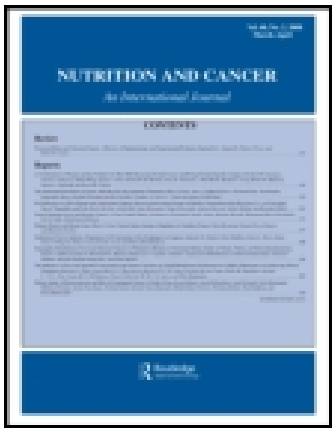

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Milk Stimulates Growth of Prostate Cancer Cells in Culture

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Concern has been expressed about the fact that cows' milk contains estrogens and could stimulate the growth of hormone-sensitive tumors. In this study, organic cows' milk and two commercial substitutes were digested in vitro and tested for their effects on the growth of cultures of prostate and breast cancer cells. Cows' milk stimulated the growth of LNCaP prostate cancer cells in each of 14 separate experiments, producing an average increase in growth rate of over 30%. In contrast, almond milk suppressed the growth of these cells by over 30%. Neither cows' milk nor almond milk affected the growth of MCF-7 breast cancer cells or AsPC-1 pancreatic cancer cells significantly. Soy milk increased the growth rate of the breast cancer cells. These data indicate that prostate and breast cancer patients should be cautioned about the possible promotional effects of commercial dairy products and their substitutes.

INTRODUCTION

The role of diet in prostate and breast cancer has been the subject of much concern. For over 30 years, it has been recognized that the incidence of these cancers is much lower in Asian countries, and that immigration of people native to these countries into the United States results in an increase in their probability of developing a hormonally related cancer. Dairy products, in particular, have been suspected of contributing to the progression of prostate and breast cancers, because the presence of hormones in milk could serve to stimulate receptor-positive tumors. Both epidemiological and experimental studies support this. Between 1950 and 1998, the consumption of milk products in Japan increased by 20-fold. This was accompanied by a 25-fold increase in prostate cancer deaths between 1947 and 1995 (1). Likewise, the incidence of breast cancer has been increasing. A meta-analysis of studies published between 1984 and 2003 resulted in a combined odds ratio for milk consumption and prostate cancer of 1.68 (2). A subsequent study found a relative risk of 1.13 (3). Several other studies have found positive correlations between dairy consumption and prostate cancer (4–6).

Milk contains estrogens E_1, E_2, and E_3 and several of their metabolized forms (7). Consumption of these estrogens could alter the delicate balance of hormones known to be implicated in breast cancer, and recent data indicate that they also could play a significant role in prostate cancer (8,9). In addition to introducing estrogens, which can stimulate growth of receptor-positive tumors, dairy products can increase circulating levels of insulin-like growth factor 1 (IGF-1) and cause upregulation of the IGF-1 receptor on prostate cancer cells (10). Data from a large-scale study involving individuals participating in the European Prospective Investigation into Cancer and Nutrition show an increase in IGF-1 levels and a decrease in insulin-like growth factor binding protein-2 (IGFBP-2) levels to be correlated with dairy consumption (11). In breast cancer, progression is known to be accompanied by an increase in the estrogen receptor and the IGF-1 receptor (12).

IGF-1 is present in milk and is known to stimulate growth of tumors, but its role in the development of prostate and breast cancers is controversial. Although overproduction of IGF-1 in transgenic animals can cause cancer (12–14), high levels of IGF-1 have been reported to increase the risk of breast cancer in premenopausal women, but not postmenopausal ones (15). A study of breast cancer in premenopausal women in the United

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States, Sweden, and Italy found a positive but not statistically significant correlation between IGF-1 levels and breast cancer (16). Likewise, Platz et al. reported a slightly increased risk of prostate cancer in men with higher levels of circulating IGF-1, but no significant correlation for invasive or metastatic cancer (17). Levels of this molecule are higher in people who consume dairy products (18) and estrogens can upregulate IGF-1 receptor concentrations on prostate cells, thereby sensitizing the cells to this growth stimulator (9,10). Thus, there could be a synergistic promotional effect involving these 2 components of bovine milk, stimulating the replication of transformed cells.

Data from studies with rodents support the link between dairy consumption and cancer. Japanese researchers have found milk to enhance the progression of prostate cancer in rats initiated with 2-amino-methyl-6-phenylimidazo[4,5-b]pyridine (19) and breast cancer in rats initiated with 7,12-dimethylbenz[a]-anthracene (20–22). These results imply that in humans consumption of dairy products could promote conversion of precancerous lesions or mutated cells to invasive cancer and enhance the progression of hormone-dependent tumors.

