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Combinatorial effect of fish oil (Maxepa) and 1α,25-dihydroxyvitamin D_3 in the chemoprevention of DMBA-induced mammary carcinogenesis in rats

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ABSTRACT

The present study demonstrates the anti-tumor effects of combined supplementations of dietary fish oil (Maxepa) and 1α,25-dihydroxyvitamin D_3 (vitamin D_3) on 7,12-dimethylbenz(α)anthracene (DMBA)-induced rat mammary carcinogenesis. Female Sprague–Dawley rats at 50 days of age were treated with 7,12-dimethylbenz(α)anthracene (DMBA; 0.5 mg/100 g body weight) by a single tail vein injection in an oil emulsion. Both fish oil (rich in EPA and DHA) and vitamin D_3 were administered orally at a dose of 0.5 ml/day/rat and 0.3 μg/100 μL propylene glycol twice a week respectively and continued to 35 weeks after DMBA administration. Fish oil in combination with vitamin D_3 resulted in a significant reduction in incidence, multiplicity and volume of mammary tumors. These supplementation also inhibited DMBA-induced mammary 7-methylguanine DNA adducts formation, which was measured by HPLC-fluorescence assay (at four sequential time points; ANOVA, $F = 42.56$, $P < 0.0001$). Immunohistochemical analysis revealed that the effect of fish oil and vitamin D_3 occurred through suppression of cell proliferation (BrdU-LI: $P < 0.0001$). Fish oil and vitamin D_3 together also reduced the mRNA expression of iNOS (84%, $P < 0.05$). In view of their natural availability, non-toxicity and acceptability; combined supplementation of fish oil and vitamin D_3 might be effective for chemoprevention of mammary carcinogenesis.

1. Introduction

Diet has a major role in the etiology of breast cancer. Different epidemiological studies indicate that breast cancer incidence rate varies among persons of various regions having different food habits [1]. Dietary fish oil (Maxepa) is a rich source of EPA and DHA. Epidemiological studies also reveal that fish oil containing n-3 fatty acids have beneficial effects to protect against colon, prostate, breast cancer [2]. One recent epidemiological study in Norway explored that cod liver oil and other dietary supplements affects survival of cancer patients with solid tumors [3]. A case control study of Korean women suggests that high consumption of fatty fish is associated with a reduced risk of breast cancer, and that the intake of omega-3 fatty acids from fish is inversely associated with post-menopausal breast cancer risk [4]. Mono- and unsaturated fats are considered as favorable indicators of breast cancer and carcinomas of other organs [5]. In recent days it has also been reported from different laboratories globally that consumption of specific fatty acids obtained mainly from marine fish is associated with lower incidences of breast cancer [6]. The inhibitory effects of fish oil (rich in EPA and DHA) on cancer development and progression are supported by many studies with cultured cells and animal models [7,8]. It has been reported from our laboratory that fish oil has protective role on early events of mammary carcinogenesis by modulation of DNA-protein cross-links, cell proliferation and p53 expression. Dietary fish oil also can induce apoptosis and modulates expression of Bax and Bcl-2 during DMBA-induced mammary carcinogenesis in rats [9].

Deprivation of sunlight or vitamin D levels in serum have been known to increase the incidence of colon, breast and prostate cancers [10–12] in human, as well as in chemically induced animal cancer models [13,14]. Vitamin D_3 was selected because it is involved in essential cell regulatory process, promotes cell differentiation and inhibits proliferation and the invasive potential of a number of different cancer cells in vitro [15]. Furthermore serum levels near the top of the physiological range of vitamin D_3 are not only associated with lower incidence but also a better prognosis of human cancer [16]. Some epidemiological reports show evidence of protection particularly against breast cancer [17] and low serum concentration of biologically active form of vitamin D has been correlated with increased breast cancer risk and metastasis. The effects of vitamin D_3 are mediated by vitamin D receptor

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(VDR). It is reported by Zinser and Welsh [18], VDR is present in over 80% of human breast cancers. They also have documented accelerated development of mammary gland in VDR knockout mice. Thus VDR impact on normal mammary gland development and tumorigenesis is very much evident [19].

Our laboratory has reported previously that the cytochrome enzymes were substantially reduced during the development of liver and mammary cancer [20]. Treatment with vitamin D_3 however resulted in the increased synthesis of these immunoreactive proteins. Further studies were also reported vitamin D_3 mediated induction of CYP3A4 mRNA in HepG2 cell line as well as in Caco-2 cells [21]. These points towards the role of vitamin D_3 on phase 1 enzyme system in controlling the process of tumorigenesis.

DMBA is the most well known polycyclic aromatic hydrocarbon that is used as a chemical inductor of model mammary carcinogenesis [22]. The main advantage of using DMBA as chemical carcinogen in rodent model is that it closely mimics human breast disease. Both of these cancers arise from ductal epithelial cells. Histopathological characterization of DMBA-induced mammary hyperplastic, premalignant and malignant lesions shows that rat mammary adenocarcinomas and the most common human breast carcinomas share several histological and morphological similarities [23].

