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Human Platelets Effectively Kill K-562 Cells, a Chronic Myelogenic Leukemia Cell Line, *in vitro*

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The cytotoxic effect of isolated human platelets on leukemic cells has been examined in order to investigate the role of platelets in host defense systems. K-562 cells (a chronic myelogenic leukemia cell line) showed significant change in their morphology and were killed when they were incubated with platelets in serum-free medium for several hours at 37°C, a condition where no killing of normal peripheral lymphocytes occurred. Some protease inhibitors inhibited the cytotoxicity of platelets against K-562 cells. Our results suggest that platelets may be involved in host defense against neoplasia and that certain proteases are implicated in the cytotoxic effect of platelets.

Key words: Platelets — Cytotoxic effect — Leukemia — Proteases

Platelets and platelet products are capable of mediating a variety of biological events not directly related to hemostasis. Recently, platelets have been demonstrated to mediate the lysis of antibody-coated erythrocytes involving complement[1,2] and also to kill *Schistosoma mansoni* larvae in an IgE- or a lymphokine-dependent manner.[3–5] Furthermore, a possible role for platelets in the host defense against neoplasia has been suggested.[6] These findings suggested that platelets may be another primary cytotoxic effector in the immunological defense systems, and thus prompted us to examine the cytotoxic effect of isolated platelets on leukemic cells in a serum-free condition. Our studies indicate that normal platelets directly exert cytotoxicity against K-562 cells, a chronic myelogenic leukemia cell line.

Normal K-562 cells are spherical in shape as shown in the sample immediately after mixing with platelets (Fig. 1A). When K-562 cells were incubated alone in serum-free medium at 37°C for 6 h, they showed no morphological change and more than 99% of them were viable as evaluated by both the eosin Y staining method and the trypan blue dye exclusion test. However, when K-562 cells were incubated with freshly isolated platelets at 37°C for 6 h, they (K-562 cells) displayed significant shape modification with membrane protrusion around the cell body (Fig. 1B). Significant numbers of the K-562 cells were dead at this time, as shown by the eosin Y staining (Fig. 1B). Besides K-562 cells, SPI802 cells, a T cell leukemia line, and THP-1 cells, a monocytic leukemia cell line, were also killed by platelets under the conditions described here. Shape modification and killing of K-562 and other target cells by platelets did not occur under 25°C (data not shown). The proper control target for these experiments to show that the effect is cancer-specific would be normal peripheral blood cells from the cancer patients from whom the cell lines were derived, which of course are no longer available. However, in order to exclude the possibility that the cytotoxic effect of platelets against these leukemic cell lines is a non-specific one, resulting from the exposure of cells to a non-physiological condition (with concentrated platelets and without plasma), normal peripheral autologous lymphocytes, against which platelets have no cytotoxicity *in vivo* under normal conditions, were examined as control targets. No killing of them was observed even after 8 h incubation under these experimental conditions, indicating that the cytotoxic effect of platelets described here is not non-specific but is target-dependent.

By examination with a scanning electron microscope, K-562 cell damage induced by platelets could be shown more strikingly. Normal K-562 cells displayed a spherical shape with many peripheral microvilli (Fig. 2A). After incubation with platelets at 25°C for 30 min, the K-562 cells still had normal morphology except that the number of microvilli was decreased, with several platelets adhering to the cell surface (Fig. 2B). Further incubation with platelets at 37°C, however, resulted in a drastic morphological change of the K-562 target cell surface (Fig. 2C, 2D). Samples after incubation for 2 h contained K-562 cells displaying a smooth surface, the microvilli having been completely lost. After 4 h of incubation, the K-562 target cells had a sponge-like appearance with many holes, and clusters of granular materials, which had probably been released from the cytoplasm of the target cell, were attached to the surface. The vast majority of target cells retained platelets adhering to their surface

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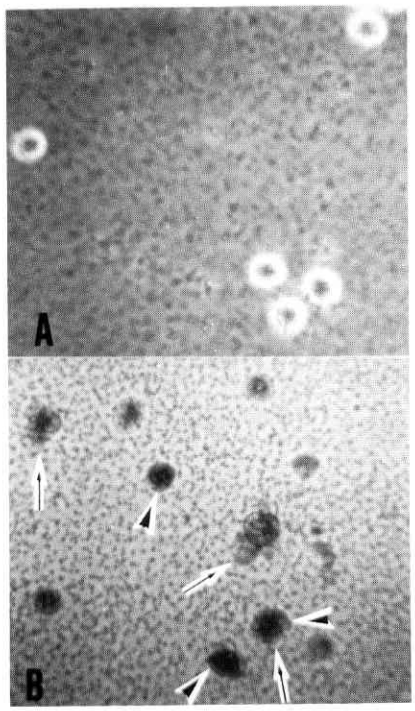


