Passive Antitumor Defense System: Hypothesis and Experimental Results

(Ph.D. Thesis)

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INTRODUCTION AND AIM OF OUR STUDIES

The well-known observations that full-blown AIDS is associated with substantial loss of virtually all cellular and humoral immune responses are unquestionably evidenced by rejection-free renal graft survival in a patient with AIDS despite the significant and prolonged withdrawal of the usual immunosuppressive agents. In spite of all these and contrary to expectation, the incidence of only a few kinds of tumor increases in patients with AIDS (mainly Kaposi's sarcoma and non-Hodgkin's lymphoma) and even in the development of tumors in question the degree of immunosuppression seems not to be a critical factor. To take also into account the similar observations made in other immune deficiency diseases and immunosuppressed patients it can be stated that the known immune system has not absolute role in the mechanism preventing tumour development. This fits in well with findings that the majority of clinically relevant tumors are not or are only weakly immunogenic.

There are two possibilities to explain that tumors do not develop in the majority of population during their lifetime despite the ineffectiveness of the known immune system against the majority of tumors. The first possibility is that there is no defense mechanism against non-immunogenic tumors; that the rise of a cancer cell is only a rare singular event but a tumor may develop from all cells arising. This assumption includes the high vitality of these cancer cells under any conditions. However, this contradicts the observations that the cell death rate is still high within non-necrotic tumor tissue and that 70% to 90% of newly produced tumor cells in humans die spontaneously by a mechanism that is yet poorly understood and only a very small percentage (<0.01 %) of circulating tumor cells can initiate metastatic colonies. The other possibility is that cancer cell formation is quite common but the majority of cells are not able to multiply to produce a tumor because they die shortly after they arise. The reasons for this may either be entirely random effects or the action of a systematic defense system. The former ones can be excluded because were cell death an absolutely accidental event, the simultaneous development of a number of primary tumors in organs should be a relatively frequent occurrence. However, the development of even double synchronous primary tumors is rather rare. Consequently, the death of non-immunogenic cancer cells can only be explained by the existence and effect of hitherto unknown defense mechanism or mechanisms.

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. These observations are widely accepted, what is more, some techniques of tumour detection (e.g., PET) use this feature of cancer cells. According to our hypothesis, this feature of tumor cells may be fatal when there are a sufficient amount of the given substances in the environment of the cells because 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 (absence of strong carcinogenic effects) or the concentrations of the required substances are not too low (healthy subjects, balanced food intake). The killing of tumor cells by the given substances form the Passive antitumor Defense System (PADS).

The aim of our studies was to evidence the above hypothesis.

METHODS

Tumor Cells and Culture

The Sp2/0-Ag14, EL4, A20, Jurkat, MCF7, Hep G2 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. The HeLa, MCF7/ADR, MDCK and LLC-MK_2 cells were grown in Dulbecco's modified Eagle's medium containing 10 % fetal calf serum. HEp-2 cells were grown in the same medium containing 5 % fetal calf serum. Vero cells were grown in Dulbecco's modified Eagle's medium and Medium 199 (1:1) containing 10 % fetal calf serum. Caco-2 cell line was cultured in Medium 199 containing 10 % fetal calf serum. The cells were incubated in a humidified atmosphere of 5 % CO_2 at 37 °C. All cell lines were free of *Mycoplasma*.

