Journal of Medical Sciences

ISSN 1682-4474

science alert

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Research Paper

JMS (ISSN 1682-4474) is an International, peer-reviewed scientific journal that publishes original article in experimental & clinical medicine and related disciplines such as molecular biology, biochemistry, genetics, biophysics, bio-and medical technology. JMS is issued eight times per year on paper and in electronic format.

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J. Med. Sci., 7 (3): 383-389
1st April, 2007

Modified Pectin Compounds Exert Different Effects on Ehrlich Ascites Tumor Cells and Lewis Lung Carcinoma and on Efficiency of Cyclophosphamide in Mice

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Pectins belonging to a group of substances termed dietary fibers were shown to have some anticancer properties. The main goal of this work was to estimate effects of low esterified pectin and calcium pectate on the Ehrlich ascites adenocarcinoma and Lewis lung carcinoma. Simultaneously the influence of polysaccharides on the efficiency of antitumor drug cyclophosphamide was assessed. Before the work the exact characteristic of the pectin samples was performed. Experiments were carried out in mice with tumor cells transplanted. The experimental mice were administered pectin preparations through gastric gavage for 7 days in experiment with Ehrlich ascites adenocarcinoma and for 9 days in experiment with Lewis lung carcinoma. Half of mice were also intramuscularily injected 150 mg kg^{-1} of cyclophoshamide. At the end of experiment animals were killed and the parameters of the tumor growth and metastasis were assessed. The results showed that calcium pectate in a dose dependent manner suppressed the growth of Ehrlich ascites tumor cells but did not affect the growth and metastasis of Lewis lung carcinoma. Low esterified pectin does not alter the growth of both tumors. At the same time, simultaneous administration of both pectin compounds and cyclophosphamide resulted in lowered growth rate of the tumors. The findings suggest that pectin compounds possess antitumor activity although different kinds of the polysaccharides do not exert similar effects on the different types of tumor cells. Also it was shown that pectins have a capacity to strengthen the efficiency of cyclophosphamide.

Key words: Cyclophosphamide, ehrlich ascites tumor, lewis lung carcinoma, mice, pectin

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INTRODUCTION

Pectins belong to a group of substances generally termed dietary fibers, which are the essential part of the plant food and were proved to have numerous beneficial effects on human health (Suter, 2005). They are widely used in food industry because of their gelling and thickening properties (May, 1990). Pectins are the ionic plant polysaccharides functioning as a hydrating agents and cementing substances for the cellulose network (Thakur *et al*., 1997). The main structural features of pectin consist of the linear chains containing more than 100 of (1→4)-linked α-D-galacturonic acid units (Thibault *et al*., 1993) presenting non-branched blocks of molecule. These smooth homogalacturonic regions are interrupted with hairy hamnogalacturonic parts, in which galacturonic acids are interspersed with (1→2)-linked α-L-rhamnopyranosyl residues carrying neutral side sugar chains (Schols and Voragen, 1996). The long linear chains of homogalacturonan are partially esterified with methanol. Natural pectins mainly are highly esterified while pectins with lower degree of esterification can be prepared (Ridley *et al*., 2001).

As it was mentioned pectins exert various pharmacological effects. Among them there is an activity of pectin substances on cancerogenesis proved through *in vitro* experiments. For example, pectins were shown to exert antimutagenic activity against nitroaromatic mutagens (Hensel and Meier, 1999) and induce apoptosis in human colonic adenocarcinoma cells (Olano-Martin *et al*., 2003). Besides, pectins were found to suppress 1,2-dimethylhydrazine-induced colon carcinogenesis in rats (Heitman *et al*., 1992). Pectin incorporated into the basal diet inhibited the growth of the transplanted tumors, belonging to two tumor lines, TLT (a transplanted liver tumor) and EMT6 (a mammary carcinoma) in mice (Taper and Roberfroid, 1999). Aberrant crypt foci are putative preneoplastic lesions that occur in the colon of both animals and man. Ten percent pectin diet significantly suppressed a formation of azoxymethane-induced aberrant crypt foci in the colon, reduced of cecal β-glucuronidase activity and increased cecal short-chain fatty acids levels 3- to 5-fold (Rao *et al*., 1998). There are also results showing that oral modified citrus pectin significantly reduces metastases of implanted prostate adenocarcinoma cell lines in rats (Pienta *et al*., 1995) and inhibit the human cancer cell growth and metastasis in nude mice (Nangia-Makker *et al*., 2002).

