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EFFECTS OF ALKYLGLYCEROLS ON CELLULAR GROWTH AND SENSITIVITY TO CHEMOTHERAPEUTIC AGENTS IN TUMOR CULTURES.

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Alkylglycerols occur in mammals mainly in bone marrow, breast milk and liver. Alkylglycerols have been used for treatment of patients with irradiation-induced leukopenia and for treatment of patients with carcinoma of the uterine cervix. The cancer patients treated with alkylglycerols had a significantly higher five-year survival than the control group. In these studies Greenland shark liver oil was used as alkylglycerol source. In addition to the usual content of alkylglycerols with alifatic fatty acid chains the shark liver oil also contain small amounts of methoxysubstituted alkylethers. In animal experiments it has been shown that the methoxysubstituted alkylethers stimulate the immune system and inhibit growth of malignant neoplastic cells. In this study we have used malignant human tumor (e.g. Breast cancer tissue) cultures which were then treated with cytostatic agents, alkylglycerols or both. Treatment with cytostatics alone did not show any marked effects. But treatment with both cytostatics and alkylglycerols showed significant killing of malignant cells in eight out of nine patients. Different possible working mechanisms are discussed. Further experimental and clinical studies should be encouraged.

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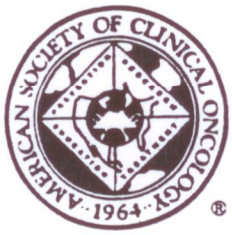

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EFFECTS OF ALKYLGLYCEROLS ON CELLULAR GROWTH AND SENSITIVITY TO CHEMOTHERAPEUTIC AGENTS IN TUMOUR CULTURES

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THE EFFECTS OF ALKYLGLYCEROLS ON CELLULAR GROWTH AND SENSITIVITY TO CHEMOTHERAPEUTIC AGENTS IN TUMOUR CULTURES

INTRODUCTION

Alkylglycerols are lipids with a glycerol backbone, to which fatty acid derivates are coupled by means of an ether bond instead of the ester bond that characterises mono-di-and triglycerides and related phospholipids. The ether lipids are present in high concentrations in human bone marrow, spleen and liver. The naturally occurring alkylglycerols are in most cases esterified with fatty acids of 16-18 carbon atoms, sometimes unsaturated[1]. Brohult and Holmberg used the unsaponifiable portion of bone marrow fat for preparations of alkylglycerols that then were used for treatment of children with leukaemia. A maturing effect of the white blood cells was observed[2]. This preliminary investigation was followed by experiments employing alkylglycerols in irradiation leucopenia[3]. In 1963, Brohult published a thesis on alkylglycerols and their use in radiation treatment[4]. The alkylethers used were isolated from Greenland shark liver (Somniosus microcephalus) by molecular destillation followed by hydrolysis. In patients with uterine cancer it was shown that the decrease in white cells and thrombocytes which usually occurs during radiation

treatment was less pronounced when alkylglycerols were administered during the treatment.

It has been shown that the incidence of injuries following radiation therapy for carcinoma of the uterine cervix was significantly decreased when the patients were treated with alkylglycerols[5, 6]. In a double-blind study performed in 1970-1972, there was a tendency toward lower stages in patients treated with alkylglycerols in comparison to controls, and a study carried out in the years 1973-1975 also showed a shift to lower stages in the prophylactically treated patients[7]. When all groups of patients with uterine cancer are put together (1964-1966, 1970-1972, 1973-1975) the total amount of patients studied consists of 841 prophylactically treated cases and 4404 control cases (=usual radiation therapy without alkylglycerol treatment). The mortality after five years in the prophylactic group was 31.0 percent while the mortality in the corresponding control group was 39.6 percent. The difference is statistically significant ($p<0.001$)[8].

About two percent of the alkylglycerols in the Greenland shark liver oil consist of methoxy-substituted alkylglycerols with the methoxy group in the 1-position. In animal experiments it has been shown that methoxy-substituted alkylglycerols stimulates cellular immunoreactivity in mice[9, 10]. It has also been

shown that methoxy-substituted alkylglycerols inhibits tumour growth in cultured cell lines[11]. Thus, these substances can stimulate the immune system and also inhibit tumour growth.

Chemotherapy for cancer has essentially remained the same for the last twenty years. There have been several variations of the standard CMF, CMFVP, CMFT, and CAF but no substantial breakthrough in new modalities or combination treatments have yet been proven more effective. Since no chemotherapy combination has markedly improved survival, it seems reasonable to attempt to modify the milieu that the tumour presided in. Burns and Spectors findings suggested a potential role for lipid nutrition in cancer therapy[12]. It has been well documented that fatty acid content of cancer cell membranes can change substantially when the cells are exposed to different types of fat. Certain physical and functional properties of the membrane are modified making the cells more sensitive to treatment of doxorubicin.

