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Isolation of Cytotoxic Proteins from Human Platelets

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Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 122, No. 8, pp. 183-185, August, 1996
Original article submitted December 5, 1995

Three protein fractions with cytotoxic activity towards lung adenocarcinoma cells are isolated from human platelets. Molecular weights of proteins from two fractions are determined. Three proteins with molecular weights of 14.5 and 22 kD are isolated from the first fraction and proteins with molecular weights of 28, 35, 44, and 65 kD from the second.

Key Words: *platelet; cytotoxic protein*

Recent data indicates that platelets participate not only in thrombus formation. They can be activated and involved as effector cells in various immunological responses to infection [7,10]. Platelets also have a role in the reaction to malignant tumor growth [1,2,8, 11,12]. Specifically, in patients with different tumor localization, increased intracellular granulation is the typical reaction of the megalokaryocyte-platelet system [2]. In addition, a tendency towards increased platelet aggregation has been observed [2,11, 12]. Platelets are known to stimulate metastasizing and endovascular invasion of tumor cells [12].

Platelets are activated by platelet-activating factor (PAF) which induces the release of biologically active substances from secretory granules [3,4]. This markedly enhances platelet cytotoxicity [5]. Similarly to T cells and monocytes, platelets exhibit killer activity towards malignant cells. It was shown that platelets lyse leukemia cells [13]. Platelets from patients with lung cancer lyse freshly isolated autologous tumor cells and allogenic ACL cells (human lung adenocarcinoma) [1]. Platelets from oncological patients display cytotoxic activity towards HeLa, K-562, and Mel-1 cells. The ACL strain proved to be the most sensitive target. Incubation of platelets from both healthy donors and oncological patients with PAF (10 pM-100 nM) markedly increases killer activity of platelets [5]. Platelet cytotoxic activity is influenced by various proteases [12]. The mechanisms underlying this activity remain obscure. Electron microscopy studies showed that platelets adsorb onto the target cells, after which ultrastructural changes typical of periodical secretion (hypertrophy of the Golgi apparatus, enlargement of secretory granules, and their orientation towards the contact zone) occur [5]. These changes are similar to those observed in T lymphocytes contacting with tumor cells [6]. From these findings it can be hypothesized that cytotoxic activity of platelets is due to their ability to secrete cytotoxic proteins with lytic activity towards tumor cells. Our aim was to isolate these proteins.

MATERIALS AND METHODS

Heparin (15 μl/ml) was added to peripheral venous blood (400 ml) of healthy donors and centrifuged for 10 min at 1550 rpm. Platelet-rich plasma was centrifuged for 10 min at 3000 rpm. Sedimented plate-

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0007-4888/96/0008-0814$15.00

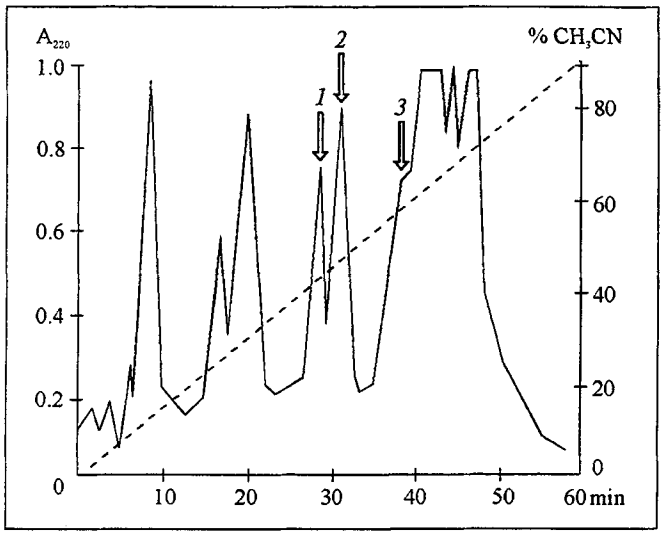

Fig. 1. Elution profile of human platelet lysate on an Ultrapore RPSC column (0-90% acetonitrile gradient in 0.1% trifluoroacetic acid, 60 min, 1 ml/min). A schematic drawing, *1-3*) active fractions exhibiting cytotoxic activity towards human lung adenocarcinoma cells.

