Assessment of Nucleosomal DNA Fragmentation in Lung Adenocarcinoma Cells Incubated with Human Platelets

T. S. Golubeva, O. Yu. Chertov, M. V. Kiselevskii, and A. R. Tuguz

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No nucleosomal fragmentation of DNA from lung adenocarcinoma cells was observed after 18 h of their incubation with normal human platelets isolated from human peripheral blood. Platelet activation with 10^{-6}, 10^{-7}, and 10^{-8} M PAF did not lead to nucleosomal fragmentation of the target cell DNA.

Key Words: *platelet; nucleosomal DNA fragmentation*

The intense development of immunodiagnosis and immunotherapy of cancer necessitated studies of cellular mechanisms of antitumor immunity and molecular mechanisms of cell death. Like T-lymphocytes, monocytes, and other immune cells, platelets exhibit killer activity toward malignant cells [1,3]. High specific cytotoxic activity of human platelets toward ACL cells (adenocarcinoma of the lungs) was revealed [1]. Receptors to adhesive thrombospondine domain were detected on the membrane of ACL cells [7].

After contact with the effector, morphological changes and events at the molecular level (DNA fragmentation, etc.), as well as metabolic changes associated with cytolysis, take place in the target cell. These changes depend on the nature of the cytolytic agent and studied cells and their metabolic status [2]. There are two types of cytolysis: apoptosis and necrosis, which differ morphologically and biochemically [4,9]. The appearance of nucleosomal DNA fragments (180-250 b.p. long) indicates that cell death occurs by apoptosis [10]. When there are no signs of nucleosomal DNA fragmentation, the type of cytolysis can be assessed only by simultaneous detection of morphological and metabolic changes and events at the molecular level occurring in the cell during its death [2,5,8,10,11].

We investigated nucleosomal DNA fragmentation during cytolysis of ACL cells after a 18-h incubation with human platelets, intact and stimulated with platelet activating factor (PAF).

MATERIALS AND METHODS

Isolation of platelets. Venous blood (400 ml) with heparin (15 μl/ml) was centrifuged for 10 min at 1500 rpm. Platelet-rich plasma was centrifuged for 10 min at 3000 rpm. Precipitated platelets were resuspended in RPMI-1640 medium and recentrifuged for 10 min at 3000 rpm to remove serum proteins. Isolated platelets were stored at -70°C until use.

Cytotoxic activity test. Before experiment, target tumor cells in a concentration of 20,000 cells per well were placed in 96-well flat-bottom culture plates (Falcon): 100 μl of the suspension in RPMI-1640 medium with 2% L-glutamine, 100 μl penicillin, and 100 μl streptomycin per 100 ml medium. For experiment, test samples (100 μl) dissolved in RPMI-1640 medium were added to wells with target cells, 3 wells per sample. RPMI-1640 (100 μl per well) with target cells served as the control. The plates were incubated for 18 h at 37°C in atmosphere with 5% CO_2. After incubation of cells with the sample, 10 μl vital stain

Oncology Research Center, Russian Academy of Medical Sciences; M. M. Shemyakin and Yu. A. Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, Moscow

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MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, Sigma) in a concentration of 5 mg/ml in phosphate-buffered saline was added to each well and incubated for 4 h at 37°C. The supernatant was collected, and 150 μl dimethyl sulfoxide was added to each well to dissolve formazane crystals. For complete dissolving of the crystals, the plate was placed in a shaker for 5-15 min. Light absorbance of the well contents was measured with a Multiscan MCC-1340P spectrophotometer at a wavelength of 540 nm. Cytotoxic activity of the samples was estimated from light absorbance of control and experimental wells.

Assessment of nucleosomal DNA fragmentation. For assessing DNA fragmentation, 10^6 cells were incubated in 400 μl serum-free medium containing the effector for 18 h in a 24-well plate. For control, cells were incubated in a serum-free medium. Cytotoxic activity was assessed as described previously. After incubation with the sample, target cells were lyzed by adding an equal volume of buffer with 20 mM Tris-HCl (pH 7.4), 0.8% Triton X-100, 0.1 M EDTA (30 min at shaking at 37°C). High-molecular-weight DNA and undissolved cell fragments were removed by 5-min centrifugation at 1000*g*. Low-molecular-weight DNA fragments in the supernatant were precipitated with isopropanol (final concentration 50%) in the presence of 0.5 M NaCl (the mixture was incubated at least 2 h at -20°C). After centrifugation (13,000*g*, 15 min), the precipitate was washed in 70% ethanol, dried, treated with RNAse A in TAE buffer (100 μg/ml, 37°C, 40 min), and analyzed by electrophoresis in 1% agarose gel at a constant voltage (100 V). The gel was stained with aqueous solution of ethidium bromide for 10 min, after which it was washed in water 3 times for 5 min. The gels were photographed in ultraviolet light (310 nm).

RESULTS

Human platelets from 4 donors were used as effectors (Table 1).

Figure 1 shows nucleosomal DNA fragmentation in ACL cells after an 18-hour incubation with platelets. The result was considered positive if DNA fragments were found in experimental but not in the control track. No nucleosomal fragmentation induced by intact human platelets was detected by electrophoresis of DNA isolated from ACL target cells.

Similar studies were carried out with PAF-activated (10^{-6}, 10^{-7}, and 10^{-8} M) platelets of one donor as effector (Table 2).

