Apoptosis of Tumor Cells Induced by Substances of the Circulatory System

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Despite global abnormalities of the immune system, such as in AIDS, the incidence of only a few kinds of tumor increases, and even in the development of these tumors the degree of immunosuppression seems not to be a critical factor. This means that the known immune system has no significant role in the tumor-preventing mechanism. Thus, the fact that tumors do not develop in the majority of the population during their lifetime, indicates the existence of an additional defense mechanism of the immune system. We demonstrated previously that this defense is produced by the synergistic action of certain substances of the circulatory system. Here we report that the substances taking part in the defense induced, but only when they were used together, the apoptosis of tumor cells, but not normal cells, as was detected by different methods. Other substances of the circulatory system did not show similar effects. These results further support the existence of the mentioned defense mechanism called by us the Passive Antitumor Defense System.

Key words: Small molecules of the circulatory system; Apoptosis; DNA fragmentation; Agarose gel electrophoresis; Flow-cytometric analysis; Passive Antitumor Defense System.

Abbreviations used: PADS - passive antitumor defense system; PBS - phosphate-buffered saline.

INTRODUCTION

In a previous paper, we expounded and proved a hypothesis about the existence of a Passive Antitumor Defense System (PADS). Briefly, the well-known observations that full-blown AIDS is associated with a substantial loss of virtually all cellular and humoral immune responses are unquestionably supported by rejection-free renal graft survival in a patient with AIDS despite the significant and prolonged withdrawal of the usual immunosuppressive agents. Contrary to expectation, the incidence of only a few kinds of tumor (mainly Kaposi's sarcoma and non-Hodgkin's lymphoma) increases in patients with AIDS and even in the development of the tumors in question, the degree of immunosuppression seems not to be a critical factor. Taking into account similar observations made in other immune deficiency diseases and immunosuppressed patients it can be stated that the known immune system has not a significant role in the mechanism that prevents the development of tumors. This fits well with findings showing that the majority of clinically relevant tumors are not, or are only weakly, immunogenic. The fact that tumors do not develop in the majority of the population during their lifetime can only be explained by the existence...
and effect of a hitherto unknown defense mechanism of the immune system.

It is obvious that the components of an antitumor defense system must be in the circulatory system. It is well known that the intake by normal cells of small molecules (aminoacids, monosaccharides, etc.) of the circulatory system is regulated but their intake by tumor cells is increased, unregulated and proportional to their availability. Thus, the tumor cells, when there is a large tumor burden having many tumor cells, have a reproductive advantage over their normal neighbors in the competition for these substrates. According to our hypothesis, this feature of tumor cells may be fatal when there are only few tumor cells and a sufficient amount of the given substances in the environment of these cells, certain substances together, if they can reach a high enough concentration in the tumor cells: can kill them. Thus, these substances can destroy the arising tumor cells in the living system, if the number of cells is not too high or the concentrations of the required substances are not too low (critical cell number). The above actions of the given substances form the Passive Antitumor Defense System.

We proved this hypothesis by being able to select experimentally thirteen substances of the circulatory system, using the synergistic tumor cell-killing effect as criteria. Their mixture had a significant toxic effect on various tumor cell lines in vitro, and on leukemia and solid tumors in vivo, but it had no toxic effect on a normal cell line in vitro, and on the animals in vivo.

Because the process of cell death can occur by either apoptosis or by necrosis, we tried to identify in the next set of experiments, if the death induced by the mixture of the selected substances was of the apoptotic or necrotic type.

MATERIALS AND METHODS

Materials

Adenine, L-arginine, L-phenylalanine, L-histidine, L-tryptophan, L-tyrosine, L-methionine, 2-deoxy-D-ribose, L(-)malic acid, d-biotin, pyridoxine, riboflavin, L-ascorbic acid sodium salt, D(+)-mannose and sodium bicarbonate (all of them were cell culture tested biochemicals) were purchased from Sigma Chemical Co. (St. Louis, MO). Dulbecco's modified Eagle's medium (No. D-3916), Medium 199 (No. M-5017), RPMI-1640, trypan blue, folic acid, hypoxanthine, D-pantothenic acid hemicalcium salt, orotic acid monosodium salt and hippuric acid sodium salt were purchased from Sigma Chemical Co. (St. Louis, MO). Amino acid Kit (AS-30), nicotinamide and thiamine hydrochloride were obtained from Serva Fcniobiochemica GmbH & Co. (Heidelberg, Germany). succinic acid disodium salt, D(-)-ribose from FLUKA AG (Buchs, SG, Switzerland) and fetal calf scurf from Sebak GmbH (Aidenbach, Germany). All other chemicals were of the purest grade available from Reanal (Budapest, Hungary).

Tumor Cells and Culture

The Sp2/0-Ag14 mouse myeloma and the K562 human erythroleukemia cell lines were kindly provided by Dr. P. Nemeth (Biotechnological Facility, University Medical School of Pecs). The Vero African green monkey kidney cell line was generously provided by Prof. Dr. J. Szekeres (Department of Microbiology, University Medic 1 School of Pecs).