The data reported here indicate that cows' milk can significantly stimulate the growth of prostate cancer cells but seems to have little effect on the growth of MCF-7 breast cancer cells or AsPC-1 pancreatic cancer cells. The E_2 present in the milk and the major milk protein casein also stimulated prostate cancer cell growth. Soy milk was found to stimulate the growth of MCF-7 breast cancer cells, probably because of the xenoestrogens present, but this milk substitute had little effect on prostate cancer cells. In contrast, almond milk significantly suppressed growth of the prostate cancer cells.

MATERIALS AND METHODS

Test Solutions

α-Estradiol and 16α-hydroxyestrone (HE) were obtained from Sigma Chemicals (St. Louis, MO). IGF was purchased from R&D Systems (Minneapolis, MN). R1881 (methytrienolone) was obtained from PerkinElmer (Boston, MA). Commercially prepared bovine milk, almond milk, and soy milk, along with fresh almonds, were purchased from a local market. The fresh almonds were prepared for in vitro digestion as follows: 3.8 g were soaked overnight in 200 mL of distilled water. The soaked almonds and water were added to a blender and pureed for 10 min. Casein and lactose were obtained from Sigma Chemicals (St. Louis, MO). Both were prepared for in vitro digestion as follows: 345 mg casein was suspended in 10 mL of distilled water and 30 mg of lactose was dissolved in 10 mL of distilled water. Pepsin, pancreatin, and bile salts used for in vitro digestion were purchased from Sigma Chemicals (St. Louis, MO).

Cell Lines

The following cell lines were purchased from the American Type Culture Collection (ATCC; Bethesda, MD): AsPC-1, LNCaP clone FGC (CRL-1740), DU145, LoVo, and MCF-7. The AsPC-1 and LNCaP cells were maintained in RPMI-1640 medium (ATCC, Bethesda, MD) supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals, Atlanta, GA). The DU145 and MCF-7 cells were cultured in Eagles Minimum Essential medium (ATCC, Bethesda, MD) supplemented with 10% FBS (Atlanta Biologicals, Atlanta, GA). The complete medium for the MCF-7 cells also contained 0.01 mg/mL bovine insulin (Sigma Chemicals, St. Louis, MO). The LoVo cells were cultured in F-12K (ATCC, Bethesda, MD) medium with 10% FBS.

In Vitro Digestion

A 10 mL aliquot of each test solution (pureed almonds, commercial milks, casein, and lactose) was digested in vitro as described below. The pH of each test solution was adjusted to 2.8 with 6 N HCl followed by the addition of 0.5 mL of a pepsin suspension (4 g pepsin/100 mL of 0.1 N HCl). The test solutions were incubated with shaking at 37°C for 2 h. Following the incubation period, the pH of the test solutions was adjusted to 5.7 with 5 M NaOH. Then 2.5 mL of a pancreatin-bile salt mixture (0.2 g pancreatin and 1.2 g bile salts suspended in 100 mL of 0.1 M $NaHCO_3$) was added to the test solutions and incubated as above for an additional 2 h. Each test solution was centrifuged at 4500 rpms for 10 min at room temperature. The supernatant was decanted into 15 mL conical tubes for storage at 4°C. An aliquot of each test solution was filter sterilized through a 0.2-μm syringe filter prior to addition to the growth medium. Controls contained equivalent amounts of sterilized phosphate buffered saline.

Cell Proliferation Assay

Cells from culture flasks were diluted as follows to give the following concentrations: 5×10^4 AsPC-1; 5×10^4 LNCaP; 2×10^5 MCF-7; 5×10^4 LoVo; and 2×10^5 DU145 cells/mL. Growth curves done in earlier experiments indicated that these concentrations produced readings in the most sensitive range of absorbance for the cell proliferation assay. For LoVo and AsPC-1 cells, 0.1 mL of this suspension was added to each well of a 96-well plate. After 24-h incubation at 37°C, 5% CO_2, both of these cell types were treated with test solutions and incubated for an additional 48 h. For the estrogen-sensitive LNCaP, MCF-7, and DU145 cells, 0.1 mL of suspended cells were added to each well of a 96-well plate and incubated for an initial 24 h. After this time, the medium was changed to medium supplemented with 10% charcoal dextran treated FBS (Hyclone Laboratories, Logan, UT) and incubation continued for another 24 h before the test solutions were added. The treated MCF-7 and DU145 cells were cultured for 48 h with the test solutions. However, because of their slower growth rate, the LNCaP cells were cultured

for 6 days. Cell proliferation was assayed by the CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay (Promega Corp., Madison, WI) (MTS) according to manufacturer's directions. The absorbance measurements at 490 nm for the 4 wells were averaged for each solution tested.