Chemical carcinogen like DMBA binds with DNA which is considered to be essential for its tumor inducing ability. Inhibitory effect of any chemopreventive agent against DMBA induced tumorigenesis correlates with decreased binding of DMBA to DNA. Cell proliferation has important role in carcinogenesis as cancer proceeds through inactivation of negative regulators of cell proliferation and activation of positive regulators of cell proliferation [24]. Histopathology of a particular tissue is the basic and reliable marker to study development of neoplasia. Increased inducible nitric oxide synthase (iNOS) expression and/or activity were also reported in human lung gynecological, prostate, breast, head and neck, skin, stomach and central nervous system tumors [25]. Over expression of iNOS is associated with the progression of DMBA-induced rat mammary tumors. It results in excessive production of nitric oxide (NO). NO causes formation of toxic intermediates, such as peroxynitrite (ONOO–) and N_2O_3, causing tissue damage and genotoxicity. It leads to induction of deaminated DNA lesions; peroxynitrite-induced adducts formation and single strand breaks, interference with DNA repair, and/or cause post-translational modifications, potentially leading to tumor initiation and promotion [26].

Nutritional supplement and/or diet modification alone may not be sufficient to control risk of mammary cancer. Thus there is a necessity to combine dietary factors/life style factors with well-known chemopreventive agents and use them in combinations for cancer prevention. A single chemopreventive agent is unlikely to be effective for the prevention of cancer. There is convincing evidence that the combination approach does indeed provide greater efficacy than the administration of individual agent at higher doses. It really holds better promise to use multiple agents that can hit multiple targets in carcinogenesis. This particular combination chemoprevention approach is more preferred because: (a) combination treatment can hit more than one critical molecular target, (b) lower dose requirement lessening the concern of associated toxicities, and (c) acceptability in human. Further it is expected that rationale for using vitamin D_3 with fish oil is to enhance the chemoprevention activities using other substance that appear to be particularly exciting. Furthermore this combination approach may act synergistically to enhance the differentiating and antiproliferating activity of vitamin D_3 and fish oil as antioxidants and its ability to alter estrogen metabolism cooperatively affect antitumorigenic actions [27].

In this present study we have designed a DMBA-induced rat mammary carcinogenesis model to determine the chemopreventive efficacy of combined supplementation of vitamin D_3 and fish oil (Maxepa) on carcinogen-DNA adduct formation, cell proliferation, expression level of iNOS mRNA. The objective is to assess the inhibitory potential of vitamin D_3 and fish oil together in chemically induced mammary carcinogenesis of rat.

2. Materials and methods

2.1. Animals and housing

The entire experiment was conducted with inbred virgin female Sprague–Dawley rats, purchased from Indian Institute of Chemical Biology (IICB), CSIR (Kolkata, India) at weaning (3 weeks of their age). Throughout the acclimatization and experimental period, the animals were housed in autoclavable polypropylene cages (Tarson, Kolkata, India) (three rats per cage) in standard laboratory conditions (humidity 50–60%, lighting (12-h light/12-h dark cycle) and temperature $25 \pm 1\,^{\circ}C$). The recommendations of Jadavpur University "Institutional Animal Ethics Committee" ("Committee for the Purpose of Control and Supervision of Experiment on Animals" (CPCSEA Regn. No. 0367/01/C/CPCSEA), India) for the care and use of laboratory animals were strictly followed throughout the study.

2.2. Chemicals

All the reagents and chemicals unless mentioned were purchased from Sigma Chemicals Co. (St. Louis, MO), E. Merck (Frankfurter) Strame, Darmstadt, Germany and Santa Cruz Biotechnology (Santa Cruz, CA, USA). The purity of vitamin D_3 was verified by high performance liquid chromatography analysis to 99.99% purity.

2.3. Diet and treatment of animals

The animals were acclimatized for 2 weeks in the laboratory conditions before experiment. They had free access to modified American Institute of Nutrition (AIN)-76-based diet (casein – 25 g, DL-methionine – 0.3 g, wheat starch – 35.7 g, cellulose – 6 g, sucrose – 27.4 g, AIN-76 vitamin mixture – 4 g and AIN-76 mineral mixture – 1.6 g) and de-mineralized drinking water ad libitum.

Different sets of rats were randomly divided into 8 groups – A, B, C, and D as experimental groups. Group a served as normal control and b, c, d as respective control groups of B, C, D. At 7 weeks of age, female Sprague–Dawley rats of Groups A, B, C and D were given a single tail vein DMBA injection at a dose of 0.5 mg DMBA/0.2 ml corn oil/100 g body weight. There was no problem with intravenous injections using corn oil as this was a very common and routine practice of our laboratory [20]. Group A served as DMBA control. In addition to single tail vein DMBA injection, animals of Groups B, C and D were treated with fish oil (Maxepa), $1,25(OH)_2$ vitamin D_3 (vitamin D_3) and both fish oil + vitamin D_3 in combination respectively 2 weeks (5 weeks of age) prior to DMBA administration and continued to 35 weeks. Fish oil supplementation was started by oral gavage at a daily dose of 0.5 ml of Maxepa (Merck, India) [a commercially available preparation of concentrated fish oil rich in ω-3 fatty acids {a gelatin capsule (1 ml) contains 180 mg EPA and 120 mg DHA}]. Vitamin D_3 was given at a dose of 0.3 μg/100 μl propylene glycol, per os, twice a week. Group D was supplemented with vitamin D_3 and fish oil in combination at the same above-mentioned doses. Doses were selected on the basis of optimum effects on several studies of our laboratory in rats taking care of toxicity as supplementations did not result any significant changes of the body weight of experimental rats [9,28]. On the day of carcinogen treatment (50 days of age) vitamin D_3 was administered to Groups C and D half an hour before DMBA injection. Rats of control groups were subjected to a similar protocol but