tumor, spread through the vasculature, and then adhere to vascular endothelium permitting subsequent invasion by malignant cells.4,5

In addition to the interactions of platelets and monocytes with tumor cells, there is evidence to suggest that these cells have significant interaction with each other. Platelets are commonly seen as contaminants in human monocyte preparations isolated from heparinized peripheral blood. Perussia et al6 described monoclonal antibodies raised against monocytes which in fact reacted with platelet antigens, due to platelet contamination of the original monocyte preparations. Cohen et al7 described adherence or fusion of platelet pseudopodia to monocyte membranes in peripheral blood samples from patients with hematologic disorders who had artificially low platelet counts due to platelet satellitism to monocytes. Further evidence suggests that the association between these two cell types is not just an artifact of anticoagulation or the separation process. Platelet factor 4 (PF4) and platelet-derived growth factor (PDGF), both α-granule constituents, are known to be potent chemotaxins for both monocytes and neutrophils.8,9 The influence of these two cell types on tumor cells and their possible interactions prompted us to examine their effects on tumor cells in an in vitro monocyte–tumor cell cytotoxicity assay. Our studies indicate that the addition of platelets to monocytes results in enhanced tumor cell cytotoxicity and that platelets are directly toxic for human tumor cell lines.

MATERIALS AND METHODS

Preparation of Human Monocytes

Fresh heparinized blood was obtained from normal human volunteer donors. Peripheral blood mononuclear cells were isolated by Ficoll-Hypaque gradient separation and allowed to adhere to culture flasks in RPMI 1640 with 10% newborn calf serum (NCS) in 5% CO2 at 37 °C for one hour. Monocytes were removed from flasks by adding phosphate-buffered saline, 0.1 mol/L EDTA (PBS EDTA), pH 7.2, and then resuspended in culture media, and plated in microtiter wells. In other experiments, the monocytes were washed twice with 100% autologous serum to remove contaminating platelets before being resuspended in culture medium, counted, and plated. This method is similar to that described by Pawłowski et al,10 except that the use of EDTA and the ten-minute incubations have been omitted. Mononuclear cells obtained by adherence were 95% nonspecific esterase-positive and 88% to 90% ingested latex. These criteria define a relatively pure population of monocytes, as previously demonstrated.11,12 Nevertheless, the adherent cell population washed by autologous serum had less than a 1:1 platelet/monocyte ratio, while in nonwashed monocyte preparations the mean platelet/monocyte ratio was 3:1, as determined by evaluation of Wright-Giemsa stained cytospin slides.
PLATELET CYTOTOXICITY OF TUMOR CELLS

Tumor Cells

Two adherent tumor cell lines, Malme, a human melanoma cell line, and 786, a human renal cell carcinoma line, were used as targets. Tumor cells were cultured in flasks overnight with (3H) thymidine, (specific activity 20 Ci/mmol/L, New England Nuclear, Boston), removed with PBS EDTA, and resuspended in culture media. (3H) thymidine-labeled tumor cell targets were plated in microtiter wells at a final concentration of 2.5 x 10^5 cells per well.

Platelets

Platelet-rich plasma (PRP) was prepared from citrated whole blood by centrifugation and further concentrated by additional centrifugation at 4°C after the addition of 0.1 mL (0.1 mol/L EDTA) per mL of PRP. Fewer than ten lymphocytes were present per milliliter of PRP. Platelets were resuspended in culture media at a concentration of 5 x 10^3/mL. Gel-filtered platelets were prepared by filtration on Sepharose 2B columns and eluted with a modified Lindon’s buffer. Platelet supernatants were obtained by allowing 1 x 10^5 platelets in 200 μL of media to adhere in microtiter wells for two to four hours in 5% CO2 at 37°C. The supernatants were aspirated, centrifuged to remove any remaining platelets, and used immediately.

Platelet Inhibition Studies

To test the effect of acetyl salicylic acid (ASA) on platelet-mediated cytotoxicity, we exposed platelets to 5 x 10^-3 mol/L ASA (Sigma Chemical Co. St Louis) for 15 minutes, washed and then assayed for tumor cell cytotoxicity. To test the effect of platelet lipoygenase inhibitors on platelet-mediated tumor cell cytotoxicity, we utilized nordihydroguaiaretic acid (NDGA) and 4, 7, 10, 13 Eicosatetraynoic acid (ETYA) (both kindly provided by Dr Gundu H.R. Rao) in final concentrations of 1.5 x 10^-6 mol/L and 10 x 10^-6 mol/L, respectively. Platelets (5 x 10^6/mL) were incubated with either inhibitor or both for 20 minutes at 37°C prior to performing the tumor cell cytotoxicity assay. Lipoygenase inhibitors remained in the system during the assay, as it is known that the effect is easily and almost immediately reversible by washing.13

In Vitro Tumor Cell Cytotoxicity Assay

To examine the effects of monocytes plus platelets, platelets alone, or their secreted products on human tumor cell lines, we utilized a previously described tumor cell cytotoxicity assay.14 In brief, human monocytes were allowed to adhere to microtiter wells (5 x 10^5 cells per well) and labeled tumor cells (Malme or 786) were added in a 20:1 effector to target (E/T) ratio. We find this ratio to be optimal for reproducible monocyte–tumor cell cytotoxicity. The cell mixtures were incubated for 48 hours at 37°C and 5% CO2, wells were washed with fresh media, and residual radioactivity counted. Percent cytotoxicity was calculated using the formula:

\[
\frac{cpm \text{ effector} + \text{target}}{cpm \text{ target}} \times 100.
\]

In some experiments, various numbers of platelets were added to the monocyte–tumor cell mixture; in others, platelets alone or platelet supernatants alone were added directly to the labeled tumor cells, and subsequent tumor cell cytotoxicity was determined.

