Catechol estrogens as biomarkers for mammary gland cancer

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Abstract. The origin of human tumors has been attributed to the exposure to several environmental chemicals and implicated in the increase of incidence in breast cancer. Progression of breast cancer follows a complex multistep process that seems to depend on various exogenous and endogenous factors. The aim of this study was to examine the effects of the organophosphorous pesticide malathion in the presence of estrogen on neoplastic transformation of rat mammary glands. Virgin female rats were sacrificed after 30, 124 and 240 days of 5-day injections twice a day. There were four groups: i) control, ii) malathion (22 mg/100 g body weight, BW), iii) 17β-estradiol (30 μg/100 g BW) and iv) combination of both. Progressive alterations in ducts were observed by the effect of malathion in comparison to control after 240 days. Ducts markedly increased in size and number of cells per square millimeter and tumors similar to ductal carcinoma were originated. The increase in number of proliferative ducts per square millimeter was significantly ($P<0.05$) higher in malathion-treated animals compared to the other groups. Progressive alterations in lobules with estrogen treatment were found after 240 days. Lobules became markedly abnormal, referred to as secretory lobules, increased in number and size and the tumors originated were similar to lobular carcinoma. The increase in number of secretory lobules was significantly ($P<0.05$) higher in estrogen-treated animals compared to the other groups. Treatment with the combination of malathion and estrogen gave rise to tumors constituted of both proliferative ducts and secretory lobules as well as formation of estrogen metabolites such as 2 and 4 catechol estrogens in the blood of the animals after 240 days. We concluded that morphological changes and alterations in the blood of the animals can be used as biomarkers for mammary gland cancer.

Introduction

The origin of many human tumors have been attributed to exposure to environmental carcinogens, pollutants, ultraviolet light, pesticides, drugs, radiation and tobacco (1). The incidence of breast tumors in women is increasing, and environmental chemicals have been implicated in this increase (2). Since environmental chemicals may also be involved in the etiology of breast cancer we studied malathion [0, 0-dimethyl S-(1, 2-dicarbethoxy-ethyl)-phosphorodithioate] an organophosphorous pesticide extensively used to control a wide range of sucking and chewing pests of field crops. It has structural similarities with naturally occurring compounds, and their primary target of action in insects is the nervous system. Exposure of the skin to this pesticide has been shown to result in a small amount of systemic absorption (3).

Estrogens are associated with carcinogenic events in both humans and animals (4-8) and the exact effect of estrogens in breast cancer remains unclear. Estrogens have generally been considered beneficial, based on a variety of hormonal effects. However, in the past 15-20 years, epidemiological studies have increasingly pointed to an increased breast risk associated with estrogens. Estrogen administration, a risk factor for humans, increases with continuous doses of estrogen and with the length of treatment (9). Slightly elevated levels of circulating estrogens are also a risk factor for breast cancer (10-13). This role of endogenous estrogen in human breast carcinogenesis is supported by risk factors of breast cancer such as high serum or urine estrogen levels (14,15). Other studies have demonstrated strong relationships between endogenous estrogen levels and breast cancer risk (12-17). It has been referred to as tumorigenesis due to the uncontrolled stimulation of mammary epithelial cell proliferation (18). The differential control of gene expression and the stimulation of proliferation of mammary epithelial cells are the biological basis for the role of estrogens. Estrogens seem to play dual roles in the induction of cancer as generation of electrophylic species that can covalently bind to DNA (19) and induce stimulation of cell proliferation by receptor-mediated processes (20). The genotoxicity studies are focused on catechol estrogen (CE) metabolites because they are hydroquinones that may readily be oxidized to DNA-reactive quinones and semiquinones. These estrogens are mainly metabolized via two main pathways such as hydroxylation of 17β-estradiol to 16-α hydroxyl estrone (16-α OHE1) and CE formation as 2 and 4-hydroxylated estrogens (2OHE2 and 4OHE2) by the cytochrome P450 (CYP) enzyme (19). In general, the major CE metabolites of the estrogens are the 2 catechol-estrogen 2-OHE2 (2-CE) and 4 catechol-estrogen 4-OHE2 (4-CE), however, 4-CE are the minor ones. The pathway to 4-CE is the one that leads to the endogenous

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carcinogenic CE 3, 4-quinones (21-24). Epidemiological evidence indicates that CE formation has a greater risk factor for breast cancer than high 16-α hydroxylation of estrogens (12). Breast biopsy tissues were analyzed from women without breast cancer and women with breast carcinoma by High Performance Liquid Chromatograph (HPLC) with electro-chemical detection for 31 estrogen metabolites and they reported that 4-CE was more common than 2-CE in breast cancer patients (24).

