Screening for Apoptosis Inducers in Microbial Products and Induction of Apoptosis by Cytostatin

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We have employed the DNA-methylgreen binding assay as a primary screening method for identifying apoptosis inducers in microbial products. Capsimycin, toyocamycin and cytostatin affect the binding of methylgreen to DNA of FS3 cells in this test system. The effect of cytostatin on apoptosis induction was confirmed by means of the ELISA system.

It is believed that apoptosis induction in tumor cells is a promising therapeutic strategy for cancer treatment[1]. However, no substance has been found which acts on the primary target as an inducer of apoptosis. We have employed the DNA-methylgreen binding assay[2] as a primary screening method for identifying apoptosis inducers in microbial products. In this way we have found that cytostatin[3] is able to induce apoptosis to tumor cells. In this paper, we report screening of microbial products for apoptosis induction and the investigation of cytostatin as an inducer of apoptosis in tumor cells.

Materials and Methods

Cell Line

FS3 mouse fibrosarcoma cells which had been cloned by us from spontaneously induced fibrosarcoma in the C3H/He mouse were maintained in RPMI1640 medium supplemented with 10% fetal bovine serum at 37°C in a 5% CO_2 atmosphere.

Cytotoxicity Test

Cytotoxicity of the test sample was determined by measuring the incorporation of [^{3}H]thymidine into the DNA of cultured cells. The amount of [^{3}H]thymidine was measured by a beta counter (Packard Instrument Co., Inc., U.S.A.).

DNA/Methylgreen Binding Assay

The assay was made in accordance with the method described by BURRES, N. S. *et al.*[2]. FS3 cells (2×10^5 cells in 200 μl/well) in a microplate were incubated with 10 μl of microbial cultured broth diluted appropriately for 24 hours or solution of test sample for 1 to 24 hours at 37°C in 5% CO_2 in air. After the incubation, the cells were fixed with 20 μl of 24~26% glutaraldehyde solution for 30 minutes and washed with tap water thoroughly. After drying, the fixed cells were stained with 50 μl of 1% methylgreen solution for 30 minutes and rinsed with tap water . The metylgreen bound to DNA of cultured cells, with or without the test sample, was extracted with 100 μl of EtOH under sonication. The absorbance at 620 nm was determined by a spectrophotometer (Flow Laboratories Inc., U.S.A.) and the inhibitory ratio was calculated as a percentage decrease by comparison with untreated cells.

Detection of DNA Fragmentation

DNA fragmentation was analyzed by means of the cellular DNA fragmentation ELISA kit (Boehringer Mannheim, Germany).

Exponentially growing FS3 cells (2×10^5 cells/ml) were incubated with 10 mM 5-bromo-2'-deoxy-uridine(BrdU) for 16 hours at 37°C in 5% CO_2 atmosphere. After incubation, the cells were centrifuged at $250 \times g$ for 10 minutes and resuspended in the culture medium. The cell suspension was adjusted to 1×10^5 cells/ml and 100 μl/well and then transferred to a microtiter plate. The cells were incubated with 100 μl of test sample solution for 1 to 24 hours at 37°C in 5% CO_2 atmosphere. After incubation, the cells were collected by centrifugation for 10 minutes at $250 \times g$ and DNA fragmentation was determined by the ELISA system.

Results

In this screening system, capsimycin[4], toyocamycin[5] and cytostatin[3], all found in microbial cultured broth, all showed cytotoxicity to FS3 cell at various concentrations. (refer Table 1) Capsimycin and cytostatin were more cytotoxic than toyocamycin. Capsimycin showed more potent inhibitory activity against the binding of methylgreen to DNA of FS3 cells than cytostatin and toyocamycin. The IC_{50} of cytostatin was almost the same as that of adriamycin (data not shown). As shown in Fig. 1, cytostatin inhibited the binding of methylgreen

Table 1. Effect of cytostatin, toyocamycin and capsimycin on FS3 cells in DNA-methyl green assay.