Fig. 1. Cytotoxicity of platelets towards K-562 cells. Platelets were obtained from citrated whole blood (usually 52.5 ml)[7] of healthy male and female volunteers. During preparation, platelets were maintained at 25°C, and plastic or siliconized tubes were used throughout. Platelet-rich plasma was centrifuged (700*g*, 10 min) and the pellet was suspended in 0.5 ml of Medium I (145 m*M* NaCl, 4 m*M* KCl, 1 m*M* $MgCl_2$, 0.5 m*M* sodium phosphate, 0.1% dextrose, 0.1% BSA, 5 m*M* PIPES, pH 6.8). This suspension was applied to a column (1×25 cm) of Sepharose CL-2B (Pharmacia) equilibrated in Medium I. The peak cell fraction eluting in the void was collected and recentrifuged (700*g*, 8 min). The pellet was resuspended in 0.5–1 ml of Medium II (145 m*M* NaCl, 4 m*M* KCl, 1 m*M* $MgCl_2$, 0.5 m*M* sodium phosphate, 0.1% dextrose, 0.1% BSA, 5 m*M* HEPES, pH 7.4). This platelet suspension (about 3×10^9/ml) was maintained at 37°C until assays were performed. This method of preparation is a modification of that reported by Wiedmer and Sim.[8] The preparation was completely devoid of contaminating leukocytes. For the cytotoxic assay, target cells which had been washed three times with Medium II were mixed with platelets in Medium II and incubated at 37°C. The concentration of target cells was 5×10^5/ml in all experiments, and that of platelets was 2×10^9/ml, unless otherwise stated. As a control, target cells without platelets were incubated. Morphology of the target cells was examined and their viability was determined by the eosin Y staining method or the trypan blue dye exclusion test. All cell lines used in this report were provided by the Japanese Cancer Research Resources Bank. A) K-562 target cells immediately after mixing with platelets have a normal spherical shape (phase microscopy). B) Samples after incubation for 6 h with platelets contain K-562 cells with modified shape (arrows). Dead K-562 cells stained with eosin Y are visible (arrowheads). Original magnification, ×268.

during the incubation period. Again, without platelets, the ultrastructural morphology of K-562 cells was not changed. These morphological observations clearly indicate that human platelets actually damage and kill K-562 cells within several hours when they are co-incubated at 37°C in the absence of any serum factors.

In further experiments, the cytotoxic effect of platelets against K-562 cells was quantified by the extent of killing, (number of cells stained with eosin Y/total cell number counted×100%), or by that of shape modification, (number of cells with modified shape/total cell number counted×100%). An optical microscope was used for these countings.

The kinetics of the cytotoxic reaction was examined at the platelet concentration of 2×10^9/ml. Fig. 3 shows three representative curves obtained by using platelets from three different donors. Significant shape modification was observed after 1 h of incubation, and a plateau level was reached by 4 to 5 h (80–95% shape modification). A lag period of 2 h (Fig. 3B and 3C) to 4 h (Fig. 3A) preceded killing. The extent of killing initially lagged behind that of shape modification but later caught up with it after several hours of incubation.

The dependence of shape modification and killing of K-562 cells on the concentration of platelets was examined using the same platelet preparation as that used in the experiment shown in Fig. 3C. The extent of shape modification after incubation for 4 h depended on the concentration of platelets, whereas significant killing was observed only at the maximum platelet concentration used (2×10^9/ml) (Table I). These results indicate that there are multiple steps before eventual cell killing. The variabilities in cytotoxic activity and in kinetics of killing in different experiments may be related to the state of platelet activation (or deactivation) in individual preparations or to the quality of platelet preparations from different donors.[9]

Preliminary results show that secretory products of activated platelets have no killing or shape-modifying activity against K-562 cells (data not shown), suggesting that platelets which directly adhere to target cells, as seen in Fig. 2, are responsible for the cytotoxic reaction. The studies of Ibele *et al.*,[6] where a melanoma cell line and a renal cell carcinoma line were used as targets of platelets and the cytotoxicity was evaluated by ^{51}Cr-release assay after incubation for 48 h at 37°C, have shown that cytotoxicity is observed at a 4:1 platelet/tumor cell ratio and that platelet supernatants are responsible for the cytotoxicity. In our studies, neither shape modification nor killing was observed unless a much higher effector/target ratio was used, and platelet supernatants had no cytotoxic activity. The difference of target cells as well as the assay methods could account for the different results obtained.