Since the progression of breast cancer follows a complex multistep process that seems to depend on various exogenous (breast irradiation) and endogenous (age, hormonal imbalances, proliferative lesions) factors the aim of this study was to examine whether an organophosphorous pesticide in the presence of estrogen was able to influence neoplastic transformation of rat mammary gland and whether estrogen metabolites were present in the blood determined by HPLC that could serve as biomarkers to predict breast cancer risk.

Materials and methods

Experimental designs. Virgin female Sprague Dawley rats (39-day-old) were obtained from the Catholic University of Chile, Santiago-Chile and housed and bred in a barrier animal facility operated in accordance with the standards outlined in the Guide for the Care and Use of Laboratory Animals (25). There were four experimental groups of five animals each injected subcutaneously for 5 days twice a day: i) saline solution as control group, ii) malathion (Fyfanon™, Cheminova, Denmark), 22 mg/100 g BW, iii) 17β-estradiol (Sigma-Aldrich Chemical Co., Milwaukee), 30 μg/100 g BW and iv) combination of both. The animals were sacrificed after 30, 124 and 240 days of 5-day injections. The LD_{50} values of the substances for malathion were 1,000 mg/kg BW. All animals were anesthetized with intra-peritoneal injections of sodium pentobarbital (8 mg/100 g BW) and opened by a midline incision from the pubis to the sub-maxillary area to remove the mammary glands. Mammary gland tissues and tumors were fixed in saline formaldehyde, sectioned at 5 μm thickness, deparaffinized and stained with hematoxylin and eosin. The blood of the animals was collected for HPLC studies to determine the presence of 2-CE and 4-CE in rats sacrificed after 240 days of 5-day injections.

Catechol estrogen determination in the blood of rats by HPLC with electrochemical detector. The presence of catechol estrogens 2-CE and 4-CE in the blood of a total of 20 animals was measured using a modified method of HPLC (26). The standard curves 2-CE and 4-CE (Sigma-Aldrich Chemical Co.) were initially prepared at a concentration of 0.1 mg/ml in acetic acid in ethanol. From this stock solution, several dilutions in methanol were prepared for both catecholes. The concentrations were 2, 10, 20, 40, 100 and 5, 20, 40, 100, 150, 200 ng/ml for 2-CE and 4-CE, respectively. The detection limit was 2 and 5 ng/ml for 2-CE and 4-CE, respectively. Changes introduced in this method having an electrochemical detector resulted in a simpler method used to separate and quantify 2-CE and 4-CE with high sensitivity. Two pairs of positional isomers of CE were resolved with chromatography on chromalith column. This monolithic column significantly reduced the pressure in comparison to those that use a porous network of macropores. This pressure reduction allowed flow rates that were unattainable with a typical particulate column and instrumentation. Furthermore, this column was used due to its selectivity, specificity and sensitivity that allowed a better separation among 2-CE and 4-CE through HPLC. Such equipment contained a CC-5 cabinet used as Faraday cage obtained from Bio-analytical Systems (BAS Inc.) that also accommodated other components of the HPLC system like cross-flow thin-layer cell stainless steel auxiliary electrode; a glassy carbon working electrode; a downstream reference electrode, a column heater system; a column chromalith performance (RP-18e 100-4, 6 mm, Merck, USA) and an injector (model 7125i Rheodyne) with a sample loop of 20 μl. The HPLC system also contained L-6200 intelligent pump (Merck-Hitachi) and an amperimetric detector controller (ClinRep L-3500A, Merck instructions) connected to an interface module HPLC manager (Hitachi D-6000) and a chromatographic data station software (Merck-Hitachi). The mobile phase contained a mixture of 21% acetonitrile and 79% of 75 mM buffer citric acid and 25 mM ammonium acetate. The mobile phase was run into the column at 1.5 ml/min at a constant temperature of 40˚C. The electrochemical potential of the detector was 0.6 V versus the reference electrode. Two pairs of positional isomers of catechol estrogens were clearly resolved under isocratic conditions with a satisfactory separation between 2-CE and 4-CE when chromatography chromalith column was used. Solid-phase-extraction (SPE) was done by using phenyl, end-capped cartridges (Phenomenex). The solid-phase was prepared with 1 ml methanol grade HPLC followed by 1 ml buffer at pH 3.0, 50 mM TRIS containing 2 mM ascorbate and 0.1% methanol (v/v). The sample was loaded onto solid-phase followed by an additional 1 ml of the buffer solution. The cartridge was then rinsed with 20% (v/v) acetone in de-ionized water. Each cartridge was dried out by evaporation under nitrogen for positive pressure for 10 min. The dried cartridges were eluted with 1 ml acetone grade HPLC. The extracts were then concentrated under nitrogen to 0.5 ml and an aliquot was injected into the instrument.