Statistical Evaluation

In almost all cases, the results of 3 or more independent experiments were averaged for each substance tested on a particular cell type. In each experiment, the MTS absorbance of the treated cells was compared to that of an identical control without the test substance. However, the absorbance of the control culture could differ somewhat from one experiment to another, most probably because of slight differences in cell numbers and in the fraction of cells in different stages of the cell cycle. Therefore, in each experiment the effect of treatment was expressed as a ratio of the absorbance of the treated culture to that of the control for that experiment. These normalized values were then averaged for all of the different experiments done on a particular cell type treated with a specific substance. Specifically if the absorbance of the control averaged over the 4 wells of the culture plate had value A_0 and the average for the 4 wells for the test solution on that plate had value A_{ts}, the ratio A_{ts}/A_0 was calculated. The values of A_{ts}/A_0 were averaged for the 3 or more independent experiments done with this test substance on the cell type being studied. For each substance tested on a particular cell type, a Student's *t*-test analysis was done to compare the average of the normalized absorbance ratios for that test solution–cell type combination to the untreated control (=1.0 by definition). *P* values less than 0.05 were considered to be statistically significant.

RESULTS

To evaluate the possibility that dairy products might act as promoters of hormonally sensitive cancers, human prostate cancer and breast cancer cells were cultured in the presence of cows' milk that had been treated with a simulated digestion procedure involving treatment with pepsin at low pH followed by pH adjustment and treatment with pancreatin and bile salts. The milk was obtained from a commercial source and was certified organic. For comparison, almond milk and soy milk purchased from a local retailer were also tested. The results are presented in Table 1. Results of Student's *t*-test analyses are presented for those cases where growth in supplemented medium is different from that for the unsupplemented control.

In each of 15 experiments, cows' milk was observed to stimulate the growth of the LNCaP prostate cancer cells. These cells are ER^+ as is characteristic of most advanced cases of prostate cancer. Growth was stimulated by an average of 31% (*P* value less than 10^{-6}). Addition of 400 ng/mL of IGF-1 did not result in further enhancement of growth. Soy milk did not significantly affect growth of these cells, but almond milk and almond milk supplemented with IGF-1 both inhibited growth by approximately 30% (*P* values of 1.7×10^{-5} and 0.01, respectively).

Bovine milk did not significantly affect growth of MCF-7 breast cancer cells, even though they are also ER^+, and it also had no effect on AsPC-1 pancreatic cancer cells. Almond milk, likewise, did not affect growth of either of these 2 cell types, but soy milk stimulated the growth of MCF-7 cells by 28% ($P = 0.002$). No additional experiments were performed with the soy or almond milks because the composition was not clearly defined, and our primary goal was to investigate the possibility that dairy products could act as promoters of prostate or breast cancer.

TABLE 1
Normalized growth[a] (relative to unsupplemented control) of cancer cell lines supplemented with milk or milk substitutes

	Milk				
Cell type	Bovine	Bovine + IGF-1	Almond	Almond + IGF-1	Soy
LNCaP	1.31 ± 0.039^b $n = 15$ $P = 8.0 \times 10^{-7}$	1.25 ± 0.058^b $n = 5$ $P = 6.8 \times 10^{-3}$	0.66 ± 0.044^b $n = 10$ $P = 1.7 \times 10^{-5}$	0.71 ± 0.065^b $n = 4$ $P = 0.011$	1.18 ± 0.13 $n = 5$ $P = 0.11$
MCF-7	0.98 ± 0.049 $n = 5$		1.07 ± 0.06 $n = 6$		1.28 ± 0.027^b $n = 4$ $P = 0.002$
AsPC-1	1.05 ± 0.090 $n = 6$		1.11 ± 0.061 $n = 6$		1.22 ± 0.072 $n = 5$ $P = 0.099$

[a]All experiments were performed in charcoal-stripped medium supplemented with each of the components indicated. Normalized growth is defined as the absorbance for cells grown in the supplemented medium divided by absorbance for cells in the unsupplemented control culture medium. *n* is the number of experiments performed in each case and *P* is the Student's *t*-test result for comparison of the control and supplemented cell cultures.

[b]Growth medium supplements that significantly affect cancer cell growth using the criterion $P < 0.05$.