Drugs	IC_{50} (μg/ml)	
	Cytotoxicity to FS3 cells	DNA-methyl green assay
Cytostatin	4.3	22.5
Toyocamycin	68.2	13.2
Capsimycin	3.1	3.1

Fig. 1. Effect of cytostatin on DNA of FS3 cells in DNA/methyl green assay.

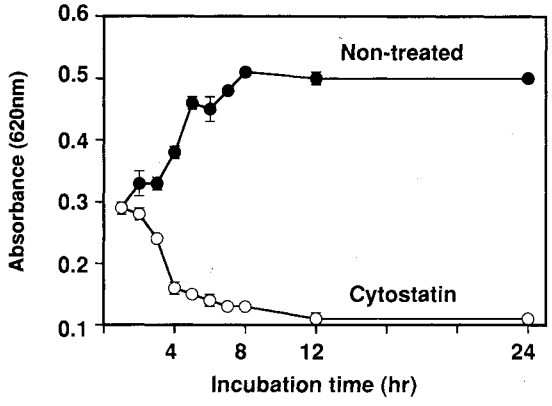
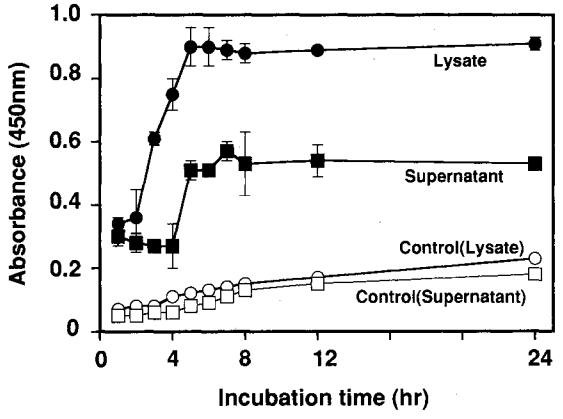


Fig. 2. DNA fragmentation of FS3 cell induced by cytostatin.



to DNA of FS3 cells within 4 hours (>90%) and inhibition was complete at 12 hours. The effect of cytostatin on DNA fragmentation was confirmed by ELISA. As shown in Fig. 2, cytostatin increased the rate of DNA fragmentation in cell lysate. It appeared in cultured supernatant within 4 hours and reached at maximum by 6 hours. The results obtained by ELISA is comparable to that for the DNA/methylgreen assay.

Discussion

The programmed cell death seen in the development of organs and in the differentiation of lymphocytes has been applied in the concept of apoptosis to cancer therapy[1]. It is obvious that most antitumor agents in clinical are inhibitors of DNA synthesis. Some antitumor agents such as actinomycin D[6], camptothecin[7], bleomycin[8], adriamycin[9] and cisplatinum[10] are known to induce DNA fragmentation.

In this context, to develop a new type of antitumor agent we have started to search microbial products for apoptosis induction on tumor cells by the DNA-methylgreen binding assay. Since this method was devised simply to detect fragments of naked DNA (BURRES, N. S. *et al.*[2]), any substance which produces this affect could be an apoptosis inducer. Instead of naked DNA, we used FS3 fibrosarcoma cells which had been cloned from spontaneously induced fibrosarcoma in the C3H/He mouse. Such FS3 cells are highly metastatic but have low immunogenicity. Although leukemic cells like HL-60 cells have been used to determine apoptotic activity, we prefered to screen for antitumor substances that could induce apoptosis in other malignant cells than those involved in leukemia.

Using this screening system, we found capsimycin, toyocamycin and cytostatin. Among them, cytostatin was found by us to be an inhibitor of cell adhesion to the extracellular matrix exhibiting antimetastatic activity[3]. The effect of cytostatin on apoptosis induction in FS3 cells was confirmed by ELISA. Microscopic observation showed that apoptotic bodies were formed in FS3 cells treated with cytostatin. In a later publication we will report the effect of cytostatin on DNA fragmentation using flow cytometry, during the cell cycle of human tumor cells.

In conclusion, we have confirmed that the DNA/methylgreen assay is valuable for the screening of apoptosis inducers and that cytostatin is an apoptosis inducer originating from a microbial source.

Acknowledgment

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