Figure 2 presents the results of assessing nucleosomal DNA fragmentation in ACL cells after an 18-hour incubation with platelets activated with PAF in different concentrations. Analysis of the image showed no nucleosomal DNA fragments in experimental tracks.

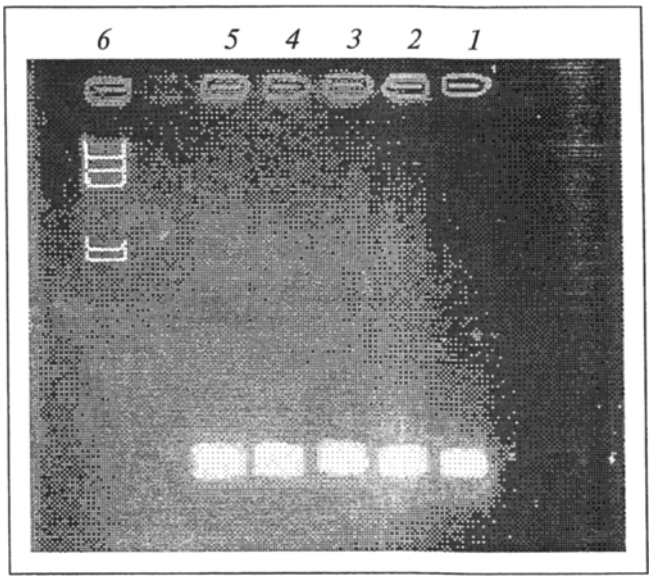

Fig. 1. Analysis of DNA fragmentation of lung adenocarcinoma target cells incubated with donor platelets (*1-4*), control (*5*), Hind III phage DNA marker (*6*).

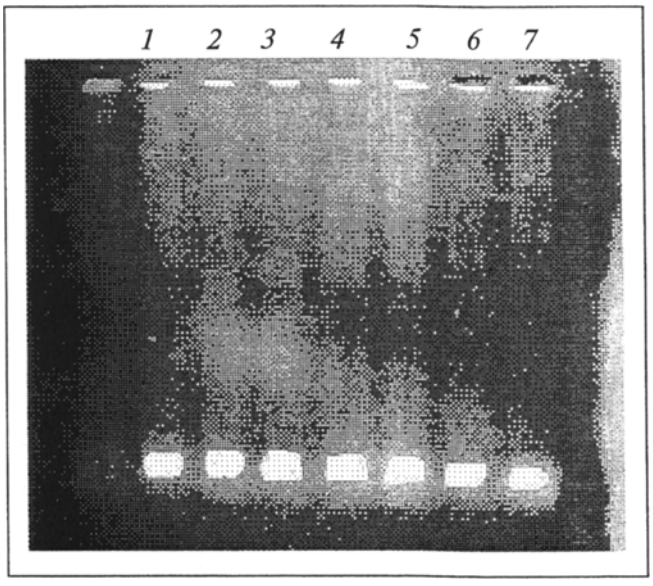

Fig. 2. Analysis of DNA fragmentation of lung adenocarcinoma target cells incubated with human platelets. *1*) PAF control, 10^{-6} M; *2*) PAF activation, 10^{-6} M; *3*) cell control; *4*) PAF control, 10^{-7}; *5*) PAF activation, 10^{-7} M; *6*) PAF control, 10^{-8} M; *7*) PAF activation, 10^{-8} M.

Thus, cytotoxic activity of platelets shown in the MTT test does not correlate with the detection of nucleosomal fragments by electrophoresis of DNA isolated from target cells after an 18-hour incubation with effectors. Activation of platelets with PAF does not lead to the appearance of nucleosomal DNA fragments under such conditions.

TABLE 1. Cytotoxic Activity of Platelets Assessed Using MTT Vital Stain

Donors	Platelet cytotoxic activity, %
1	10.1±3.3
2	15.4±5.7
3	9.6±3.8
4	11.7±5.1

TABLE 2. Cytotoxic Activity of Effectors Towards ACL Cells in Test with MTT Vital Stain

Effector	Cytotoxic activity, %
Platelets	11.2±2.2
PAF, 10^{-6} M	0.2±1.8
Platelets+PAF, 10^{-6} M	11.8±3.1
PAF, 10^{-7} M	6.0±3.8
Platelets+PAF, 10^{-7} M	18.0±3.7
PAF, 10^{-8} M	1.6±2.1
Platelets+PAF, 10^{-8} M	13.4±3.2

The peak of nucleosomal DNA fragmentation may not coincide with the 18-hour mark. It is possible that the death of target cells during their interaction with platelets does not involve nucleosomal DNA fragmentation. Presumably, the cytolysis associated with it involves DNA disintegration to large fragments (50 b.p.-long and longer), single-strand ruptures of DNA, or no fragmentation at all. As for morphological changes in target cells under the effect of platelets, we revealed [6] that exposure of K-562 cells to platelets results in wrinkling of the cell surface and the appearance of protrusions on the plasma membrane. The cells lose microvilli as early as after 2-hour incubation with platelets, and after 4 h they look "washed" with many fossae and clusters of granular material [6].

We intend to investigate nucleosomal DNA fragmentation in target cells in various periods of incubation with platelets and to study the kinetics of appearance of large fragments and single-strand ruptures of DNA during death of target cells. Together with our previous results [6], these data help us elucidate the mechanisms of malignant tumor cell cytolysis under the effect of human platelets.

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