The cell lines were maintained under previously described culture conditions. Briefly, the Sp2/0-Ag14 and K562 cells were cultured in RPMI 1640 medium containing L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 μg/ml) and 10 % fetal calf serum. Vero cells were grown in Dulbecco's modified Eagle's medium and Medium 199 (1:1) containing 10 % fetal calf serum. The cells were incubated in a humidified atmosphere of 5 % CO₂ at 37°C. All cell lines were free of Mycoplasmo.

DNA Gel Electrophoresis

The effect of the mixtures was assessed by adding the indicated concentrations of the components dissolved in the applied medium to cultures. 16 × 10⁴ Sp2/0-Ag14, 4 × 10⁵ K562, 8 × 10⁴ Vero cells per 1000 μl medium. The composition of control mixture and active mixture are given in the legend to Fig. 1. The cells were allowed to proliferate for 24 h. Untreated cells were collected at the same time as treated samples. The number of viable cells was then counted microscopically with the trypan blue dye exclusion method. DNA fragmentation was
monitored by gel electrophoresis as described previously. Briefly, the untreated control mixture or active mixture treated cells, were collected by centrifugation, washed in PBS (140 mM NaCl, 3 mM KCl, 8 mM Na$_2$HPO$_4$, 1.5 mM KH$_2$PO$_4$, pH 7.1), resuspended (5 x 10$^6$ cells) in 0.5 ml of 45 mM Tris-borate buffer-1 mM EDTA, pH 8.0, containing 0.25 % Nonidet P-40 and 0.1 % RNase A, incubated at 37°C for 30 min and then treated with 1 mg/ml of protease K, and incubated for an additional 30 min at 37°C. After incubation, 0.1 ml of loading buffer (0.25 % bromophenol blue, 30 % glycerol) was added and 40,40 of the tube content were transferred to the gel. Electrophoresis was performed on 1.6 % agarose gel containing 0.5 % ethidium bromide at 80 V for 1-2 h with a TAE (40 mM Tris-acetate, 1 mM EDTA) running buffer. DNA was visualized under ultraviolet light and photographed.

Flow Cytometric Analysis

Flow cytometric analysis was performed to identify apoptotic cells as described earlier. Briefly, cells were fixed in 70 % ethanol overnight at 4°C. Cells after fixation were incubated in PBS containing 50 μg/ml RNase for 1 h, and stained with 65 μg/ml propidium iodide for 1 h at 4°C and then analyzed by a FACSort flow cytometer.

RESULTS AND DISCUSSION

Testing other eighteen compounds of the circulatory system (data not shown) using the same method described earlier, we could select three additional substances (orotic acid, hippuric acid, D(+)-mannose). In the subsequent experiments, the mixture of the sixteen substances (called the active mixture) was used (described in the legend to Fig. 1).

Apoptosis and necrosis can usually be distinguished morphologically and biochemically. Characteristics of apoptosis include cell shrinkage, chromatin condensation, apoptotic body formation, and DNA degradation. These properties differ significantly from characteristics of necrosis.

Cells with characteristic features of apoptosis were observed by light microscopy after 24 hours treatment of Sp2/0-Ag14, and K562 tumor cells by the active mixture. Drastic morphological changes occurred in the mentioned cells in comparison with Vero normal cells or tumor cells untreated or treated with control mixture (results are not shown). The control mixture (given in the legend to Fig. 1) contained also sixteen compounds having similar characteristics as the substances of the active mixture at a concentration that ensured the same osmolarity as the active mixture. The components of the control mixture were chosen from the substances.
of the circulatory system that were found ineffective in the previous experiments in comparison with the components of the active mixture. Thus, the possibility that any effect induced by the active mixture was the result of an osmotic effect or an aspecific overload of nutrients or an amino acid imbalance or ammonium toxicity could be excluded. The death of cells undergoing apoptosis is preceded by chromatin cleavage at the linker regions between nucleosomes by specific endonucleases, which results in a number of 180-200 base pair fragments and multiples of it. In general, the DNA fragments can be demonstrated by agarose gel electrophoresis, wherein a "ladder" develops. The cells treated with the active mixture show fragmentation of DNA into endonucleosome-sized units characteristic of apoptotic cell death in the case of Sp2/0-Ag14 mouse myeloma (Fig. 1A) and K562 human erythroleukemia cells (Fig. 1B).

In contrast, a ladder-like pattern of DNA fragmentation can not be seen in the case of untreated cells and in the case of cells treated with the control mixture. No fragmentation is visible in the case of Vero normal cells (Fig. 1C) treated with active mixture under the same experimental conditions, although it was demonstrated earlier that degradation of DNA in Vero cells giving rise to the typical ladder pattern on gel electrophoresis can be induced. These results are consistent with the previous findings demonstrating that the active mixture has cytotoxic effect on the above cell lines except for normal cells. Thus, the result of the gel electrophoresis repeatedly demonstrates the selectivity of the active mixture. It is important to emphasize that K562 has been shown to be relatively resistant to a variety of apoptotic stimuli (diphtheria toxin, etoposide, etc.) but the given substances of the circulatory system together could induce apoptosis of K562. On the other hand, other substances of the circulatory system (see control mixture) had not effect even together.