Because it is generally recognized that although pectins may act as anticarcinogens but their effects are significantly less pronounced than that of antitumor drugs, it appeared worthwhile to examine if pectins may influence the efficacy of the antitumor drugs. Therefore, the two main goals of the present work were investigation of the possible anticarcinogenic effect of pectin preparations on the growth of two lines of transplantable tumors and assessment of the influence of them on the anticarcinogenic activity of the antitumor drug in rats. The research was carried out through two experiments. In the first experiment the effects of pectin preparations on the growth of Ehrlich ascites adenocarcinoma and efficacy of cyclophosphamide was studied. In the second experiment the influence of pectin compounds on the growth and metastasis of the Lewis lung carcinoma and antitumor and antimetastasis efficacy of cyclophosphamide was investigated.

MATERIALS AND METHODS

Chemicals, drugs and cell lines: In both experiments was used approved preparation of cyclophosphamide (Cyclophosphane. Lens-Pharm, Co., Ltd., Moscow. Russia).

Ehrlich ascite adenocarcinoma cells and Lewis lung carcinoma cells were provided by Laboratory of Biomodeling (Scientific Research Institute of Pharmacology, Tomsk, Russia). Ehrlich ascites adenocarcinoma cells were transplanted with ascites fluid obtained in donor-mice on 7th day after inoculation, which was diluted with physiologic solution. Prepared suspension containing 7.5×10^6 tumor cells in 0.2 mL was injected intraperitoneally in healthy mice. Donor animals with solid Lewis lung carcinoma were killed under slight ether anesthesia by cervical dislocation; the tumor parts with no necrotic signs were removed, cut into small pieces in a blender. Tumor tissue was homogenized in sterile physiologic solution. Prepared suspension containing 5×10^6 tumor cells in 0.1 mL was transplanted into healthy mice. Before transplantation the number of cells was counted using a microscope.

High-esterified citrus pectin without additives was obtained from Copenhagen Pectin A/S, Lille Skensved, Denmark. The stated degree of esterification of this preparation was 60.0%. The pectin preparation contained no acetyl or amide groups. The sample of high esterified pectin was used for preparation of the samples of low esterified pectin and calcium pectate. All other chemicals were of the highest quality available. Distilled water was used throughout. Initially pectin with the degree of esterification approximately 1.0% was prepared. During this process 100 g of high esterified citrus pectin was de-esterified in 1600 mL 50% ethanol containing 20 g NaOH and 20 g KOH (30 min at 20°C). After acidification, pectin was isolated from ethanol by filtration. For the preparation of calcium pectate 100 g of low esterified pectin was suspended in 500 mL 70% ethanol. Gradually

100 mL of 70% ethanol solution containing 22.6 g $CaCL_2 \cdot 6H_2O$ was added. After the process was finished, calcium pectate was separated through a glass filter, rinsed with 800 mL 70% ethanol and dried at 60°C.

Pectin analysis: The galacturonan content of the pectin preparation was determined colorimetrically by the m-hydroxydiphenyl method (Blumenkrantz and Asboe-Haunsen, 1973). The degree of esterification was characterized using titrimetric analysis (Afanasyev *et al*., 1984). Intrinsic viscosity of pectin was determined in 0.05 M NaCl/0.005 M Na-oxalate at 25.0°C and pH 6.0 using an Ubbelohde viscosimeter. The intrinsic viscosity is related empirically to the molecular weight by the Mark-Howink relation (Anger and Berth, 1986).

Test animals and diet: Healthy young female mice weighing 20 g each were obtained from Laboratory of Biomodeling (Scientific Research Institute of Pharmacology, Tomsk, Russia). The animals were housed in plastic cages (in groups of six-eight per cage) and kept in an isolated room under standard conditions of temperature (20-22°C), ambient humidity (50-55%) and 12 h artificial light-dark cycle (8.00-8.00 pm). Animals were first adapted to the facility for 1 week. The mice had free access to food and water. The composition of the standard diet was as follows (g/100 g): casein, 21.0; cellulose, 5.3; sunflower oil, 7.0; cholesterol, 1.0; sucrose, 15.0; starch, 45.9; methionine, 0.3; minerals, 3.5; vitamin mixture 1.0. The use of animals in this study was in accordance to the principles and guidelines of the Scientific Research Institute of Pharmacology which is following the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

Experimental design: There were two experiments carried out, in which for a modeling of cancer growth the viable neoplastic cells of two lines of transplantable mouse tumors were transplanted into mice.