Results

Malathion and estrogen presented significant progressive morphological alterations at cellular level in ducts and lobules of rat mammary glands in comparison to control animals after 240 days of the 5-day treatment as seen in Figs. 1 and 2, respectively. Fig. 1A shows a normal duct of the control rat mammary gland. In malathion-treated rats the density of terminal end buds disappeared as time progressed (Fig. 1B-E). The ducts markedly increased in size and number of cells per mm^2. Those structures are referred to as proliferative ducts. Sections of the ducts were filled with increased number of cells with dark nucleus. Furthermore, such structures increased in number and size per mm^2, until tumors started to appear with similar type of cells as seen in Fig. 1F, where a cross section of a mammary gland tumor is seen. After 240 days of pesticide, treated animals developed mammary gland tumors. Histological diagnosis of the mammary tumors revealed that the tumors originated following the pesticide injection were encapsulated, and the pathology was similar to ductal carcinoma. The increase in number of proliferative ducts per mm^2 was significantly ($P<0.05$) higher in malathion-treated animals after

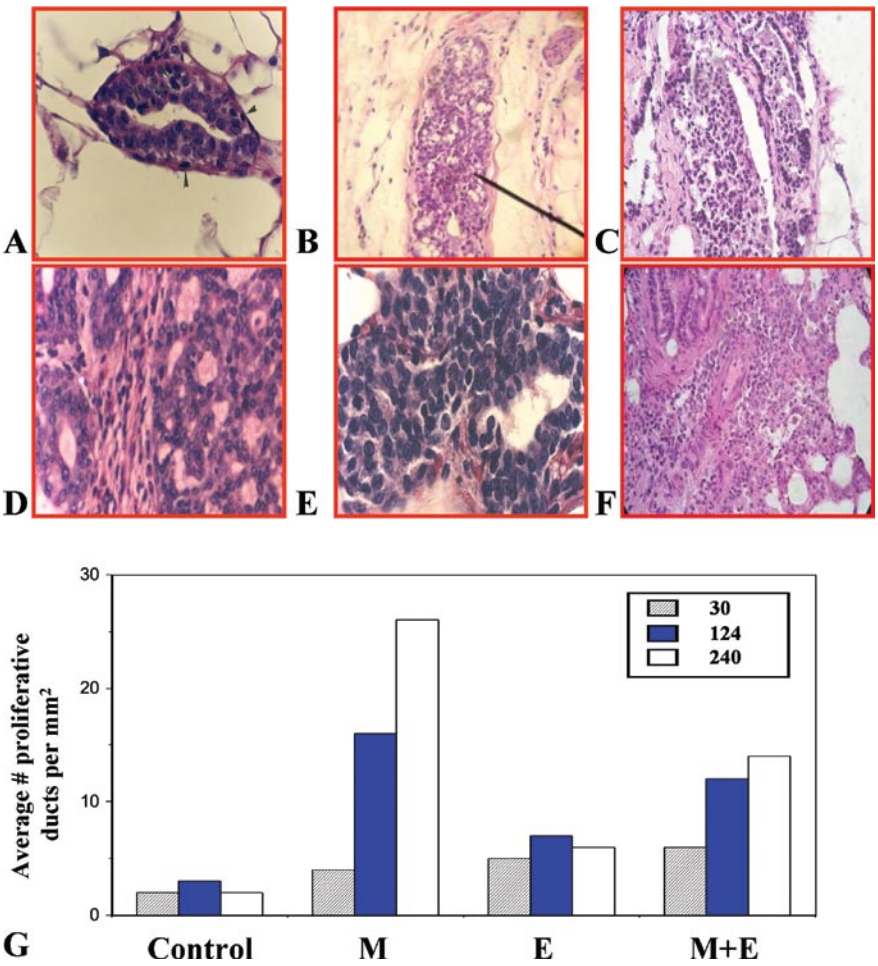

Figure 1. Representative images of histological sections of rat mammary glands treated with malathion after 240 days of the 5-day treatment. (A) Cross section of a normal duct of a control rat mammary gland (magnification x400). (B and C) Cross section of rat mammary gland with altered ducts filled with transformed cells in malathion-treated animal (magnification x100). (D and E) Higher magnification of a cross section of malathion-treated rats (magnification x400). (F) Section of mammary gland tumor of malathion-treated rat constituted with ducts filled with increased number of cells corresponding to ductal carcinoma (magnification x400). (G) The average of proliferative ducts per mm^2 by the effect of malathion (M), estrogen (E) and combination of both (M+E) after 30, 124 and 240 days of the 5-day treatment is shown.

240 days of 5-day injections than in the other treated groups as seen in Fig. 1G.

