Gene and Protein Expressions Induced by 17β-estradiol and Parathion in Cultured Breast Epithelial Cells

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Cancer of the breast is the most common form of malignant disease occurring among women of the western world and environmental substances seem to be involved in the etiology of this disease. Many studies have found an association between human cancer and exposure to agricultural pesticides and among them parathion, the organophosphorous pesticide used in agriculture to control mosquito plagues. The association between breast cancer and prolonged exposure to estrogens suggests that this hormone also may have a role in such process. However, the causative factors for breast carcinogenesis remain an enigma. The objective of this study was to determine the effects of 17β-estradiol (E2) and parathion on cell transformation of human breast epithelial cells in vitro. The results of this study showed that parathion alone and in combination with E2 induced malignant transformation of an immortalized human breast epithelial cell line, MCF-10F, and the malignant feature was 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, nine genes involved in the regulation of cell cycle were altered. These included cyclins (A1, A2, C, G1, G2, and H), cyclin-dependent kinases (CDKs), and minichromosome maintenance deficient (MCM). Results suggest that parathion has the potency to cause malignant transformation of breast epithelial cells through modulation of expression of cell cycle regulated genes.

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INTRODUCTION

Breast cancer is increasingly common among women throughout the world and this increased incidence has been partially attributed to environmental agents (1). Breast cancer is the leading cause of death among woman in the age group of 40–45 (2,3) and is a complex disease involving numerous genetic alterations. Epidemiological and clinical evidence indicate that breast cancer risk is associated with prolonged exposure to female ovarian hormones (4–7). The association between breast cancer and prolonged exposure to estrogen suggests that this hormone may have some role in breast carcinogenesis.

A complex series of cellular and molecular changes that occur during cancer development can be mediated by diverse endogenous and environmental stimuli. These changes convert normal cells to latent tumor cells by altering the genetic material that facilitates proliferation of such cells (7). Therefore, knowledge of these specific genetic changes is critical for understanding the molecular basis for breast carcinogenesis. Exposure to carcinogens plays an etiological role in breast cancer initiation. Organophosphorous compounds are of great health concern due to their extensive use in agriculture, medicine, and industry-based applications (8–11). Such compounds are the most widely used pesticides by virtue of their biodegradable nature and short persistence. Parathion, in particular, is used to control mosquitoes, insect plagues, head lice, and mites in humans (11). These compounds are known to cause significant biochemical alterations in cells (12).

It is currently accepted that breast carcinogenesis is a multi-step process involving a complex cascade of cellular and molecular alterations leading to the conversion of normal cells to malignant cells. Activation and/or enhanced expression of cell cycle regulators and oncogenes have been implicated in the process (13–17). Some of these changes involve specific genetic loci that contribute directly to one or more attributes of transformation, i.e., upregulated proliferation and invasion, while other changes confer genetic instability that increases the possibility of acquiring subsequent, specific lesions relevant to tumorigenesis (18,19). Transformation of breast cells occurs through loss or mutation of tumor suppressor genes, or activation or amplification of oncogenes, leading to an abnormal amplification of growth signals, and aberrant expression of genes

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that ultimately transform the cells into invasive cancer (17,20,21). Transformed cells are identified by morphological changes, lack of anchorage independency, and invasive characteristics (19,22–26).

In vitro model systems have been used extensively in the study of initiation in cancer (19,27–30). Spontaneously immortalized human breast epithelial cell line MCF-10F was used in these studies. This cell line originated from a mortal human diploid breast epithelial cell line from the breast tissue of a 36-year-old female with a near diploid karyotype and luminal epithelial origin (31). This cell line retains all the characteristics of the normal epithelium in vitro, including anchorage independence, non-invasiveness, and non-tumorigenicity in the nude mice and has the morphological characteristics of normal breast epithelial cell lines (19,22).

Methods to define patterns of gene expression have applications in a wide range of biological systems. Several molecular biological techniques are used to study expression patterns during the neoplastic progression of breast epithelial cells. A total of 96 genes from the human cell cycle array were examined. The genes represented in this array are related with G1 phase, G1/S transition, S phase, DNA replication (minichromosome maintenance deficient, MCM), and regulation of the cell cycle genes as cyclins A1, A2, C, G1, G2, H, and CDKs.

