Evaluation of Selected Chemopreventive Agents Present in Common Foods in Mouse Mammary Gland Organ Culture

Michael Hawthorne¹, Vernon Steele² and Rajendra G. Mehta¹

¹Department of Surgical Oncology, College of Medicine, University of Illinois at Chicago, Chicago, USA and ²Chemopreventive Agent Development Group, National Cancer Institute, Bethesda, Maryland, USA

Abstract

Prevention of cancer by natural and synthetic non-toxic chemopreventive agents has become a major research area in the past 15 years. The naturally occurring chemopreventive agents from the herbal medicine and edible plants can be evaluated in a variety of bioassays and identified for their activity as cancer preventive agents. We have adapted a mouse mammary gland organ culture assay (MMOC) for evaluating chemically pure chemopreventive agents for their activity to inhibit 7,12-dimethylbenz(a)anthracene (DMBA)-induced mammary alveolar lesions (MAL). Here, we report a list of 32 agents that are found in the herbs or edible foods and showing inhibition of more than 55% in MMOC. From the studies reported in the literature it appears that there is a good correlation between the effects in MMOC and effects observed with in vivo carcinogenesis models. Recently, we have modified the MMOC assay to evaluate efficacy of chemopreventive agents specifically the ones that may have anti-estrogenic activity. Thus, MMOC provides a valuable tool for preliminary evaluation of chemopreventive agents prior to conducting a long-term animal carcinogenesis studies.

Keywords: Mammary glands, organ culture, chemoprevention, natural products, preneoplastic lesions.

Introduction

In recent years the concept of chemoprevention has received considerable attention. The term chemoprevention was coined by Sporn while introducing preventive properties of retinoids, vitamin A analogs in a variety of experimental systems (Sporn et al., 1976; Sporn & Hong, 1997). The term was defined as a chemical that prevents or delays the development of any cancer at a non-toxic concentration. Since the introduction of the concept of chemoprevention, it has become one of the major fields of cancer research. The main difference between the prevention and therapy can be summarized as that prevention is most suited for people at a high risk of developing cancer or for the entire population if the agent is present in everyday edible food and can be consumed by general population, which may protect a person from developing cancer (Kelloff et al., 1999; Pezzuto, 1997). On the other hand, chemotherapy has to be reserved for patients with existing tumors. The major reason is that chemotherapeutic agents are often toxic and have unpleasant side effects such as loss of hair and nausea (Shanholtz, 2001; Perrone et al., 2002). However, the risk to benefit ratio is lower, in the sense the benefits outweigh the unwarranted side effects and therefore the treatment with chemotherapeutic agents is considered acceptable. Many, if not most, chemopreventive agents have shown antiproliferative effects against cancer cell growth in culture. This suggests that the chemopreventive agents have a potential as a chemotherapeutic agent or as an adjuvant to an established chemotherapeutic agent (Kelloff et al., 1999; Alberts et al., 1999).

Herbal or alternative medicine is being revisited in recent years (Wargovich et al., 2001). Throughout the world, the food habits have been largely considered as an important source of information for the cancer incidence of various target organs. For example, a classical case is often represented by Japanese women with much lower breast cancer incidence in Japan. However, when Japanese women migrate to the United States, their breast cancer incidence increases to the level of American women (Pineda et al., 2001). Lower incidence in Japanese women in Japan is largely attributed...
to food habits, which include soy, seaweeds, fish and low fat diet. Similarly, in India and China, consumption of herbs and spices have also recently been considered to have chemopreventive properties (Craig, 1999; Surh, 1999). On the other hand, chewing of betel nuts and tobacco is a proven risk factor for oral cancer (Thomas & Wilson, 1993). There are a few programs in the world, including the one at the University of Illinois, where edible food components are systematically evaluated in numerous bioassays and chemopreventive agents are isolated by a synchronized process of activity-guided fractionation (Kinghorn et al., 1998; Pezzuto et al., 1998). The recent examples of new chemopreventive agents include brassinin in Chinese cabbage (Mehta et al., 1997a,b,c), resveratrol in grapes (Jang et al., 1997) and deguelin from a non-edible African plant (Gerhauser et al., 1995).