SUBJECT RECRUITMENT AND SELECTION: Ten (10) patients were studied, 8 female and 2 male between the ages of 43 and 70 years old, with an average age of 55. Patients were drawn from a pool of cancer patients who were undergoing surgery and follow-up chemotherapy. Consent forms were obtained. Tumours had to be of sufficient size to provide at least one gram of viable

tumour tissue. A biopsy was taken from each and analysed using *in vitro* cultures.

METHOD - FLUORESCENT CYTOPRINT ASSAY: The fluoroscent cytoprint *in vitro* assay was designed to measure the effectiveness of specific chemotherapy drugs in destroying individual patient's cancer cells[13, 14, 15, 16, 17]. Tumour tissue fragments were "sandwiched" between two thin papers coated with collagen and supported by small grids at the surface of the culture medium. This technique assured the tumour fragments, called "micro-organs" (300-500 viable tumour cells having the same structure and function of the original tumour) would be stationary and could be monitored over time under the microscope and photographed. The tissue samples were then exposed to a panel of chemotherapeutic agents and examined to see how many and which micro-organs had been killed. Drugs were also tested in varying concentrations.

A sample of 1 gram of viable tumour tissue was sufficient for assay of the treatments at three different concentrations. The specimen was centrifuged, washed with fresh medium, and after mincing, collagenase was added. The culture was then incubated for 18-24 hours. Following the initial incubation, the micro-organ cultures were prepared. Tumour fragments were collected by centrifugation, washed, and resuspended in media. After 30 minutes in the dark,

large fragments (100-1500 cells) were planted in a matrix of cellulose fibres impregnated with collagen. These micro-organ cultures were placed on stainless steel screen supports located in each well of a 24-well tissue culture plate. Medium was added so that the culture sat at the liquid gas interface and was fed by capillary action through the cellulose matrix. Cultures were returned to the incubator for 24 hours. Following the 4 hour incubation, the initial cytoprint was prepared. Fluorescein acetate in serumfree medium was added. Viable tumour cell clusters or micro-organs with intact cell membranes retained fluorescein released from the substrate and became fluorescent. After 30 minutes in the dark, cultures were washed and the patterns of fluorescent micro-organs (cytoprints) were recorded photographically under a dissecting microscope. This record served as the baseline, i.e., each culture served as its own baseline when cytoprinting was repeated at the end of the assay period. Cultures were then returned to the medium to allow viable tumour cell clusters to expel the fluorescein.

Drug treatment

In the initial studies we determined tumour susceptibility following:

a) the addition of lipid-based emulsions (alkylglycerols) alone, (b) chemotherapeutic agents alone and (c) lipid-based emulsions (alkylglycerols) plus chemotherapeutic agents. The drug groups were run concurrently within

any one assay. All samples including control (no drug) were carried out in duplicate.

The chemotherapeutic drugs used were Doxorubicin (Adriamycin®) and Fluorouracil. They have been dosed to give a concentration in the cultured tumour cells similar to the concentration of these drugs in the patients being chemotherapeutically treated. Two concentrations of the lipid-based emulsion (alkylglycerols) were used; 5 and 20 micrograms per millilitre and these concentrations are similar to the concentrations in patients that are treated with alkylglycerols.

Evaluation and Drug Effects

Cytotoxicity (loss of fluorescent micro-organs) was assessed by comparing photographic and fluorescent cytoprints taken before and after treatment. Results of the cytotoxicity was reported as "sensitive" (greater than 90% cell death); "intermediate" (between 25 and 90% cell death), and "resistant" (less than 25% cell death). Tumour growth and viability was indicated by comparing changes in shape and size of the micro-organs following drug treatment with the initial cytoprints of untreated cultures of the same specimen (control).

RESULTS

The purpose of this study was to evaluate the use of alkylglycerols in combination with cytotoxic agents in the treatment of cancer by examining their ability to kill tumour cells and augment the cytotoxic effects of chemotherapeutic agents. The *in vitro* assay we used (FCA) has been shown previously to be predictive of a response to chemotherapy in patients whose tumours scored "Sensitive" (>90% cell kill) in the assay[18]. Patients' tumours were tested *in vitro* against (i) alkylglycerols alone, (ii) chemotherapy alone (Doxorubicin or 5-FU) and (iii) chemotherapy in combination with alkylglycerols (Doxorubicin+AG or 5-FU+AG).

In our study group, one patient's tumour tissue sample was inadequate for comparison testing. Of the nine remaining patients, none scored a "Sensitive" when exposed *in vitro* to chemotherapy agents alone. However, when alkylglycerols were added to the *in vitro* chemotherapy regimen, 6 of the 9 patients' tumours' response were increased, and scored "Sensitive", i.e. the cell kill was >90%. With the 3 remaining tumour specimens the sensitivity was also increased, though the cell kill in these instances was not sufficient to merit a "Sensitive" score. In two instances, when the tumours were exposed to alkylglycerols alone, an inhibitory effect with an intermediate score was noted.