Cytotoxicity Assay

The toxicity was assessed by adding the tested compounds dissolved directly in the applied medium to cultures in 96-well micro plates. In the case of Sp2/0-Ag14, EL4, A20, Jurkat and K562 lines, the logarithmically growing cells were harvested from the medium and resuspended to a final concentration of 4×10^4 cells of Sp2/0-Ag14, EL4, Jurkat and 2×10^4 of K562, A20 in 250 µl appropriate medium per well containing the tested materials. In the case of HeLa, HEp-2, Vero, MCF7, MCF7/ADR, Hep G2, MDCK, LLC-MK_2 and Caco-2 cell lines, the cultured cells were harvested from 75 % confluent tissue culture flasks with 0.2 % trypsin, 0.025 % versene solution and resuspended in the appropriate medium at a density of 10^5 cells/ml. Aliquots (100 µl) - in the case of Caco-2, 100 µl and 50 µl - were dispensed into 96-well micro plates, made up to 250 µl with the appropriate medium and incubated for 24 h. Then the medium was gently discarded and replaced with 250 µl fresh medium containing the tested compounds. All kinds of cells were allowed to proliferate for 48 h. The number of viable Sp2/0-Ag14, EL4, A20, Jurkat and K562 cells was then counted microscopically with the trypan blue dye exclusion method. The survival of HeLa, HEp-2 and Vero cells was measured by assessing endogenous alkaline phosphatase activity of cells. Briefly, after incubation period the culture medium was removed from the well, the cells were rinsed with sterile PBS. Then 150 µg of alkaline phosphatase substrate dissolved in 150 µl fresh 10 % diethanolamine buffer (pH 9.8) was added to each well. Plates were incubated at 30 °C until the absorbance in the case of untreated cells reached a value of about 1. The reaction was stopped by adding 50 µl of 3 M NaOH to each well. The absorbance was measured at 405 nm with the aid of a Dynatech ELISA reader. Peripheral wells of each plate were utilized for blank (N = 3) background determinations. Background values were subtracted from each reading. The viability of Caco-2, MCF7, MCF7/ADR, Hep G2, MDCK and LLC-MK_2 cells was assessed with the MTT colorimetric assay. Because of disturbing effect of the tested compounds the modified assay was used. In brief, after 48 h incubation the medium was removed from the wells and the cells were washed with sterile PBS. To the cells in each well was added 50 µl of a 5 mg/ml sterile filtered solution of MTT in the applied medium. After incubating the plate for 4 h in 5 % CO_2 at 37 °C, the untransformed MTT was removed from the wells and the cells were washed with PBS. Then 50 µl isopropanol was added to all wells of the plate and thoroughly mixed in order to solubilize the formazan crystals. The quantity of formazan product formed was assessed by its absorbance at 550 nm on a Dynatech MR7000. Peripheral wells of each plate were utilized for blank (N = 3) background determinations. Background values were subtracted from each reading.

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, $16x10^4$ Sp2/0-Ag14, $4x10^4$ K562, $8x10^4$ Vero cells per 1000 µl medium. The cells were allowed to proliferate for 24 h. The untreated, control mixture or active mixture treated cells, were collected by centrifugation, counted, washed in PBS, resuspended (5×10^6 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 proteinase 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 µl of the tube content were transferred to the gel. Electrophoresis was performed on 1.6 % agarose gel containing 0.5 µg/ml ethidium bromide at 80V 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. The 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.

Treatment Schedule

The solution used for treatment of animals was prepared immediately before use and injected at 6.00, 9.00, and 12.00 a.m.; 3.00, 6.00, and 9.00 p.m. on each treatment day in a volume of 0.2 ml.

Evaluation of Antitumor Activity in i.p. Tumor Model

BALB/c mice (10 mice/group) were injected i.p. with 5×10^4 Sp2/0-Ag14 mouse myeloma cells suspended in 0.2 ml RPMI 1640 on Day 0. The solution used for treatment was given i.p. daily according to the defined schedule from Day 1 after tumor inoculation for 10 days. Tumor-bearing control mice were given injections of 0.2 ml PBS. Mice were monitored daily for mortality. Efficacy of the solution used for

treatment was expressed as the percentage increase in median survival time of treated over control tumor-bearing mice (T/C%).

Evaluation of Antitumor Activity in s.c. Tumor Model

A cell suspension of 5×10^7 HeLa cells/ml was prepared in Eagle's minimum essential medium and 0.1 ml of the cell suspension (5×10^6 cells) was implanted subcutaneously in the lower extremities of the BALB/c nude mice (5 mice/group). At the start of treatment all tumors had a volume of 30-60 mm^3. The first day of treatment was indicated as day 0. The solution used for treatment was administered i.p. according to the defined schedule for 10 consecutive days. Tumor-bearing control mice were given injections of 0.2 ml PBS only. Digital calipers were used to measure the length (L), width (W), and height (H) of each tumor or each lobe in multi-lobed tumors twice weekly and the tumor volumes were estimated by the formula of $0.5 \times L \times W \times H$. Because of the variation in size at the initiation of treatment, volumes were converted to the initial tumor volume. The relative tumor volume was expressed by the formula V_t/V_0, where V_t is the tumor volume on a given day of measurement, and V_0 is the initial volume of the same tumor at the start of the treatment. The ratio of the mean relative volume of treated tumors over that of control tumors multiplied by 100, (T/C%), was calculated at each evaluation. The criteria for effectiveness were the percentage of T/C value with 42 and less. Mean growth delay was measured based on the number of days required for mean relative tumor volume to reach nine fold of the initial volume.