Activity of chemopreventive agents is generally evaluated by using in vitro and in vivo experimental models. The in vitro models often use already transformed commercially available cancer cell lines for the studies. A true chemoprevention potential can be best described if the agent could inhibit the transformation or the progression of transformed cells. Very few in vitro models are available that would satisfy this requirements. We have established a mouse mammary gland organ culture (MMOC) model for such purpose in our laboratory (Mehta & Banerjee, 1975; Mehta et al., 1988). Here, mammary glands from normal young female Balb/C mice can be exposed to a carcinogen 7,12-dimethyldibenz(a)anthracene (DMBA) for a short period. With appropriate hormonal changes during a 24-day experimental period, the mammary epithelial cells attain a transformed phenotype (Lin et al., 1976) which has the potential of forming adenocarcinoma when transplanted in syngeneic mice (Telang et al., 1979). In MMOC, mammary glands develop ovarian steroids-independent mammary alveolar lesions (MAL) in response to DMBA, if the incubation medium is supplemented with aldosterone and hydrocortisone during the growth phase of the first ten days of culture. However, if the medium contains estradiol and progesterone instead of adrenal hormones, then the glands develop atypical mammary ductal lesions (MDL) (Mehta et al., 2001). Incubation of the glands with efficacious chemopreventive agents, during the first ten days of culture decreases the incidence and multiplicity of the MAL or MDL in the glands, depending on the hormones present during the first ten days of growth period. This can be used as a measure to determine the efficacy of the test agent. There is a very good correlation between the efficacy observed in vitro in MMOC and its effects in experimental mammary carcinogenesis models in vivo (Steele et al., 1997). We have used this MMOC model to evaluate efficacy of more than 300 chemopreventive agents. Unlike naturally derived and chemically isolated pure chemopreventive agents in an activity-guided fractionation, as a part of this study we evaluated synthetic pure chemicals with some chemopreventive potential. In this report we describe the effects of only the pure synthetic chemopreventive agents which are also found in common foods on the DMBA induced MAL in MMOC.

**Materials and methods**

The procedure and evaluation of chemopreventive agents in MMOC is previously described and schematically represented in Figure 1 (Mehta et al., 1997a,b,c). Briefly, BALB/c female mice (4 weeks old; Charles River, Wilmington, MA) were pretreated for 9 days with 1 μg of estradiol and 1 μg of progesterone. On the tenth day, the mice were sacrificed and the second thoracic mammary glands were dissected on silk and transferred to 60 mm culture dishes containing 5 mL of Waymouth’s 752/1 MB medium supplemented with 100 units of streptomycin and penicillin and 35 μg/mL glutamine. Fifteen glands were used per group. The glands were incubated for 10 days (37°C, 95% O2 + 5% CO2) in the presence of growth-promoting hormones (5 μg of insulin, 5 μg of prolactin, 1 μg of aldosterone, and 1 μg of hydrocortisone per mL of medium). Glands were exposed to 2 μg/mL DMBA between 72 and 96 h. After the exposure, glands were rinsed and transferred to new dishes with fresh medium. The fully differentiated glands were then permitted to regress by withdrawing all hormones except insulin for 14 additional days. Test compounds were present in the medium during days 1–10 of culture at five concentrations ranging from 10^{-8}M to 10^{-4}M. Control glands did not receive any chemopreventive agents, but only the solvent. At the end of the experiments, the glands were fixed in formalin and stained with alum carmine as described previously (Lin et al., 1976; Mehta et al., 1997a,b,c). The glands were scored for lesions and percent glands with MAL were calculated for every group. Results were subjected to χ² analysis to determine statistical significance.

**Experimental design for chemoprevention in mouse mammary gland organ culture**

![Figure 1. Schematic diagram showing experimental protocol for induction of mammary lesions in response to DMBA and effects of chemopreventive agents.](image-url)

1. **DMBA**
2. **Hormones + CPA**
3. **Days in Culture**
4. **Regression**

DMBA + IPEPg = Ductal Lesions (MDL) = E2 Dependent
DMBA + IPAF = Alveolar Lesions (MAL) = E2 Independent
Results and discussion