The various kinds of tumor cells differ from normal cells differently and therefore it is presumable that the kind and amount of the substances effective against them also differ to a certain extent. However, the mixture containing many compounds is effective against several kinds of tumor cells as was demonstrated above and earlier. Obviously, the mixture having the most universal effect is the fluid of the circulatory system (of course, the number of the cells has to be below a critical value). For practical reasons the Sp2/0-Ag14 cell line was used in the previous experiments to select the components of the active mixture. Consequently, this mixture is probably the most potent against Sp2/0-Ag14 cells from all the cell lines. Therefore the Sp2/0-Ag14 cell line was used in the following experiments to investigate the characteristics of the apoptosis-inducing effect of the active mixture.

We demonstrated previously that the
cell-killing effect of the active mixture was not caused by the individual toxicity of any components but was caused by the synergistic action of the given substances. Therefore, we investigated if the components of the active mixture can induce apoptosis singly or only together. As shown in Fig. 2, these substances, when they were used singly in exactly the same concentration as in the active mixture, could not induce apoptosis of the tumor cells. The DNA ladder appeared only when the cells were exposed to the simultaneous effect of the substances. This demonstrates the synergism in an early phase of the way leading to cell death and proves together with the former results that the synergistic action is a fundamental feature of these substances.

When cell cultures were incubated with different amounts of the active mixture, subsequent DNA flow-cytometric analysis revealed a number of cells with low DNA stainability, resulting in a sub-G1 peak, designated as apoptotic cells (Fig. 3A). There is circumstantial evidence that this reduced DNA stainability may be the consequence of progressive loss of DNA from the cells, due to the activation of endogenous endonuclease, and subsequent leakage of the low-molecular-weight DNA products. The dilution of active mixture and control mixture was expressed in percentages of the starting mixtures given in the legend to Fig. 1 and called 100% active mixture and 100% control mixture. It means that all components of the mixtures changed by the same proportion with dilution. The percentage of fluorescent events detected in the sub-G1 region began to increase at the 80% mixture amount (Fig. 3A). On the other hand, the number of S-phase cells continuously decreased as a result of an elevation of the active mixture dose (Fig. 3A). The DNA fragmentation into oligonucleosomal sized units detected by gel electrophoresis (Fig. 3B) and the cell death detected by cell count fit in well with the result of the flow-cytometric analysis. Fluorescence in the sub-G1 region, a ladder-like pattern of DNA fragmentation, and cell death, cannot be detected in the case of untreated cells and in the case of the control mixture (Fig. 3). Giving the same results after reiteration: the above experiments corroborate the importance of the proper proportion between the number of tumor cells and the amount of the defense molecules (critical cell number). It could be excluded that the apoptosis was induced by...
detection techniques or sample preparation procedure because the two different detection methods gave the same result.

Intermucleosomal DNA fragmentation was first detected after treatment for 1.5 h (the first time point examined) with the active mixture and became more prominent with longer treatment (Fig. 4A).

There was no detectable DNA fragmentation in untreated, and control mixture treated cells after 24 h. The change of cell number as a function of time (Fig. 4B) detected by cell count supports these results.

According to our hypothesis, the arising tumor cells are extinguished by the simultaneous action of the mentioned substances of the circulatory system. These cells undergo apoptotic cell death as shown by the above results. In this contest it is of interest that preneoplastic cells exhibited much higher apoptotic activity than the surrounding normal cells and single initiated cells should have a relatively high risk of elimination by apoptosis."

Both mathematical and biological analyses suggest that 80-90% of the initiated cells induced by chemical carcinogens are eliminated by apoptosis.

PADS must still have some influence even in the presence of a growing tumor because the tumor cells are always subjected to some effect of the "defense" molecules. It has been reported that apoptosis may be an important pathway for cell loss even in untreated tumors and that spontaneous regression may also be due to apoptosis, induced by unknown stimuli."

The observations that the majority of clinically relevant tumors are not immunogenic and that in some cases spontaneous regression of tumors occurs in AIDS patients suggest that in the above mentioned reports the PADS is the reason of the apoptosis (the "unknown stimuli").

In conclusion, the above results fit in well with our theory, with our previous results and with the data of other reports.

The investigations of the mechanism whereby the substances mentioned above trigger the programmed cell death are currently under way in our laboratory.

ACKNOWLEDGMENTS

I thank Prof. B. Sümegi, Dr. L. Lex, Dr. M. Bors for continuous support; H. Halász for excellent technical assistance; Prof. A. Sándor for critical reading of the manuscript; Prof. J. Szekeres for a gift of Vero cells; Prof. L. Pajor for flow-cytometric analysis that was supported by a grant from the Ministry of Health of Hungary, ETT T-07 632193.
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