Toxicity Testing

Toxicity was monitored by weight loss and toxic death. A weight loss nadir of 20 % per mouse or greater or 20 % or more toxic death is considered an excessively toxic dosage of the given substances.

Statistical Analysis of Data

The two-tailed Student's t test was used to determine the statistical significance of any changes observed.

THE NEW RESULTS OF THIS DISSERTATION

1. We selected five molecules (L-tryptophan, L-tyrosine, L-methionine, L(-)malate and L-ascorbate) as possible participators of the hypothetical defense system on the bases of literary data. Examining the effect of them singly and in combination on Sp2/0-Ag14 mouse myeloma cells *in vitro* we found that the mixture of them is really toxic for tumor cells and they increase the effect of each other synergistically.

2. Then we selected experimentally nine additional substances that could potentiate synergistically the effect of the former five. Of the 66 compounds examined, 9 compounds (adenine, L-arginine, L-phenylalanine, L-histidine, 2-deoxy-D-ribose, d-biotin, pyridoxine, riboflavin and oxaloacetate) potentiated significantly ($P<0.001$, for oxaloacetate $P<0.01$) the effect of the five-component mixture.

3. It was found that treatment of the cells with different amount of mixture containing thirteen (the first five substances plus the next eight, without oxaloacetate) components caused significantly larger effect to decrease cell proliferation than mixture containing the five substances. The control mixture was not cytotoxic for Sp2/0-Ag14 cells at any amount.

In this and in all the other experiments we used control mixtures. In this experiment the control mixture contained thirteen compounds of similar characteristics (succinate, hypoxanthine, ribose, amino acids, etc.) as the thirteen-component active mixture at a concentration that ensured the same osmolarity as the thirteen-component active mixture. The components of control mixture were chosen from that part of 66 compounds that were found in the previous experiment ineffective in potentiating the cell killing effect of the five component mixture. Thus, the possibility that the measured effect in the experiments was a result of an osmotic effect or an aspecific overload of nutrients or an amino acid imbalance or ammonium toxicity could be excluded.

4. We investigated the effect of the thirteen-component active mixture and control mixture as a function of time on the growth of Sp2/0-Ag14 mouse myeloma cell line compared to untreated cells. The number of untreated cells and cells treated with the control mixture increased exponentially in 48 hours. At the same time, the number of

cells treated with thirteen-component active mixture decreased compared to starting value. The death of about 100,000 tumor cells proved that the synergistic interaction of the given substances did not only cause an inhibition of cell proliferation but it really killed the cells.

5. There was no significant difference at all between active mixtures containing the same thirteen compounds but different counter-ions (calcium or potassium instead of sodium and sulphate instead of chloride).

6. The effect of active mixtures did not change when it was complemented by different amounts of control mixture. This demonstrated besides others that the cell death was not caused by an imbalance. This finding also evidenced that the other compounds of the circulatory system could not antagonize the effect of active substances.

7. The thirteen-component active mixture was found also significantly effective *in vitro* against K-562, HEp-2, HeLa and Caco-2 tumor cell lines compared to the control mixture. The same active mixture had no cytotoxic effect against the Vero normal cell line.

8. We examined and found that the mixture of the thirteen components significantly increased the survival time of mice injected i.p. with Sp2/0-Ag14 mouse myeloma cells by killing more than 2 logs (99 %) of the cells. The difference between mean survival time of control (12.9±0.6 days) and treated (18.9±0.5 days) group is highly significant ($P<0.001$). The T/C % calculated from the median survival time of control (13.5 days) and treated (20 days) group is 148.1 %.

9. The finding that the number of cells in the ascitic fluid of treated animals was significantly ($P<0.001$) lower (10.8×10^5) than in ascitic fluid of control animals (9.68×10^7) excludes the possibility that the increase of survival time was caused by a simple roborating effect of the above substances.