In the first experiments 7.5×10^6 Ehrlich ascites adenocarcinoma cells were intraperitoneally transplanted into 73 young female inbred mice. Then all animals were randomly divided into 10 groups. The control group was administered 0.3 mL of distilled water through a gastric gavage daily for 7 days beginning at the second day of experiment. On the 4th day of experiment 0.1 mL of physiological solution was injected intramuscularly. Four groups of mice were daily given through a gastric gavage from the second day of the experiment a suspension containing 50 and 100 mg kg^{-1} of calcium pectate and solution containing 50 and 100 mg kg^{-1} of low esterified pectin, respectively. The remaining five groups of mice were treated the same way but all animals on the 4th day of the experiment were injected intramuscularly 0.1 mL of physiological solution containing 150 mg kg^{-1} of cyclophosphamide. At day 8 of experiment the mice were given light ether anesthesia and killed by cervical dislocation. Ascites was collected from abdominal cavity and its volume was estimated. Total volume of the tumor cells in the ascites was assessed after the five minute centrifuging at 3000 rpm.

In the second experiment 5×10^6 Lewis lung carcinoma cells were intramuscularly transplanted into 95 female C57B1/6 mice. Then all animals were divided into 10 groups. The control group was administered 0.3 mL of distilled water through a gastric gavage daily for 12 days beginning at the eighth day of experiment. On the 14th day animals of this group were injected intramuscularly 0.1 mL of physiological solution. Similarly to the first experiment, four groups of mice were daily given through a gastric gavage from the eighth day of the experiment a suspension containing 50 and 100 mg kg^{-1} of calcium pectate and solution containing 50 and 100 mg kg^{-1} of low esterified pectin, respectively, for 12 days. The remaining five groups of mice were treated the same way but all animals on the 14th day of the experiment were injected intramuscularly 0.1 mL of physiological solution containing 150 mg kg^{-1} of cyclophosphamide. At day 21 the mice were ether anaesthetized, then killed by decapitation and autopsied. Primary tumor nodule was removed from the lung and weighed. The number of metastasis on the internal pleural surface was counted, two perpendicular metastasis dimensions were measured with a vernier caliper and the mean metastasis surface in mm^2 was calculated for each mouse. Also there was calculated frequency of metastasis as the ratio between number of the mice with metastasis and the total number of mice with transplanted Lewis lung carcinoma.

Statistical analysis: The results are expressed as mean±SEM (Standard Error of Mean). Results obtained at the end of the study were analyzed using one-way ANOVA using post hoc Tukey's test and p values were determined. Differences were considered significant at $p<0.05$. Statistical analysis was performed using software package SPSS (Statistical Package for Social Sciences) for Windows, version 11.0.

RESULTS

Physico-chemical properties of pectin preparations: The main physico-chemical parameters are shown in Table 1. The pectin preparations studied were not differing in molecular weight, anhydrogalacturonic acid content and the degree of esterification. But the differences were in the calcium content and the water solubility.

Experiment 1: Calcium pectate suppressed the growth of Ehrlich ascites tumor cells. In mice given 50 mg kg^{-1} of the body weight of calcium pectate ascites volume and total volume of the tumor cells was 37.5 and 26.7%, respectively, lower than in the control group. In mice given 100 mg kg^{-1} of the calcium pectate preparation the volume of the ascites and total volume of the tumor cells was 44.3 and 47.5%, respectively, lower than in the control group. Both doses of low esterified pectin did not contribute to the alterations of the ascites volume and total volume of the tumor cells (Table 2).

In mice administered cyclophosphamide the ascites volume and total volume of the tumor cells was averagely 48.9 and 41.7%, respectively, lower than in the control group. After the course of combined administration of cyclophosphamide and calcium pectate used in both doses tested there were no noted any changes of the cyclophosphamide efficiency. In contrast in mice given simultaneously cyclophosphamide and 100 mg kg^{-1} of low esterified pectin the total volume of the tumor cells was 41.4% lower than in the group of animals given cyclophosphamide only ($p<0.05$).

Table 1: Physico-chemical properties of tested pectin preparations

Properties	Calcium pectate	Low-esterified pectin
Molecular weight	39300	39300
Anhydrogalacturonic acid	67.3%	69.0%
Degree of esterification	1.2%	1.2%
Calcium	38 (mg g^{-1})	nd
Protein	nd	nd
Methyl ester	nd	nd
Acetyl ester	nd	nd
Water solubility	Insoluble	Soluble

nd - not determined

Experiment 2: There were no differences detected in the weight of the primary nodule of the lung Lewis carcinoma, frequency of metastasis, number of metastasis and the metastasis area between the control group and groups of mice given both doses of calcium pectate and low esterified pectin (Table 3).