Rat mammary gland presented significant progressive alterations at cellular level in lobules with estrogen treatment in comparison to control animals after 240 days of the 5-day treatment. The control rat mammary tissue showed the presence of a single primary or main lactiferous duct that branches into secondary ducts from which terminal end buds and the alveolar buds are formed as shown in Fig. 2A. Estrogen-treated rats showed that the density of the number of terminal end buds per mm^2 decreased as time progressed and lobules became markedly abnormal, while large and dilated congested structures increased in size and number per mm^2 (Fig. 2B-F). Such structures were referred to as secretory lobules. Sections of the mammary gland showed that ducts were lined by flattened cuboidal epithelial cells. These congested tubules were filled with pink eosinophylic deposits. Furthermore, such structures increased in number per mm^2 and also in size until tumors started to appear (Fig. 2F). Histological diagnosis of the mammary tumors revealed that the tumors originated following estrogen injection alone were grossly nodular and encapsulated, and pathologically similar to lobular carcinoma. The increase in the number of secretory lobules was significantly ($P<0.05$) higher in the estrogen-treated animals after 240 days of the 5-day injections than in the other treated group (Fig. 2G).

Representative images of calibration curves of 50, 100 and 200 mU/ml of 2-CE and 4-CE studied by HPLC expressed in minutes are shown in Fig. 3A-C. Concentrations of 2-CE and 4-CE present in the blood of control animal after 240 days is shown in Fig. 3D. Fig. 3E-H correspond to representative images of rats treated with malathion alone (Fig. 3E), estrogen alone (Fig. 3F), and combination of both substances (Fig. 3G-H). There was no formation of any characteristic peak of 2-CE and 4-CE in the control and malathion treated animals. The treatment of estrogen induced the formation of the two peaks found with 2-CE and 4-CE in the standard curves but it was very small. However, the combination of malathion and estrogen induced the two peaks corresponding to 2-CE and 4-CE, respectively. Control-, estrogen- and malathion-treated rats did not form mammary gland tumors with the doses given. However, the animals treated with a combination of malathion and estrogen formed mammary gland tumors after 240 days of the 5-day injections. It is noteworthy that the animals that formed