10. The treatment with mixture of the thirteen components decreased the growth of tumors in the BALB/c nude mice injected s.c. with HeLa cells. The mean relative tumor

volumes of the control and treated groups differed significantly ($P<0.05$) in all cases. The T/C % was less than 42 % at each evaluation. The least value was 35.7 %. The mean growth delay was 14 days.

11. The change in average body weight during above treatment was not significant between the control and the treated group and toxic death was not observed either during or after treatment, meaning that the substances in the given amount were not toxic.

12. Testing other seventeen compounds of the circulatory system we could select experimentally three additional substances (orotic acid, hippuric acid, D(+)-mannose) for the active mixture.

13. We demonstrated that the sixteen-component active mixture containing orotic acid sodium salt, hippuric acid sodium salt and D(+)-mannose additionally to the thirteen-component active mixture has significantly higher toxic effect on the Sp2/0-Ag14 cell line than the thirteen-component active mixture.

14. Investigating the effect of different ions (Fe^{2+}, Cu^{2+}, Zn^{2+}, Cr^{3+}, SeO_3^{2-}), it was found that only Cu^{2+} potentiated significantly ($P<0.001$) the effect of the sixteen-component mixture. This effect could be prevented completely by catalase. On the other hand, the Cu^{2+} could not potentiate the effect of active mixture when the mixture did not contain ascorbate. This findings made improbable that Cu^{2+} could play a role under physiological conditions and could take part in the PADS, therefore it was not used in our further experiments.

15. The sixteen-component active mixture showed significant cell-killing effect *in vitro* on A20, EL4, Jurkat, Hep G2, MCF7, and MCF7/ADR tumor cell lines compared to the sixteen-component control mixture. On the contrary, the sixteen component active mixture had no cytotoxic effect on the MDCK and the $LLC\text{-}MK_2$ normal cell lines. The degree of cell death was especially high in case of the A20 and EL4 lymphoma cells. It is an important result because the active mixture could have therapeutic value to prevent lymphomas which develop frequently in the case of AIDS, in other immune deficiency

diseases or in immunosuppressed patients. It is also worthy of note that the active mixture had cytotoxic effect on both MCF7 human breast adenocarcinoma cells and its adriamycin-resistant variant, MCF7/ADR cells. The sixteen-component control mixture was not cytotoxic for any cell lines in any amount.

16. To decide if the active mixture kill the normal cells or only decrease the proliferation of them, the change of cell number as a function of time with or without treatment by active mixture or control mixture was investigated. It was found, that the normal cells either untreated or treated with control mixture had a fairly high proliferation rate similarly to the tumor cells. The number of normal cells treated by 100 % active mixture also increased as a function of time and dying cells could not be observed microscopically in contrast to tumor cells treated by the same active mixture whose number decreased during the 48 hours incubation period in consequence of cell death. The inverse change of cell number in the case of normal and tumor cells treated by the active mixture is an unquestionable evidence for the selectivity of the sixteen-component active mixture. This experiment also demonstrated that the A20 and EL4 lymphoma cell lines were highly susceptible (more than the other cell lines) to the effect of the active mixture, since all the cells were killed after 12 hours of incubation.

17. The maximal and minimal concentration of the substances of active mixture in the blood can be found in the scientific literature. The active mixture, when the final concentration of its components corresponded to their maximum concentration existing in the blood, killed all the K562 cells under 125 cells/ml initial cell concentration whereas the untreated cells proliferated to 5×10^5. The minimal active mixture was ineffective to destroy the cells even at 60 cells/ml initial cell concentration. The control mixture did not show cell killing effect at all. The results were similar in the case of Sp2/0-Ag14 cells. The maximal active mixture was a model of a fairly operating and the minimal active mixture of a poorly operating defense system. It results from these experiments that the selected substances may act in physiological conditions, too. These experiments also demonstrates the importance of the optimal amount of the above substances in the blood.

18. We demonstrated, that the length of time needed for death of the same amount of cells decreases as the concentration of the active mixture increases. This means that the rate of cell death depends on the concentration of the active mixture.

