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Enhanced tolerance to high doses of ionizing radiation or chemotherapy by oligoelements plus phospholipase A_2

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SUMMARY

In the present work we evaluated the tolerance induced by the sc daily administration of oligoelements (Zn. Se. Mn) plus phospholipase A_2 (O-PA_2) to treatment with high doses of chemotherapy or ionizing radiation. Combined action of cyclophosphamide, methotrexate and 5-fluoruracil (CMF) was evaluated in male Sprague-Dawley rats in doses ranging from 1 to 20-fold the dose used in human patients. Range of whole body radiation doses was 2 to 15 Gray. Results indicated a significantly higher global survival in O-PA_2 treated rats. For irradiation studies LD_{50} value was 9.6 Gray in O-PA_2 treated rats versus 5.8 Gray in control groups. LD_{50} in O-PA_2 treated rats was 18.5 fold the basic CMF dose versus 14.1 in controls. All control rats showed severe bone marrow aplasia, sepsis and secondary leukemia. Present data demonstrated the protective effect of O-PA_2 in rats receiving large doses of radiation or chemoterapeutic drugs.

INTRODUCTION

Diverse investigations have postulated an important role of some oligoelements such as Zinc (Zn), Selenium (Se) and Manganese (Mn) in cellular resistance against free radicals, suggesting a potential usefulness for cancer prevention (1). Se, Zn, magnesium and calcium show modulatory effects in experimental carcinogenesis and many of these effects have also been reported in human epidemiological studies (2). Tobey demonstrated that pretreatment with Copper (Cu), Zn and Se induces an increase in the survival of normal cells challenged with alkylating agents, while no protection is observed in tumor cells. Zn enhanced from 7 to 9 fold the chemotherapeutic dose of melphalan to be applied in pretreated versus non pretreated cultures and up to 16 times such dose when associated with Se and Cu (3). Zn and Se are able to inhibit secondary carcinogenesis produced by antineoplastic drugs employed in human patients and many organoselenium compounds are effective chemopreventive agents for various chemically induced tumors in animal models (4). Mn acts in the prevention of cell damage induced by oxidizing agents, particularly the superoxide ion. It has been postulated that alteration in the regulation of manganese superoxide dismutase (Mn SOD) plays a critical role in the development of many types of tumor (5). Also, many radioprotective drugs have been providing substantial protection against radiation damage. Glutathion radioprotective action is attributed to the capture of free radicals produced by ionizing radiation (6). Besides, it has been demonstrated that phospholipase A_2 (PLA_2), acts as an effector system for certain enzymes as cyclo-oxigenase and lipooxigenase, cytokines as tumor necrosis factor (TNF), interleukin-1 (IL-1) and interleukin-6 (IL-6) (7) and also produces histamine release from activated mast cells (8). It is well known that one of the effects of histamine is the modulation of tumoral and cellular immune response, in part through the stimulation of IL-6 production by B cells (9). The objective of this work was to evaluate the tolerance induced by Zn, Se and Mn plus PLA_2 (OP-A_2) to treatment with high doses of ionizing radiation or chemotherapeutic drugs.

MATERIALS AND METHOD

In vitro studies: cell cultures. Cell lines derived from human pancreatic carcinoma (SOJ-6), and from normal mammary epithelium (HBL-100) were cultured in RPMI 1640, 10% FCS at 37°C in a 5% CO_2 humidified atmosphere.

Experimental protocol: Cells were seeded in 24 well plastic dishes and incubated for 24 hs; afterwards, a proportion of the cultures was treated with freshly thawed 5-fluoruracil (5-FU) in concentrations ranging from 1 to 100 µM and further incubated for another 24 hrs. Then the overlayer medium was removed, the cell monolayers were washed and a fresh complete medium was added. 48 hs later half of the cultures were trypsinized, viability assayed by Trypan Blue dye exclusion and cells counted. Remaining wells were fixed and stained with O-toluidine Blue.

In identical parallel experiments, cell cultures were grown in complete medium containing Zn, Se and Mn 10^{-6} g/l and phospholipase A_2 (PLA_2) 10^{-12} g/l during the whole experimental period (96 hs). The treatment with 5-FU was performed under the same experimental conditions as mentioned above. Control cultures were grown in complete medium. All experimental points were performed in quadruplicate.

In vivo studies

Animals: Male Sprague-Dawley rats, inbred in our laboratory and weighing 380-

420 g were employed. One hundred and twenty eight animals were used in the chemotherapy experiments and ninety-six (96) for irradiation. Animals were kept in groups of 4 per cage, with water and food *ad libitum*, temperature at 22-23ºC, humidity at roughly 56% and a 12 hours light cycle. Body weight was monitored every day.

Treatments with chemotherapeutic drugs: For cytostatic tolerance studies, a combination of cyclophosphamide, methotrexate and 5-fluoruracil (CMF) was given as a single intraperitoneal dose. The lowest CMF dose given, indicated as 2-fold, was calculated by extrapolating the double value of that one used in human patients: cyclophosphamide 500 mg/m^2, methotrexate 40 mg/m^2 and 5-fluoruracil 600 mg/m^2 (12). The range of employed doses were multiples of the latter and were indicated as: 2-, 4-, 10-, 12-, 14-, 16-, 18- and 20-fold, respectively.