As expected administration of cyclophosphamide to the mice resulted in reduction of the tumor weight and frequency of metastasis in comparison with the control group by 30.2 and 12.5%, respectively, whereas metastasis number and metastasis area was 4.8 times and 15.0 times, respectively, lower than in the control. Combined administration of the preparations studied and cyclophosphamide showed that the pectin compounds do not influence the tumor weight, number of metastasis and metastasis area. At the same time there were found differences in the frequency of metastasis. In the groups given 50 mg kg^{-1} of calcium pectate and 100 mg kg^{-1} of low esterified pectin the frequency of metastasis of averagely by 20.0 and 11.1% lower, respectively, than in mice given monotherapy with cyclophosphamide.

Table 2: Effects of pectin preparations on the Ehrlich adenocarcinoma growth in mice and efficiency of the treatment with cyclophosphamide

Group of animals	Ascites volume (mL)	Total volume of the tumor cells (mL)
Control, n = 5	5.26±0.48	1.20±0.09
Calcium pectate (50 mg kg^{-1}), n = 7	3.29±0.38† **	0.88±0.11†*
Calcium pectate (100 mg kg^{-1}), n = 7	2.93±0.52† **	0.63±0.17†*
Low-esterified pectin (50 mg kg^{-1}), n = 7	5.80±0.49	1.30±0.12
Low-esterified pectin (100 mg kg^{-1}), n = 8	4.38±0.42	1.26±0.16
Cyclophosphamide, n = 7	2.69±0.39† **	0.70±0.12†*
Cyclophosphamide + Calcium pectate (50 mg kg^{-1}), n = 9	2.75±0.57	0.67±0.17
Cyclophosphamide + Calcium pectate (100 mg kg^{-1}), n = 7	2.79±0.26	0.63±0.14
Cyclophosphamide + Low-esterified pectin (50 mg kg^{-1}), n = 8	2.99±0.77	0.85±0.24
Cyclophosphamide + Low-esterified pectin (100 mg kg^{-1}), n = 8	2.16±0.22	0.41±0.06‡*

† Compared with the control group, ‡ Compared with the mice treated with cyclophosphamide, *$p<0.05$, **$p<0.01$

Table 3: Effects of pectin preparations on the lung lewis carcinoma growth in mice of C57BL/6 line and efficiency of the treatment with cyclophosphamide

Group of animals	Tumor weight (g)	Frequency of metastasis (%)	Metastasis number	Metastasis area (mm^2)
Control, n = 9	6.29±0.25	100.0	24.0±0.4	45.1±11.2
Calcium pectate (50 mg kg^{-1}), n = 9	5.44±0.61	100.0	24.7±4.0	36.2±11.7
Calcium pectate (100 mg kg^{-1}), n = 11	5.06±0.43	100.0	21.2±2.2	33.7±10.0
Low-esterified pectin (50 mg kg^{-1}), n = 9	6.21±0.27	100.0	18.4±4.8	31.7±10.6
Low-esterified pectin (100 mg kg^{-1}) , n = 11	5.58±0.24	100.0	25.6±3.4	53.0±1.4
Cyclophosphamide, n = 8	4.39±0.46† **	87.5	5.0±1.7† ***	3.0±1.6† ***
Cyclophosphamide + Calcium pectate (50 mg kg^{-1}), n = 10	4.75±0.30	87.5	4.1±1.5	2.1±1.6
Cyclophosphamide + Calcium pectate (100 mg kg^{-1}), n = 8	4.29±0.36	70.0‡*	5.8±1.8	4.1±1.4
Cyclophosphamide + Low-esterified pectin (50 mg kg^{-1}), n = 11	3.81±0.37	81.8	3.9±1.5	3.6±1.6
Cyclophosphamide + Low-esterified pectin (100 mg kg^{-1}), n = 9	5.19±0.37	77.8‡*	4.4±1.9	2.7±1.0

† Compared with the control group, ‡ Compared with the mice treated with cyclophosphamide, *$p<0.05$, **$p<0.01$, ***$p<0.001$