19. It was investigated visually (by photos) using the active mixture as a model of defense system, what happens with the tumor cells when the effectiveness of defense is shifted from the well operating to the poorly operating condition. It was found that as the amount of active mixture decreased, in other words, as the effectiveness of the defense system declined, the division of the K562 human erythroleukemia cells compared to the cell death became more and more dominant. However, the photos also demonstrate that the defense mechanism has a cell-killing effect even after a tumor developed and even if its effect is not enough to kill all the tumor cells.

20. The cells treated with the sixteen-component active mixture showed fragmentation of DNA into endonucleosome-sized units characteristic of apoptotic cell death in the case of Sp2/0-Ag14 mouse myeloma and K562 human erythroleukemia cells. In contrast, a ladder-like pattern of DNA fragmentation could not be seen in the case of untreated cells and in the case of cells treated with the control mixture. No fragmentation was visible in the case of Vero normal cells 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. It is important to emphasize that K562 has been shown to be relatively resistant to a variety of apoptotic stimuli but the given substances of the circulatory system together could induce apoptosis of K562. On the other hand, other substances of the circulatory system (control mixture) had not effect even together.

21. The components of active mixture, 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.

22. When cell cultures were incubated with different dilution 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. The DNA fragmentation into oligonucleosomal sized units detected by gel electrophoresis fit in well with the result of the flow-cytometric analysis. Fluorescence in the sub-G_1 region and a ladder-like pattern of DNA fragmentation cannot be detected in the case of untreated cells and in the case of the control mixture. On the basis of the above results, 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.

23. Internucleosomal 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. There was no detectable DNA fragmentation in untreated, and control mixture treated cells after 24 h.

SUMMARY AND CONCLUSIONS

In conclusion, considering the essentially direct proportion between the amount of mixture and the cell-killing effect in the *in vitro* experiments, the synergistic interaction of the substances, the number of killed cells at the given concentrations, the fact that the cell-killing effect is not antagonized by other substances of the circulatory system, the different effect of substances observed *in vitro* on normal and tumor cells, the non-toxic antitumor effects of the selected substances *in vivo*, the cell-killing effect of the mixture using the components in the same concentration as they occur in the blood, the apoptosis inducing effect on tumor cells by the synergistic action of the components, it can be stated that these substances existing together in the living system can really destroy a certain number of cancer cells in the body under physiological condition when their concentrations are in physiological range. This supports our hypothesis that in the living systems a Passive Antitumor Defense System exists and the compounds found by us participate in this defense system.

The operation of the Passive Antitumor Defense System can be explained in the following manner. The amount of substances of the defense system continuously changes

in the circulatory system depending on nutrition, age, life-style, etc. If the number of arising cancer cells remains under a critical value then the concentration of substances of the defense system existing in the environment of the given cells is satisfactorily high in order to destroy all the arising cells. This happens in the majority of the population during their lifetime. If the number of tumor cells arising simultaneously exceeds a critical value because of some reason (e.g., strong carcinogenic effects, viruses, hereditary predisposition to cancer, etc.), then the tumor develops because above the critical value the divisions of the cells overcompensate for the killing of cells by the defense mechanism. The other way to develop a tumor is the decrease of the concentration of the above mentioned substances in the circulatory system in consequence of some reasons (e.g., malnutrition, disease, stress, etc.) and thus the impairment of the effectiveness of the PADS. Although in this case no more tumor cells arise than in the majority of the population, the number of cells can still reach the critical value because of the low concentration of the defense molecules. Obviously, the level of defense never decreases to zero since the majority of the substances taking part in the operation of the PADS have endogenous sources, only the defense cannot always operate optimally.

According to our experiments, the rate of cell death depends on the concentration of substances of the defense system. Obviously, the rate of the cell division is conditioned by the cell concentration. On the bases of the experimental results, it can be stated that the cell division and the cell death caused by the defence system compete with each other ceaselessly. The balance of them determines that the tumour may or may not develop. At the critical value the rate of division and the rate of death are equal. Below the critical value the rate of cell death is higher than the rate of division and so all the cells die, but above the critical value the rate of cell division is higher than the rate of death and the tumour develops.

The existence of PADS is also supported by many epidemiological and clinical observations, as well as other literary data as detailed in the complete version of the dissertation.

PUBLICATIONS BEING THE BASIS OF THIS DISSERTATION

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