Mammary glands develop mammary alveolar lesions in response to DMBA in the culture medium for 24 h between days 3 and 4 of the culture. A representative photograph showing presence of MAL in response to DMBA and its comparison with that of solvent control and effect of a chemopreventive agent (Figure 2). Typically 9–10 glands from a group of 15 glands (9/15) develop MAL, yielding an incidence of 60%. In the current collective data of 32 chemoprevention experiments 495 glands were used for controls (15 glands per group). The average incidence of 63% (302/480 glands) was observed for control glands. All experiments were carried out with 15 glands per group and each chemopreventive agent was evaluated at five different concentrations. Percent suppression of MAL formation by a chemopreventive agent was calculated by comparing the MAL incidence in control gland with that of chemopreventive agent treated glands. Thus, each experiment was supported by its own control. Results generated from each experiment were used to determine IC₅₀, dose required for maximum inhibition, toxic concentration, and an efficacy score (Steele et al., 1997). A typical analysis for a chemopreventive agent is shown in Figure 3. Here, ascorbigen inhibited MAL development by 62% at 10⁻⁵M concentration. These results are then subjected to an analysis score to generate an arbitrary score for each compound. From numerous experiments it has been concluded that when a compound inhibits the MAL development by more than 60% in a group of 15 glands and with the control exhibiting at least a 60% incidence the statistical analysis results in a p-value of <0.05. Thus, in this report we have included compounds present in edible plants and fruits and exhibited at least 55% inhibition.

In recent years considerable attention has been diverted toward understanding the role of chemicals present in the herbs and commonly used edible plants in different countries. The process of identifying new chemicals that may have chemopreventive efficacy is a very involved process. A variety of classes of agents have been reported in the herbs including flavanoids, saponins, sterols, curcumin, terpenoids, sulfides, lignans, carotenoids, polyphenolics, etc. (Craig, 1999). It is not possible to review all the chemicals present in the herbs and edible plants for their efficacy in MMOC. However, we have evaluated more than 300 pure potential chemopreventive agents for their efficacy in MMOC. A few of these agents are also found in plants. In this report, we have summarized a list of agents which have shown efficacy in MMOC. Chemopreventive agents that are present in edible plants but having less than 55% inhibition of MAL development, such as diallyl disulfide from garlic, genistein from soy, or conjugated linoleic acid from meat and milk fat, have not been included. Table 1 shows analysis of 32 chemopreventive agents present in a variety of fruits and edible plants. The results suggest that many of the commonly consumed fruits and vegetables could be a powerful resource for chemopreventive agents. These natural functional foods largely include the plants with antioxidant

Figure 2. Morphology of alveolar lesions (MAL). In the absence of DMBA the glands regress back to the ductal stage with very few alveolar structures and no end buds. The middle photograph shows alveolar lesions in response to DMBA. In response to chemopreventive agents the glands treated with DMBA and chemopreventive agent structurally resemble the control non-DMBA treated glands. This shows the effectiveness of the chemopreventive agent.

Figure 3. Effect of ascorbigen on the development of MAL in MMOC. Glands were incubated with increasing concentrations of ascorbigen. Percent inhibition was calculated from determining glands with MAL in each group. Each group consisted of 15 glands.
properties, citrus fruits, flavanoids, green and black tea, cruciferous vegetables, garlic, rhubarb, grapes, soybean, carotenoids, licorice, coffee beans and selenium from walnuts and brazil nuts.

Recently, we reported that in the presence of estrogen and progesterone DMBA induces estrogen-progesterone dependent ductal lesions (Mehta et al., 2001). It would be extremely important to evaluate efficacy of many of the herbal agents and ovarian steroid hormone antagonists in this newly developed system. For example we observed that tamoxifen a classical anti-estrogen failed to show effectiveness against MAL, but was highly effective against MDL (Mehta et al., 2001). Thus chemopreventive agents such as genistein and daidzein, which did not show efficacy against MAL may be effective against DMBA-induced MDL development.

Among the agents listed in Table 1 many of them have already proven to have anticarcinogenic activity in experimental in vivo models such as EGCG, quercetin, retinoic acid, selenium analogs, vitamin D analogs, and resveratrol. This study also shows a wide array of compounds that may show chemopreventive activity in experimental carcinogenesis models. For example, indole-3-cabinol, naringenin, ascorbigen, rhapontin, theaflavin, fumaric acid and rosmerinic acid have not been evaluated in mammary carcinogenesis models. Since many of the chemopreventive agents identified by the MMOC assay have shown to be effective in either DMBA- or \( \text{N}-\text{methyl-N-nitrosoure} \)
induced mammary carcinogenesis models, it may be interesting to evaluate the agents showing positive effects MMOC for in vivo carcinogenesis experiments.

Acknowledgements

This work was supported by CN-55135, CN-65114, CN-85138, and CN-95102 from the Chemopreventive Agent Development Group, DCP, National Cancer Institute, Bethesda, MD. This paper is dedicated to the memory of Dr. Mihir R. Banerjee, Tumor Biology Laboratory, University of Nebraska, Lincoln, in whose laboratory the procedure of MMOC was extensively characterized.

References