DISCUSSION

The use of dietary fibers having protective and/or preventive effects on cancer progression and metastasis is an important emerging field of research. Identifying of the new food supplements and understanding of their mechanisms of action are some of the main challenges in the usage of functional foods as a cancer therapeutics modality. Since the works of Burkitt (1969) who first have related the low incidence of colon cancer in parts of Africa to the diets with high contents of plant foods and dietary fibers, there were numerous papers published showing controversial results (Obrador, 2006). A combined analysis as well as meta-analyses of case-control studies indicated an inverse association between fiber intake and colorectal cancer (Trock *et al*., 1990; Howe *et al*., 1992; Bingham *et al*., 2003), whereas some prospective studies have largely failed to support this association (McCullough *et al*., 2003; Michels *et al*., 2005; Otani *et al*., 2006). There is the report suggesting a decreased risk of colon, but not rectum, cancer with increasing intakes of insoluble dietary fiber (Wakai *et al*., 2006). Some epidemiological data suggest that increased intake of fiber may be associated with the diagnosis of breast cancers of more favorable prognosis (Giles *et al*., 2006). Some works were focused on pectin compounds, in which it has been shown that modified pectin, whether administered orally (Pienta *et al*., 1995) or intravenously (Platt and Raz, 1992), reduces both spontaneous and experimental lung colonization of tumor cells, while intravenous injection of the murine B16-F1 melanoma cells with the natural citrus pectin results in a significant increase (up to threefold) in the appearance of tumor colonies in the lung (Platt and Raz, 1992). Daily oral administration of modified citrus pectin reduced the growth of intramuscularly transplanted mouse tumors from tumor cell lines TLT and EMT6 (Taper *et al*., 1997) as well as inhibited the tumor growth and metastasis of orthotopically grown breast and colon cancer cells (Nangia-Makker *et al*., 2002). The diet supplemented with 20% apple pectin significantly decreased the number and incidence of dimethylhydrazine- or azoxymethane-induced colon tumor in rats. Alongside there is an opinion suggesting that foodstuffs containing high amounts of indigestible polysaccharides such as pectin may be an additional risk for cancer patients (Noack *et al*., 2000).

In the present study, we have investigated the effects of two pectin compounds differing in their calcium content and, hence, water solubility on the growth of two cell lines of transplantable tumors. The results obtained were different. Insoluble calcium pectate inhibited growth of Ehrlich ascites adenocarcinoma cells but did not influence the Lewis lung carcinoma. Soluble low esterified pectin with no calcium content did not affect the growth of the both tumor lines. And both pectin compounds had no effects on the metastasis of the Lewis lung carcinoma. At the same time both pectin compounds have significantly proved beneficial influence on the efficiency of cyclophosphamide, although this influence was not the same in regard two tumor species.

The results suggest that different tumor cells have a different sensitivity regarding pectins and at the same time, pectin compounds with different physicochemical properties do exert not similar effects on the same tumor cells.

The exact mechanisms of antitumor activity of polysaccharides are not clear yet. Though there are several hypothetical mechanisms that may be involved in the inhibitory and/or anticarcinogenic effect of pectins on tumor growth and appearance. Pectins like other dietary fibers are not digestible by endogenous enzymes, but they are fermented by colon microflora and act as prebiotics, thus modifying the composition of colonic microflora (Gibson and Roberfroid, 1995). Such alterations of the colonic microflora have been reported to have an inhibitory action on tumor incidence and/or growth (Reddy, 2000). Pectins increase the number of lactobacilli in a colon, which were shown to have antigenotoxic and anticarcinogenic effects (Rumney and Rowland, 1995). Products of the dietary fiber fermentation short-chain fatty acids including butyrate, propionate and acetate were proved to be associated with suppressed proliferation in transformed cells and do not change proliferation of the normal cells (Comalada *et al*., 2006). In addition, apoptosis may be increased in transformed cells but inhibited in normal cells when butyrate is present (Marchetti *et al*., 1997).

In *in vitro* experiment was shown that dietary pectin and its degradation products induce apoptosis in human colonic adenocarcinoma cells (Olano-Martin *et al*., 2003). Besides, soluble pectin regulates expression, function and distribution of apoptotic-related proteins in the crypt during colon carcinogenesis, induced by dimethylhydrazine in rats and these changes probably induce a reduction in tumor volume (Avivi-Green *et al*., 2000). The enhancement of apoptosis associated with pectin feeding may be caused by modulation of the redox environment that promotes reactive oxygen species-mediated apoptosis (Sanders *et al*., 2004).

However the mentioned mechanisms can not explain all aspects of the pectin antitumor activity due to the found specificity of these effects regarding various tumor cells. Therefore, more profound studies of the mechanisms involved in antitumors influence may lead to

a considerable improvement in the understanding of action of all substances included into the dietary fiber group, thus providing their application as food components reducing the risk of cancer.

The results presented in the current study are the first report showing a possibility of enhancing efficiency of cytotoxic drugs with the use of modified pectin compounds. The mechanism of this influence is not clear yet, but application of non toxic and safe functional food components purposed for increased efficiency and reduced dosage of generally highly toxic antitumor drugs is considered as perspective direction in anticancer therapy.

ACKNOWLEDGMENTS

The present study was supported by Grant No. 05-II-CM-05-006 from the Presidium of the Far East Branch of Russian Academy of Sciences and Presidium of the Siberian Branch of Russian Academy of Medical Sciences.

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