Protection with oligoelements and phospholipase A$_2$: Half of the rats (64, divided in 8 rats each group) were daily treated (0.5 ml sc) with oligoelements Mn, Se and Zn (1.5 µg/Kg BW each) plus phospholipase A$_2$ (0.1 ng/Kg BW). Protective treatment started 10 days before CMF ip administration and continued for 70 days. Each group received one dose of 2-, 4-, 10-, 12-, 14-, 16-, 18- and 20-fold CMF, respectively. *Controls*: Sixty-four rats divided in 8 groups were daily treated (0.5 ml sc) with NaCl 0.9%. Each group received one dose of 2-, 4-, 10-, 12-, 14-, 16-, 18- and 20-fold CMF, respectively.

Treatments with ionizing radiation: For ionizing radiation tolerance studies, a range of whole body radiation doses from 2 to 15 Gray was assayed. A ^{137}Cs source of 5.100 Ci (1.1x10^{16}Bq.) (8.57 Gy/min), calibrated with a TLD 700 dosimeter, was utilized. *Protection with oligoelements and phospholipase A$_2$*: Half of the rats (48, divided in 8 rats each group) were daily treated with O-PA$_2$ as above indicated. Protective treatment started 10 days before irradiation and continued for 70 days. Each group received one dose of 2, 5, 8, 10, 12 or 15 Gray, respectively.

Controls: Forty-eight rats (divided in 8 rats each group) were daily treated (0.5 ml sc) with NaCl 0.9%. Each group received one dose of 2, 5, 8, 10, 12 or 15 Gray.

Parameters recorded on in vivo studies. a) *LD$_{50}$ determination*; b) *body weight*; c) *observation of collateral macroscopic effects*; d) *necropsy*; e) *histopathological study of organs and tissues*. For histopathological studies, all animals were autopsied at the time of death or at the end of the experiments (day 60th post-CMF injection or irradiation). Viscera and the sixth dorsal vertebrae were removed and fixed in 10% formaldehyde buffer. Specimens of lung, liver, heart, kidney, spleen, genitalia and bone marrow were harvested for histological studies. Tissues were embedded in paraffin and stained with hematoxylin-eosin.

RESULTS AND CONCLUSIONS

Results of the in vitro experiments

In vitro experiments indicated that the cytostatic effect of 5-FU was significantly enhanced by O-PA$_2$ pretreatment in transformed cells; on the contrary, in normal cells O-PA$_2$ exerted a protective effect with a significant decrease of toxicity. Exposure of transformed and normal cells to different doses of 5-FU resulted in a dose dependent cytotoxic effect, with an EC$_{50}$ value of 5±2 µM. The combined treatment with O-PA$_2$ produced an opposite response in transformed than in normal cells. Addition of O-PA$_2$ to SOJ-6 cells enhanced the cytotoxic effect of 5-FU showing an EC$_{50}$ value for 5-FU when combined with O-PA$_2$ of 0.8±0.2 µM. On the contrary, when normal HBL-100 cells were treated with 5-FU in the presence of O-PA$_2$, a protective effect was observed indicating a significant higher

EC_{40} value of 20 ± 3 μM.

Results of the in vivo experiments

Tolerance to chemotherapeutic drugs: All parameters showed enhanced tolerance to systemic CMF treatment in O-PA_2 treated groups. Results indicated a significantly higher survival in protected versus non protected animals ($p<0.004$, logrank test). The groups treated with 14-, 16- and 18-fold CMF showed the most significant difference vs controls ($p<0.0064$, $p<0.0052$ and $p<0.0023$, respectively, Wilcoxon test). Results of LD_{50} showed a significant increase of tolerance to cytostatic drugs in O-PA_2 protected rats; the interpolated LD_{50} value for CMF in control rats was significantly lower than the value in rats protected with O-PA_2: 14.1-fold versus 18.5-fold the basic CMF dose ($p<0.01$ Lifchield-Wilcoxon). With respect to body weight evolution, in batches receiving 2- to 10-fold basic CMF dose, O-PA_2 protected rats showed a significant higher increase in whole body weight than control animals ($p<0.05$ Hotelling's test). Collateral macroscopic effects as nasal and gut hemorrhage, fur discoloration and hair loss were invariably milder in protected batches compared to controls. Histopathological results indicated marked differences between protected and control rats: whereas at a 16-fold CMF dose, 80% of control rats died of sepsis and exhibited bone marrow aplasia, only 20% of protected rats showed signs of sepsis or aplasia. On the other hand, studies performed on surviving rats sacrificed 60 days post-CMF injection indicated that all control animals exhibited chronic myeloid leukemia. In contrast, O-PA_2 treated rats showed normocellular or slightly hypercellular bone marrow.

Tolerance to ionizing radiation: Animals irradiated with 8, 10 and 12 Gray showed the most significant difference versus controls ($p<0.0001$, 0.013 and 0.0083 respectively, logrank test). Rats irradiated with 2 and 5 Gray did not show significant difference. Results of $^{30}LD_{50}$ showed a significant increase of tolerance to high irradiation doses in O-PA_2 protected rats: the $^{30}LD_{50}$ value was 9.6 Gray in protected rats versus 5.8 Gray in controls. ($p<0.02$, two way ANOVA). Similarly, batches receiving 2 or 5 Gray indicated a significant increase in whole body weight in O-PA_2 protected rats with respect to control animals ($p<0.05$ Hotelling's test). Macroscopic observation indicated that gut hemorrhage was most severe in control than in protected rats. Histopathological studies indicated that rats irradiated with 10, 12 and 15 Gray showed more severe generalized hemorrhage and bone marrow aplasia than protected ones.

The present studies clearly demonstrated a protective effect exerted by the combination of oligoelements Mn, Se and Zn plus PLA_2 on *in vivo* high-dose of chemotherapeutic drugs and ionizing radiation. Furthermore, *in vitro* O-PA_2 showed a different action between normal and transformed cells.

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