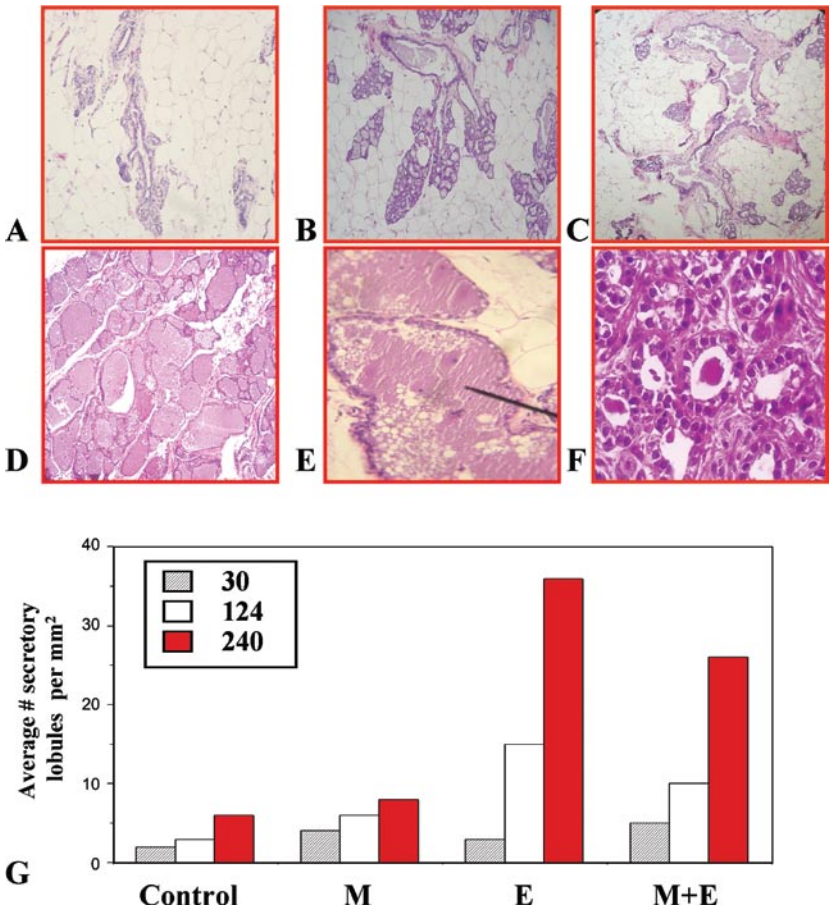

Figure 2. Representative images of cross sections of estrogen-treated rat mammary glands after 240 days. (A) Cross section of a normal lobule of a control rat (magnification x100). A main lactiferous duct branches into secondary ducts with lobules. (B and C) Cross section of lobules of estrogen-treated rat mammary gland (magnification x100). (D and E) Representative images of higher magnification of lobules markedly abnormal (magnification x400). (F) Cross section of mammary gland tumor of estrogen-treated rat constituted with lobules corresponding to lobular carcinoma. (G) The average of secretory lobules per mm^2 by the effect of malathion (M), estrogen (E) and combination of both (M+E) after 30, 124 and 240 days of the 5-day treatment is shown.

mammary tumors had specific peaks that corresponded to 2-CE and 4-CE. It can be concluded from these studies that the morphological changes originated by the effect of an organophosphorous substance such as malathion and an endogenous substance such as estrogen can be complemented with alterations in the blood of the animals. In these animals the presence of specific peaks of 2-CE and 4-CE can be used as a biomarker in blood for mammary gland cancer.

Discussion

To understand the morphological changes that occur during mammary gland carcinogenesis an adequate animal model system is needed. The susceptibility of the mammary gland to environmental substances, such as malathion, an organophosphorous pesticide in the presence of estrogen was analyzed in a rat model. Rat mammary gland presented significant progressive alterations at cellular level in ducts and lobules by the effect of both substances in comparison to controls after 240 days of the 5-day treatment. When animals were treated with malathion the terminal end buds disappeared as time progressed by the effect of malathion alone and induced changes exclusively at the level of ducts that increased in size and number of cells per mm^2 after 240 days. Those structures, referred to as proliferative ducts, were filled with an increased number of cells until mammary gland tumors started to appear after 240 days of pesticide effects. Histological diagnosis of the rat mammary gland tumors revealed a similarity to ductal carcinomas as described by pathologists (27) and the World Health Organization. Mammary tumors formed in various strains of rats induced by several carcinogens have been classified by several authors (27,28). Several studies (29-31) have addressed the association between cancer in humans and agricultural pesticide exposure, both occupational (e.g., farmers) and non-occupational, exposed to contaminated clothing, soil, ground and surface water, as well as drifts from aerial spraying of pesticides.