TABLE 2
Normalized growth (relative to unsupplemented control) of LNCaP cells supplemented with milk components[a]

Supplement	Growth	n	P
Casein	1.27 ± 0.12	7	0.18
Lactose	0.96 ± 0.07[b]	10	2×10^{-4}
E2 (10^{-8}M)	2.01 ± 0.14[b]	15	2×10^{-6}
IGF-1	0.92 ± 0.12	7	0.26
E2 (10^{-8}M) + IGF-1	2.07 ± 0.25[b]	6	4×10^{-3}
E2 (10^{-9}M)	1.86 ± 0.19[b]	7	3×10^{-3}
E2 (10^{-9}M) + IGF-1	1.96 ± 0.34	3	0.11
HE (10^{-8}M)	1.00 ± 0.06	10	0.48
HE (10^{-8}M) + IGF-1	0.98 ± 0.30	2	
HE (10^{-9}M)	0.92 ± 0.09	6	0.38
HE (10^{-9}M) + IGF-1	0.94 ± 0.39	2	

E2 indicates estradiol; IGF-1, insulin-like growth-factor-1 (400 ng/mL); HE, 16α-hydroxyestrone.

[a]All experiments were performed in charcoal dextran treated FBS medium supplemented with each of the components indicated. Normalized growth is defined as the absorbance for cells grown in the supplemented medium divided by absorbance for cells in the unsupplemented control culture. n is the number of experiments performed in each case and P is the Student's t-test result for comparison of the control and supplemented LNCaP cell cultures.

[b]Growth medium supplements that significantly affect cancer cell growth using the criterion $P < 0.05$.

Because of the clear stimulation of the LNCaP prostate cells by bovine milk, the milk components casein, lactose, and estradiol (E_2) were tested. Since it has been suggested that 16α-HE could stimulate breast cancer development, experiments were also done on LNCaP cells to test the effects of HE and HE combined with IGF-1. The results are given in Table 2. Casein had a stimulatory effect when averaged over 7 experiments, but this was found to vary from one experiment to another, with growth rates ranging from 0.90 to 1.70 times that of the control. A possible explanation for this variability could be that the sensitivity of the cells to casein supplementation might change with the fraction of cells is S phase at the time of the switch from FBS-supplemented medium to charcoal stripped medium plus casein.

E2-stimulated growth of the LNCaP prostate cells at concentrations of 10^{-8} M and 10^{-9} M, with an approximate doubling of growth at both concentrations. In preliminary dose-response experiments, IGF-1 did not affect LNCaP cell growth for concentrations up to 400 ng/mL, so this concentration was tested in combination with the estrogens. However, IGF-1 had no significant effect in the extent of stimulation observed for E_2. In 2 experiments, the androgen R1881 at 5nM stimulated LNCaP cells by approximately 75%, but 400 ng/mL of IGF-1 had no effect here either. In one experiment, the extent of stimulation by 5nM R1881 and by 5nM R1881 + IGF-1 were 79% and 76%, respectively. In the second experiment, these values were 71% and 70%. Likewise, concentrations of IGF-1 ranging from 50–400 ng/mL of IGF-1 had no effect on the growth of DU145 androgen insensitive prostate cancer cells. As a comparison, the same concentrations of IGF-1 were used to supplement the growth medium of LoVo colon cancer cells. Here, concentrations of 300, and 400 ng/mL stimulated growth by 30% and 34%, respectively ($P = 0.003$, 0.001). In each case, the data were normalized over 4 separate experiments. 16α-HE had no effect on the growth of LNCaP cells either alone or combined with IGF-1 (Table 2). Two experiments were done with E_2 and HE combined. The concentrations of E_2 and HE used were, respectively, 10^{-8}, 10^{-8}; 10^{-8}, 10^{-9}; 10^{-9}, 10^{-8}; and 10^{-9} and 10^{-9}. In all cases, the extent of stimulation observed for the mixture was not significantly different from that found for E_2 alone. We did not observe any evidence of apoptosis in any of the cell lines after application of any of the substances tested.