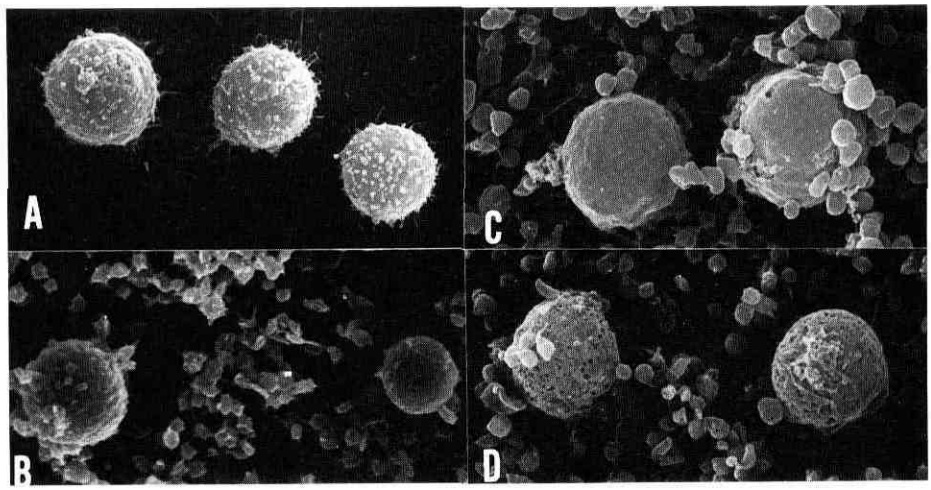
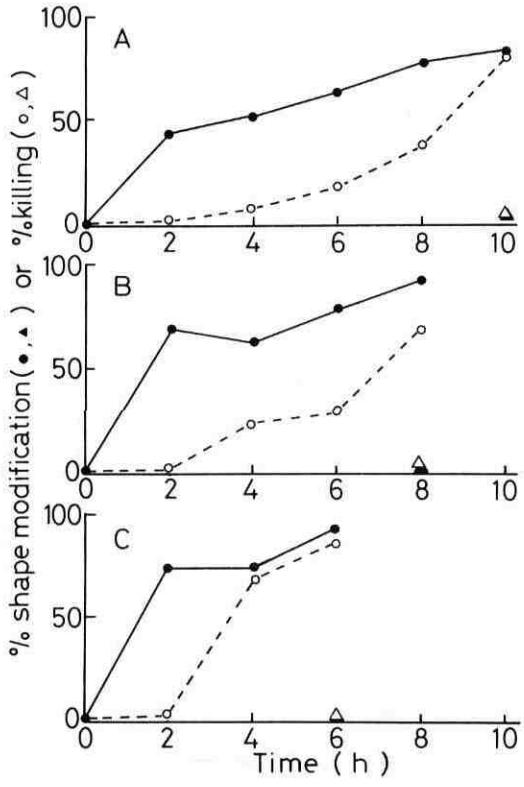


Fig. 2. Platelet-mediated ultrastructural change of K-562 cells. K-562 cells incubated with platelets in Medium II were overlaid on coverslips coated with poly-L-lysine and allowed to settle for 30 min at 25°C. The coverslips with samples were washed three times with phosphate-buffered saline (pH 7.4), and the samples were prefixed with 0.75% glutaraldehyde (in Medium II without dextrose and BSA) and postfixed with 2% OsO_4 (in Medium II without dextrose and BSA). The specimens were washed three times with distilled water, dehydrated with ethanol, and dried by the critical point method using liquid CO_2. Observations were made with a scanning electron microscope (Hitachi HS500). A) Intact K-562 cells. B) Samples settled on a coverslip immediately after mixing. C) Samples after 2 h of incubation at 37°C. The target cell surface has become smooth. D) Samples after 4 h of incubation at 37°C. The target cells show a peculiar sponge-like morphology. Original magnification, ×2500.