It has been demonstrated that mammary carcinoma formation in Sprague-Dawley rats can be induced by chemical carcinogens (32-36). Dimethylbenz[α]anthracene (DMBA) induced mammary carcinomas in 100% of the animals with a latency period of 86 days (32-34). Another widely used experimental system to study mammary tumorigenesis is the model in which tumors were induced in the Fischer 344 rats by a single dose of N-nitrosomethylurea (NMU) (35,36). On the other hand, Miyamoto *et al* (37) observed neoplastic transformation of mouse mammary epithelial cells by *in vitro* exposure to NMU. Furthermore, the highest number of tumors per animal

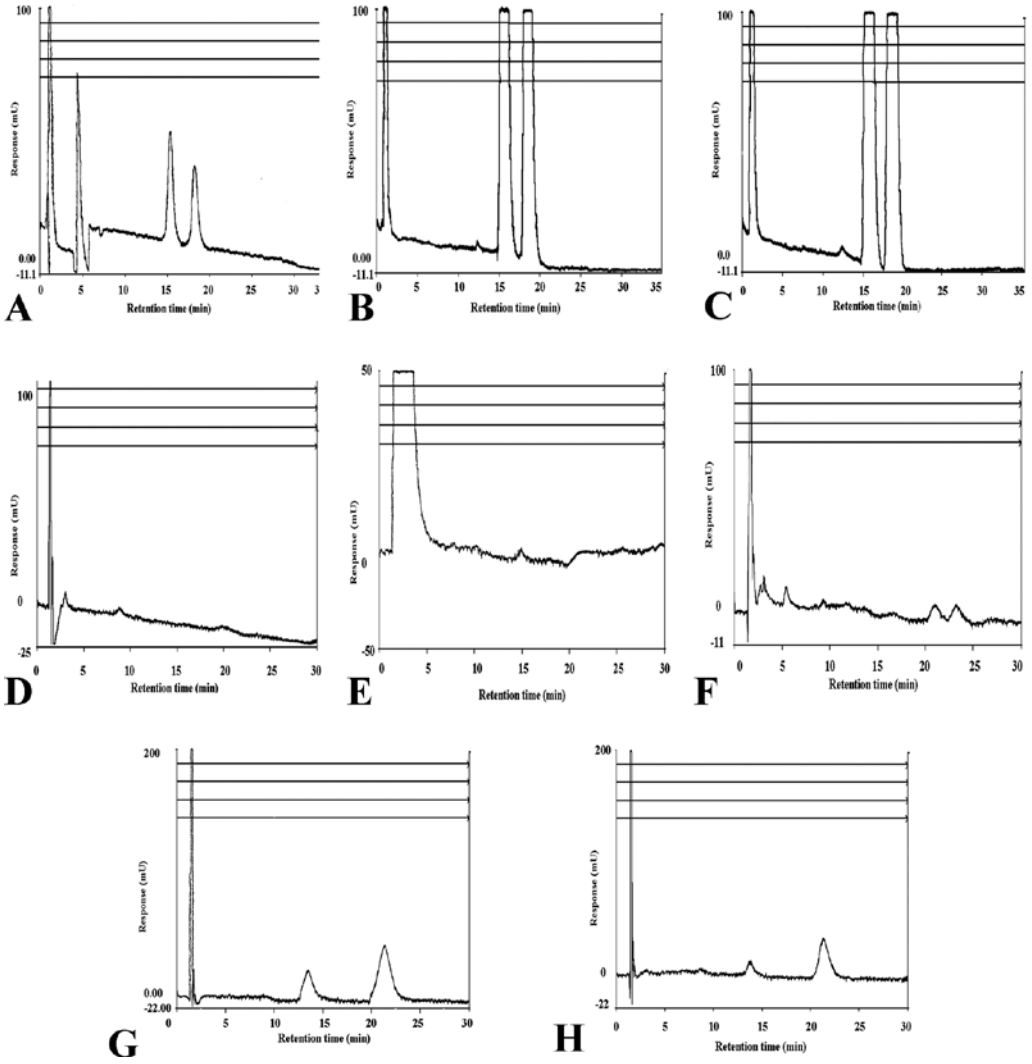

Figure 3. Curves of catechol estrogens (2-CE and 4-CE) observed with an HPLC with electrochemical detector with 50 (A), (100) (B) and 200 (C) ng/ml of 2-CE and 4-CE. (D-H) Representative images of curves corresponding to blood of control (D). Malathion- (E), estrogen- (F) and malathion-plus-estrogen (G) (H) -treated animals after 240 days of the 5-day treatment.