The patients possessed the following characteristics: Six patients had breast cancer, one had metastatic adenocarcinoma of the lung, one had mesothelioma, one had colon cancer and one had renal cancer. In the breast cancer group five of the patients had infiltrating ductal carcinoma, and one had adenocarcinoma. Three of the patients with infiltrating ductal carcinoma showed increased sensitivity levels at or above 90% when chemotherapy was used in combination with alkylglycerols. Another patient had an improvement in their sensitivity from Resistant to Intermediate when alkylglycerols were added to the chemotherapeutic agents, and in one case there was minimal improvement. The patient with adenocarcinoma in this group had an inadequate tissue sample, and we were unable to compare results. One of these patients was also tested with a specific fraction of alkylglycerol, methoxyglycerol. When this compound was added to one of the tumour cultures in combination with doxorubicin the highest response rate was seen, and the tumour went from approximately 90% sensitivity to greater than 90% sensitivity. With fluorouracil alone, the tumour was Resistant, and exhibited Intermediate sensitivity when used in combination with the methoxyglycerol. In the mesothelioma patient the sample was resistant to all chemotherapeutic agents when given alone. When the tumour was exposed to a combination of doxorubicin and alkylglycerol the tumour response was at 90%. The second lung cancer patient sample was a metastatic lesion from a primary colon cancer. This sample was Resistant to doxorubicin and

showed an Intermediate sensitivity to fluorouracil. When exposed to doxorubicin in combination with alkylglycerols the sensitivity increased to Intermediate level, and further increased to Sensitive when alkylglycerols were added to the fluorouracil. This was found to be the case in both the mid dose and the high dose groups. In the clear cell-predominant renal cell carcinoma patient the tumour showed an Intermediate sensitivity to both doxorubicin and fluorouracil, and was resistant to an additional chemotherapeutic agent, vinblastine. In the mid dose alkylglycerol / doxorubicin combination, more than 80% of the tumour was killed.

DISCUSSION

The increasing incidence of cancer may be related to our diet which is high in saturated fats and vegetable oil. Lissner et al. Have shown that the type of fat consumed influences the occurrence of endometrial cancer[19]. Shu et al. found in a study in China that diets high in animal fat may play an important role in the epidemiology of endometrial cancer[20]. Zhang et al. concluded that high fat intake is associated with reduced survival in post menopausal women with breastcancer[21]. Alkylglycerols may exert their beneficial effects by modifying membrane structure and function and by altering signal transduction. Membrane fatty acids can be altered by diet in animals. Such modifications can alter the membrane fluidity and possibly alter the cellular transport

mechanisms. Sebokova concluded in rat models that the changes in the type of oil administered to rats changed plasma membrane contents and binding capacities of the gonadotropin receptor[22].

It has been shown by Das et al. that dietary ether lipids can be directly utilised by mammals to synthesise membrane alkyl glycerolipids and plasmalogens in most tissues[23]. Several studies have shown that the amount of alkyldiacylglycerols is much higher in neoplastic cells than in normal cells[24]. The amount in tumourous tissue can be 10-100 times higher as compared with normal tissue. The explanation is the tumourous tissue contains extremely low amounts of ether cleavage enzyme.

The German research groups have shown that alkyllysophospholipids without the methoxygroup in the glycerol part can activate macrophages in the bone marrow[25, 26]. This shows that ordinary glycerolethers, after incorporation into phospholipids, can activate the body's immune defence system. Tumour cells have only a low activity of enzymes which can break down ethers. This means that alkylethers are incorporated into the cell membrane's phospholipids which are then recognised and attacked by macrophages which have a high activity of ether catabolic enzymes. Since no macrophages were involved in our study on the effects of alkylglycerols on cellular growth in the tumour cultures, there

must also be other explanations for the effects on the tumour cells. The shark liver oil preparation that we used contained 2-3 percent methoxy-substituted alkylglycerols. The methoxy group may block and disturb the cell membranes more than the ordinary alkylglycerols. One theory is that we are dealing with a selective competitive inhibition that is disrupting malignant cells more than normal cells. The effected tumour tissue will then be more easy to treat with chemotherapeutic agents. They may also prevent replication of tumour cells by altering the structure of the lipid membrane thus causing increased fragility of tumour cells.

Interactions between different types of alkylglycerols and human neutrophil granulocytes have been studied by Palmblad, Samuelsson and Brohult (1990)[27]. Platelet activating factor (PAF) was the most potent with regard to the ability to produce an oxidative response followed by the methoxy-substituted alkylglycerols. The study shows that there is a dissociation between the ability of an alkylglycerol to initiate oxidative and calcium responses, indicating strict structure-activity relationships for the different alkylglycerols studied.

CONCLUSION

Alkylglycerols have demonstrated the potential to inhibit tumour cell growth, and in combination with chemotherapeutic agents, specifically, doxorubicin

(Adriamycin®) and fluorouracil, inhibit tumour cell growth and augment the cytotoxic effects of chemotherapeutic agents in tumour cell culture. Further evaluation of the role of alkylglycerols in cancer therapy based on the results of this study may prove beneficial.

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