lets were resuspended in RPMI-1640 medium and washed from serum proteins by repeated centrifugation for 10 min at 3000 rpm. For isolation of platelet proteins the pellet was resuspended in buffer containing 2 M NaCl and 0.1% aqueous trifluoroacetic acid. The specimen was frozen-thawed three times and centrifuged for 5 min at 5000 rpm. High-performance liquid chromatography (HPLC) was carried out in a System COLD chromatograph (Beckman). After the platelets were lysed, the supernatant was applied to an Ultrapore RPSC (4.6×75 mm, Beckman) column and eluted with a 0-90% acetonitrile gradient in 0.1% trifluoroacetic acid for 60 min at an elution rate of 1 ml/min. Spectrophotometrical determination was performed at 220 nm. An aliquot of each fraction was lyophilized for the cytotoxic activity tests. The ACL strain cells were suspended in 100 µl RPMI-1640 medium with L-glutamine and antibiotics (100 µl penicillin and 100 µl streptomycin per 100 ml medium with 2% L-glutamine). The cells (20,0000 cells per well) were transferred to 96-well flat-bottom plates (Falcon) prior to experiment. Lyophilized samples were dissolved in RPMI-1640 medium and incubated with the cells (100 µl per well, 3 wells for each sample) for 18 h at 37°C in an atmosphere containing 5% CO_2. After the incubation, the vital dye MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, Sigma, MTT, M2128) was added in a concentration of 5 mg/ml in isotonic phosphate buffer (pH 7.4) and incubated for 4 h at 37°C. The supernatant was collected, and 150 µl DMSO was added to each well to dissolve formazan crystals. For complete dissolution of the crystals the plates were placed in a shaker for 5-15 min. Light absorbance was measured in a MULTISCAN MCC 1340P spectrophotometer at 540 nm. The percentage of dead cells after the incubation, i.e., the cytotoxic activity, was calculated from the light absorbance values for control and experimental samples. Electrophoresis was performed by the method of Laemmli [9] in 12% polyacrylamide gel 0.75 mm thick and 10 cm long. Electroblotting onto an Immobilon PVDF membrane (Millipore) was performed in an Ancos apparatus for semidry electroblotting. The membrane was placed in methanol for 2 min and equilibrated in a blotting buffer (840 mg sodium bicarbonate in 500 ml water). Electroblotting was carried out for 1.5 h at 1 mA/cm^2. The membrane was then washed with water and stained with 0.1% Coomassie R-250 (Sigma). Excessive stain was washed with 50% methanol, and the membrane was washed with water for 10 min.

RESULTS

Figure 1 shows the HPLC elution profile of human platelet lysate. Peaks *1*, *2*, and *3* eluted at an acetonitrile concentration of 43, 47, and 58%, respectively,

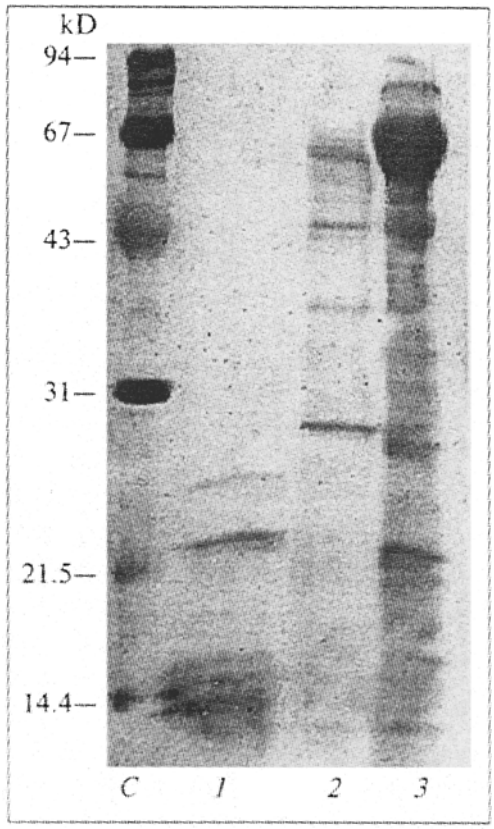

Fig. 2. SDS electrophoresis in 12% polyacrylamide gel with subsequent electroblotting onto Immobilon membrane of fractions with cytotoxic activity isolated from human platelets. *C*) standard protein kit for electrophoresis (LMW-kit, Bio-Rad), fractions *1*, *2*, and *3*.

contain substances exhibiting a cytotoxic activity of 15.3±1.1, 20.3±2.2, and 21.5±2.7%, respectively. Analysis of active fractions by SDS electrophoresis in polyacrylamide gel with subsequent electroblotting onto an Immobilon membrane and staining showed that they contain proteins (Fig. 2). Fraction *1* contains three proteins with a molecular weight of about 14.5 kD and one protein with a molecular weight of 22 kD. Fraction *2* contains proteins with molecular weights 28, 35, 44, and 65 kD. Fraction *3* contains a number of proteins requiring further fractionation.

Isolation of protein fractions with cytotoxic activity from human platelets corroborates the secretory hypothesis of platelet cytotoxicity. Each protein from active fraction may possess cytotoxic activity. The determination of their primary amino acid sequence may lead to the identification of the known cytotoxic proteins. Expression of such proteins in prokaryotic cells and determination of the activity of expressed proteins may broaden the spectrum of known cytotoxic proteins produced by immunocompetent cells.

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