induced by DMBA was observed when the carcinogen was given to animals when they were 40 to 46 days of age, a period when terminal end buds were most actively differentiating into alveolar buds (38). According to these authors a tumor incidence of 94-100% was obtained when DMBA was administered at 39 days of age. In our previous work (39) we reported that parathion and malathion induced 14.3% and 24.3% of rat mammary tumors, respectively, that correlated with the greater density of terminal end buds in the mammary gland present in the 44-day-old treated animals. In contrast to those potent carcinogens, which induced mammary carcinomas in 100% of intact females, organophosphorous pesticides seem to have a slow and less infiltrating and potent effect.

Estrogen treatment induced significant progressive alterations at cellular level in lobules in comparison to control in the rat mammary gland after 240 days of the 5-day treatment. The density of the number of terminal end buds per mm^2 decreased as time progressed and lobules became markedly abnormal, while large and dilated congested structures increased in size and number after 240 days of the 5-day estradiol treatment. Such structures, referred to as secretory lobules, were congested tubules filled with pink deposits until tumors started to appear and revealed that the tumors originated were pathologically similar to lobular carcinomas according to pathologists (27). It can be suggested that the alterations induced by estrogen observed in the terminal end buds may have induced the formation of mammary lobular carcinomas after 240 days. The combination of malathion and estrogen triggered mammary tumor formation after 240 days in conjunction with the concomitant presence of CE in the blood of the same animals. Several morphological changes that were found by the effect of either malathion or estrogen alone as proliferative ducts and secretory lobules, respectively, were observed in these animals.

An estrogen well established/induced hamster renal tumor model of hormonal carcinogenesis that shares several characteristics with human breast cancer has been shown to have 80-100% tumor incidence (22). Different estrogens differ in their estrogenic, carcinogenic, and metabolic activation potentials. The 17β-estradiol is a good catechol progenitor and a potent estrogen and its use results in 80-100% tumor incidence in the

hamster kidney (22). These data indicated that oxidant stress plays a crucial role in estrogen-induced carcinogenesis (40). Previous studies (41) have examined the effect of eserine, an acetylcholinesterase inhibitor, as are the organophosphorous compounds malathion and parathion, in the presence of 17β-estradiol on cell proliferation and tumor formation in rat mammary gland. These studies showed that eserine and 17β-estradiol induced carcinogenesis in the epithelium of rat mammary glands. There was a significant increase in the number of cells per duct of the 44-day-old rat mammary gland after the 10-day eserine treatment, compared to the control. A higher increase was observed in the animals treated for 10 days with eserine followed by 30 daily injections of estrogen in comparison to control animals. In 12 animals, two mammary tumors directly developed in response to 17β-estradiol injected at 39 days of age with a latency period of 180 and 245 days, respectively. These results suggested that terminal end buds were the major targets related to rat mammary carcinogenesis and 17β-estradiol can be an initiator and promoter in this process.

Parallel studies (42) also analyzed the morphological and molecular alterations induced by malathion and 17β-estradiol in rat lung tissues. Morphometric analysis indicated that the combination of these two substances also showed higher incidence of alveolar proliferative lesions, preneoplastic lesions in bronchiolar epithelium as hyperplasia, metaplasia, carcinoma *in situ* and invasive carcinoma than malathion or estrogen alone-treated and control animals after 240 days. Molecular biology studies indicated that c-ErbB2 and Rho-A protein expression was higher in the presence of the combination of the two substances than in control and either malathion or estrogen alone. Such a combination sharply induced pathological lesions in lung alveolar parenchyma and bronchiolar epithelia in comparison to control animals or in animals treated with either substance alone. These results indicated an increase in the risk of rodent lung tumor formation by environmental and endogenous substances.