RESULTS

Effects of Monocytes, Monocytes Plus Platelets, and Platelets Alone on Tumor Cells

Monocytes alone resulted in a mean tumor cell cytotoxicity of 32% ± 1.5 (n = 10). When additional platelets (5 x 10^5 per well) were added to these monocytes, cytotoxicity increased to 61% ± 3.2 (n = 6) (P < .01, Fig 1). Dose–response curves were obtained by adding increasing numbers of platelets to constant numbers of monocytes (5 x 10^6 per well) and tumor cells (2.5 x 10^6 per well). Cytotoxicity increased with the addition of increasing numbers of platelets and reached a maximum of 78% at a 1:1 platelet/monocyte ratio. A representative experiment is shown in Fig 2.

In some experiments, various numbers of platelets were added to the monocyte–tumor cell mixture; in others, platelets alone or platelet supernatants alone were added directly to the labeled tumor cells, and subsequent tumor cell cytotoxicity was determined.

Fig 1. Percentage of cytotoxicity of monocytes, monocytes plus platelets, monocytes washed with autologous serum, and washed monocytes plus platelets (n = 6). Data are expressed as mean ± SEM.
observed with NDGA. Preincubation of platelets with both ASA (5 × 10^-4 mol/L) and NDGA (1.5 × 10^-6 mol/L) resulted in a significant decrease in cytotoxicity to 20% ± 6.0% (n = 7; P < .01). Variable inhibition was observed with ETYA, a problem noted by other investigators when using this inhibitor.13

**DISCUSSION**

These observations indicate that platelets and/or their secreted products are toxic for human tumor cell lines. Monocyte preparations free of platelet contamination result in less tumor cell kill than monocyte preparations from which platelets have not been removed. Platelets alone, as PRP or gel-filtered platelets, and supernatants obtained from either of these platelet preparations were also effective mediators of tumor cell lysis. The use of a cyclooxygenase inhibitor (ASA) or both cyclooxygenase and lipoxynegase inhibitors (ASA and NDGA) result in decreased platelet-induced cytotoxicity. These results suggest that arachidonate metabolites may be involved in this toxic event.

Although considered important primarily as mediators of hemostasis, there is evidence to suggest that blood platelets and/or platelet products are capable of mediating a variety of cellular effects not directly related to hemostasis. In addition to the well-known cellular growth stimulation induced by platelet-derived growth factor,13 cellular growth inhibition, killing, and lysis have been attributed to platelets and platelet products. Inhibition of tumor growth in vitro has been observed following the addition of prostaglandins D2, E1, and E2, products of the cyclooxygenase pathway of platelet prostaglandin metabolism. Lipooxyngase pathway products have also been implicated in cellular growth inhibition and lysis. The lipoxynegase pathway product, 12 hydroxyeicosatetraenoic acid (12 HETE), has been shown to be inhibitory for human neuroblastoma cells.19 Cell lysis by natural killer (NK) cells may also involve lipoxynegase pathway metabolites. Inhibition of lipoxynegase by BW775C and NDGA resulted in inhibition of NK-mediated lysis of K562 and Molt 4 cells, without influencing target cell binding.13 No inhibition was noted at levels sufficient to inhibit cyclooxygenase but not lipoxynegase.13

More direct evidence in support of our observations of platelet-mediated cytotoxicity is the fact that platelets have been found to be capable of lysing complement-sensitized human erythrocytes.20 Also of interest is the observation that platelets from patients infested with schistosoma mansoni, but not from normal controls, kill schistosomes in vitro.21 This study extends previous observations that indicate platelets are capable of exerting significant effects on cell growth and viability. Although the studies completed to date do not precisely define the mechanism by which platelets mediate tumor cell cytotoxicity, they strongly suggest that secretory products, perhaps arachidonate metabolites, are responsible. The apparent additive effect of cyclooxygenase and lipoxynegase inhibitors is of interest in view of the fact that lipoxynegase inhibitors have been shown to inhibit platelet adhesion and primary wave aggregation in ASA-incubated platelets.22 In addition, our study

![Fig 2. Effect of adding increasing numbers of platelets to a fixed concentration of nonwashed autologous monocytes (5 × 10^6 per well) on observed tumor cell cytotoxicity (n = 5). Data are plotted for a representative experiment and expressed as mean of triplicates.](image1)

![Fig 3. Tumor cell cytotoxicity observed with addition of increasing numbers of platelets to a fixed number of tumor cells (2.5 × 10^4 per well). Data are plotted for a representative experiment.](image2)

![Fig 4. Effect of adding increasing amounts of platelet culture supernatant to wells containing a fixed number of tumor cells (2.5 × 10^4 per well). Data are plotted for a representative experiment.](image3)
Platelet Cytotoxicity of Tumor Cells

highlights the possible contribution of platelets in in vitro monocyte–tumor cell lysis assays. Platelets generally have not been removed from monocyte preparations used in tumor cell cytotoxicity assays, presumably because they have not previously been known to possess effector functions in such situations. Our results indicate that it is important to consider the contribution of the platelet when analyzing monocyte–tumor cell cytotoxicity studies. In addition, they suggest the possibility that platelets play a role in host defense against neoplasia.

REFERENCES

Human platelets exert cytotoxic effects on tumor cells

GM Ibele, NE Kay, GJ Johnson and HS Jacob