DISCUSSION

Based on the fact that cows' milk contains estrogens and several of their metabolites, it has been hypothesized that milk could stimulate the growth of estrogen-sensitive tumors, particularly those of breast and prostate tissues. In addition to the hormones, milk also contains IGF-1, a known mitogen for some cancer cells. Epidemiologic data clearly indicate a lower incidence of hormone-stimulated tumors in cultures in which milk consumption is low, but interpretation of these data is complicated by the fact that in those cultures the diets consist of large quantities of vegetables and small amounts of meat. Several studies on the tumorigenic properties of milk have been done in Japan, where fish consumption is high, resulting in increased intake of omega-3 fatty acids. Epidemiology indicates the possible implication of dairy products as promoters of cancer, but an experimental approach is complicated by the fact that the hormones and growth factors present in milk are natural growth regulators in test animals, and their effects are extremely concentration sensitive, depending not only on their independent concentrations but also on the ratios of the numerous steroids and growth factors to one another. The optimal ratios for maintaining health are very likely different for different species and even different individuals of a particular species or the same individual at different times. Perturbation of this delicate balance by consumption of dairy products could be detrimental to some individuals or to individuals in a particular subgroup (e.g., sex, age group, physical condition), but not to others. Also, differences in the metabolism of these components after ingestion probably alters their effects, resulting in another difficulty in comparing results from different individuals.

Experiments clearly show that in rodents treated with initiating carcinogens, cows' milk acts as a promoter of tumor growth and metastasis (18–22). No data are available regarding the possible effects of dairy consumption on humans from populations known to have been exposed to higher than average levels of

initiating agents; leaving the promoting effects of dairy products in humans an unresolved problem.

A recent case control study by Raimondi et al. of Canadian men found a twofold increase in prostate cancer risk as a result of milk consumption (18). In addition, experiments clearly show that in rodents treated with initiating carcinogens, cows' milk acts as a promoter of tumor growth and metastasis (19–23). Although no data are available regarding the possible effects of dairy consumption on humans from populations known to have been exposed to higher than average levels of initiating agents, an enhanced effect of dairy consumption might be found in these individuals.

In the study reported here, cows' milk, milk components, and 2 commercial milk substitutes were subjected to a simulated digestion process and tested for their effects on human cells isolated from hormone-sensitive cancers and known to have receptors for estrogens. Most of the experiments were performed with LNCaP prostate cancer cells. E2 alone at a concentration of 10^{-8} M caused a doubling of the growth rate of these cells. In each of 15 experiments, the digested cows' milk stimulated growth of the cells. The casein component of the milk also stimulated growth almost as much as the whole milk digested. Neither digested milk nor casein affected the growth of MCF-7 breast cancer cells or AsPC-1 pancreatic cancer cells.

IGF-1 was found not to affect growth of LNCaP cells, either alone or in combination with digested cows' milk or almond milk. It is possible that under these growth conditions, an autocrine effect overwhelms any stimulation resulting from supplemental IGF-1.

Two interesting results were obtained with the milk substitutes: 1) Almond milk suppressed growth of the prostate cancer cells, reducing their growth rate by 34%, but had no effect on the breast cancer or pancreatic cancer cells 2) Although soy milk showed a small, but not statistically significant, growth enhancement of the prostate and pancreatic cancer cells, it clearly stimulated growth of the breast cancer cells. This is in agreement with earlier studies reporting stimulation of MCF-7 growth by soy extracts and purified phytoestrogens. Wang, Sathyamoorthy, and Phang found concentration-dependent growth stimulation of these cells by genistein, a major isoflavone component of soy, at concentrations as low as 10^{-8} M. This was accompanied by increased expression of pS2, an estrogen-responsive gene (24). Hsieh et al. also observed stimulation of MCF-7 cell proliferation and enhanced expression of pS2 by genistein. In addition, these investigators demonstrated that genistein caused increased growth of tumors produced by implanting MCF-7 in ovariectomized athymic mice (25). Using the same system, Allred et al. reported dose-dependent growth enhancement of tumor growth in mice fed soy diets containing different amounts of genistein (26). Dip et al. studied the changes in global gene expression of MCF-7 cells treated with a mixture of phytoestrogens isolated from soy milk and compared these with changes induced by 17β-E2 and cow's milk. They found the soy-induced genetic fingerprint to be indistinguishable from that produced by E2 (27). Also, Qin et al. found commercial soy milk to stimulate the growth of tumors in rats initiated with 7,12 dimethylbenz[a] anthracene (28).

The results reported here support the hypothesis that consumption of milk products could promote the growth of prostate cancer. Although they could have a similar effect on breast cancers, these data do not support this. Only one type of breast cancer cells was tested. It is quite possible that other breast cancer cells might show sensitivity. It is clear that consumption of milk products by prostate cancer patients or of soy products by breast cancer patients should be undertaken with caution; however, there is no evidence at present indicating that these products could affect development or growth of other types of cancers.

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