Cancer progression has been associated with an increase in genomic instability indicated by inactivation of tumor suppressor genes and activation of oncogenes. We (43) evaluated the effects of parathion and malathion in the presence of estrogen on malignant transformation as well as on genomic instability, that is in the frequency of loss of heterozygosity (LOH) and microsatellite instability (MSI). MCF-10F, an immortalized human breast epithelial cell line, was treated with parathion or malathion alone and in combination with estrogen. These studies indicated that either pesticide alone or in combination with estrogen induced malignant transformation as shown by anchorage-independent growth capability and invasive characteristics in comparison to control. Such malignant phenotypic characteristics were corroborated by an increase in p53 and c-Ha-ras protein expression. Different microsatellite markers indicated different degrees of allelic imbalance in the form of LOH or MSI. With the use of a marker for *p53* tumor suppressor gene at loci 17p13 MSI was found in malathion and estrogen-treated cells. The same combination of substances presented MSI with a marker for *c-Ha-ras* mapped in chromosome 11p14.1. LOH in the presence of estrogen or malathion alone. The combination of parathion and estrogen induced MSI in codon 61 was observed. Furthermore, *c-Ha-ras* mutation was observed in codons 12 and 61.

The genes and protein expression alterations we found induced by parathion alone or in combination with 17β-estradiol in cultured breast epithelial cells (44) induced malignant transformation of MCF-10F confirmed by anchorage-independency and invasive capabilities. Parathion alone efficiently elevated the expression of EGFR, c-kit, Trio, Rac 3, Rho-A, and mutant p53 proteins. Analysis of gene expression using commercially available human cell cycle array revealed transcriptional alterations in 22 out of a total of 96 genes. Among them, genes involved in the regulation of cell cycle were cyclins (*A1*, *A2*, *C*, *G1*, *G2* and *H*), cyclin-dependent kinases (*CDKs*), and minichromosome maintenance deficient (*MCM*). We (45) identified differentially expressed genes encoding enzymes that are important to drug transport and metabolism by analysing human drug metabolism genes in parathion- and estrogen-treated MCF-10F cell lines either alone or in combination. Seventeen genes were found altered either by parathion or estrogen alone, or the combination of both and among them were several *CYPs* as *CYP2F1*, *CYP3A7* and *CYP4F3*, *metallothionein*, *methyltransferase*, *glycosyltransferases*. Other studies (46) indicated that several genes were up-regulated by the effect of 17β-estradiol combined with either malathion or parathion, such as the *cyclins*, *cyclin D1* and *cyclin-dependent kinase 4*, and *keratin 18* among others. The *c-Ha-ras* oncogene was up-regulated by the effect of malathion alone and with the combination of estrogen and either malathion or parathion. The *DVL1* gene was up-regulated only with malathion and the combination of parathion with estrogen. Expression of the *HSP 27*, *MCM2* and *TP53* inducible protein 3 genes was up-regulated with malathion alone and with the combination of estrogen and either malathion or parathion while *TP53* (Li-Fraumeni syndrome) was up-regulated by estrogen alone and malathion alone.

Malathion treatment did not induce formation of any peak of 2-CE and 4-CE in the blood of these animals. Estrogen induced the formation of two peaks of 2-CE and 4-CE with a small area of surface. Control, estrogen and malathion-treated rats did not form tumors with the doses given in these experiments. Animals treated with a combination of malathion and estrogen formed mammary gland tumors after 240 days of the 5-day injections. The animals that formed mammary tumors had specific peaks that corresponded to 2-CE and 4-CE. It can be concluded from these studies that morphological changes originated by the effect of malathion and estrogen can be complemented with alterations in the blood of the animals. Thus, specific peaks of CE can be used as a biomarker for mammary gland cancer.

The use of organophosphorus insecticides has significantly increased in agricultural environments and in urban settings. Since malathion has been extensively used in Latin American countries as well as in many others, the present studies are relevant to understanding the possible effects of other combined agents in this process. On the other hand, previous *in vitro* studies indicated that malathion and parathion in the presence of estrogen induced changes at the level of the tumor suppresor gene *p53* and the oncogene *c-Ha-ras* and was considered pivotal to breast carcinogenesis. Collectively, these results allowed us to conclude that the presence of environmental substances such

as the organophosphorous pesticides parathion and malathion, and endogenous substances such as estrogen can increase the risk for mammary gland cancer.

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