In view of previous findings that proteases are implicated in the cytotoxic reactions mediated by macrophages, neutrophils, and cytolytic T cells,[10-12] the effects of protease inhibitors on the cytotoxicity of platelets were examined by co-incubating these inhibitors with K-562 target cells and platelets. As can be seen in Fig. 4, SBTI, TPCK, and aprotinin inhibited the shape modification in a dose-dependent manner. Pepstatin also had a significant inhibitory activity, although the effect did not depend on the dose within the range used. By contrast, TLCK and leupeptin had no inhibitory activity. These observations indicate that certain proteases are also implicated in the cytotoxic reaction mediated by platelets. Further studies are under way to determine whether proteases trigger the cytotoxic activity of platelets or are themselves the effector molecules.

All our experiments described so far were carried out under serum-free conditions, but preliminary studies have shown that similar results could be obtained when the medium contained 3% fetal calf serum. The concen-

Fig. 3. Kinetics of platelet-mediated shape modification and killing of K-562 cells. (○), % killing; (●), % shape modification; (△), % killing without platelets; (▲), % shape modification without platelets. Panels A, B, and C show representative curves obtained by using platelets from different donors.

Table I. Dependence of Extent of Shape Modification and Killing of K-562 Cells on the Concentration of Platelets

Concentration of platelets ($\times 10^9$/ml)	% Shape modification[a)]	% Killing[a)]
2	77.8±4.5	73.3±3.9
1	30.0±6.6	3.0±2.7
0.5	21.9±10.2	2.3±2.9
0	0.3±0.6	0.2±0.3

K-562 cells were incubated with platelets in Medium II at 37°C for 4 h. At 0 time, more than 99% target cells had a normal spherical shape and were viable. At least 200 target cells were counted per assay.

a) Values are mean±SD of triplicates.

tration of platelets effective in the cytotoxic reaction (2×10^9/ml) was much higher than that in normal plasma ($\sim 3\times 10^8$/ml) under the experimental conditions described here. However, since platelets are aggregated *in vivo* by some stimuli under abnormal conditions, platelets may be concentrated locally to such a high concentration and they may exert cytotoxicity in a localized manner.

Platelets have been demonstrated to be involved in the development of some tumor metastatic lesions.[13-16] Although the relevance of our observations to the physiological role of platelets has yet to be established and specific targets of platelets remain to be elucidated, the report by Ibele *et al.*[6] and the results reported here may bring to light a new aspect of platelet involvement in neoplasia.

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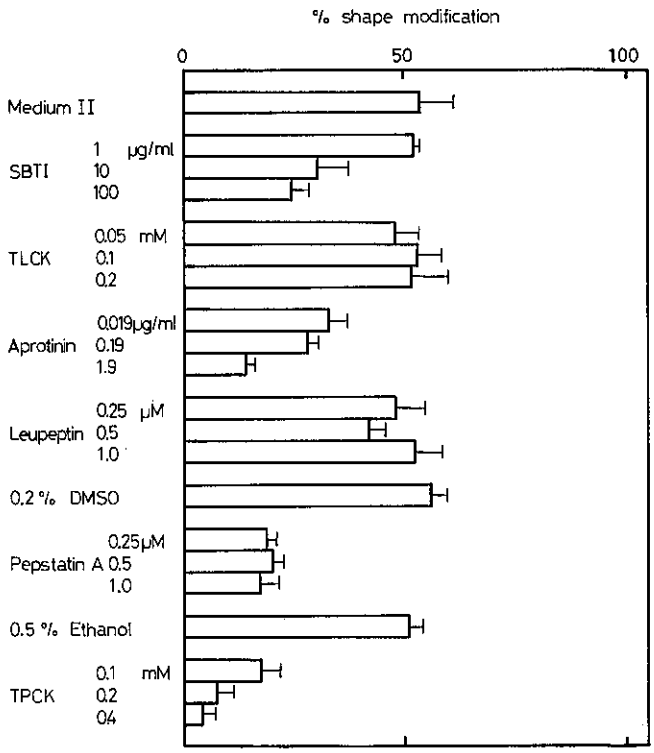

Fig. 4. Effect of protease inhibitors on platelet-mediated shape modification of K-562 cells. After co-incubation of K-562 cells and platelets with inhibitors for 2 h at 37°C, morphological change of target cells was examined. The effects of soybean trypsin inhibitor (SBTI), Nα-*p*-tosyl-L-lysine chloromethyl ketone (TLCK), aprotinin, and leupeptin were tested in Medium II, and those of pepstatin A and N-*p*-tosyl-L-phenylalanine chloromethyl ketone (TPCK) were tested in Medium II containing 0.2% dimethyl sulfoxide (DMSO) and 0.5% ethanol, respectively. Values are mean±SD of triplicate determinations.

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