Available information on cell transformation induced by the effects of estrogen and pesticides is very limited. Further studies are warranted to understand the molecular basis for estrogen- and pesticide-induced malignant transformation. The present study was undertaken to determine the effect of estrogen and the organophosphorous pesticide parathion on cell transformation using the well-established MCF-10F cell line as an in vitro model system. A combination of cellular and biochemical techniques was used in this study to identify the potential molecular causes for cellular transformation.

MATERIALS AND METHODS

Chemical Treatments of Cells

The spontaneously immortalized breast epithelial cell line MCF-10F (31) was used in these studies. Cell viability was determined by calculating the number of cells after treatment by trypan blue exclusion method. Effects of both estrogen and methyl parathion were studied on cell viability. Previous studies (32) indicated that 17β-estradiol (E2) (Sigma-Aldrich Chemical Company, St Louis. MO, USA) at 10^{-8} M dissolved in the culture media was the best stimulatory dose when analyzed from 10^{-6} to 10^{-10} M. MCF-10F cell line was treated with several doses of methyl parathion (Sigma-Aldrich Chemical Company) dissolved in culture media. Cell proliferation was analyzed with 0, 100, 200, 400, and 800 ng/mL of methyl parathion for 14 days. Cells treated with this substance had the maximum effect with 100 ng/mL and reached the maximum plateau after 12 days (1×10^7 cells per mL) starting with 1×10^3 cells per mL. Toxicity was observed with 400 ng/mL of parathion after 12 days.

Anchorage-independent and Cell Invasion Assay

To test for cell growth in semi-solid medium, cells in passage 20 after the treatments were trypsinized and plated at a density of 2×10^5 cells in 10 mL 0.35% agarose over a 0.7% agar base in 100 mm culture dishes. Media were replenished every other day and colonies with more than 50 cells were scored after four weeks in culture. Invasiveness was carried out as described previously (18,25) using modified Boyden's chambers (Transwell, Costar, Cambridge, MA, USA). Briefly, 8 mm diameter filters (8 μm pore) of cell culture inserts were coated with 60 μg/filter basement membrane matrigel (Collaborative Research, Bedford, MA, USA) reconstituted with 100 μL of MEM with 0.1% bovine serum albumin (BSA) (Collaborative Research). Such filters were coated and dried overnight. Exponentially growing control and treated cells were trypsinized, harvested, suspended in DMEM plus horse serum, and 3×10^4 cells/chamber added to the upper chamber. Fibronectin (Collaborative Research) was used as a chemoattractant at a concentration of 1 μg/chamber in 0.5 mL of MEM with 0.1% BSA and placed in the lower chamber. The cells on the upper surface of the filter were kept for 20 h at 37°C in a 5% CO_2 and, after that period, cells from the top were removed by wiping with a cotton swab. The total number of cells that crossed the membrane was counted under a light microscope. Experiments were performed three times. Statistical analysis was done with the ANOVA *F*-test (Randomized Block) and comparisons between groups with the Orthogonal-*t*-test with significance P value of < 0.05.

Determination of Protein Expression by Immunocytochemistry Coupled with Confocal Microscopy

Exponentially growing control and treated cells were plated on a glass chamber slide (Nunc Inc., Naperville, IL, USA), as previously described (25,33–35). Cells were plated at a density of 1×10^4 in 1 mL of medium and allowed to grow for two to three days until they reached 70% confluency. The cells were incubated with 1% H_2O_2 in methanol for 30 min to block endogenous peroxidase, washed twice with buffer solution and fixed with buffered paraformaldehyde in PBS (pH 7.4) at room temperature. Cells were covered with normal horse serum for 30 min at room temperature and incubated with the corresponding primary antibodies [EGFR (sc-03) rabbit polyclonal, c-Kit (sc-168) rabbit polyclonal, Trio (sc-6061) rabbit polyclonal, Rac 3 (sc-16698) goat polyclonal, Rho-A (sc-418) mouse monoclonal, and mutant p53 (Pab240, sc-99) mouse monoclonal] at a 1:500 dilution overnight at 4°C. After washing in buffer solution, cultures were incubated for 60 min at room temperature with anti-mouse Rhodamine-conjugated secondary antibody (Jackson ImmunoResearch Lab., West Grove, PA, USA) at a 1:1000

dilution. Following several washes of 5 min each with buffer solution, slides were mounted with Vectashield mounting media (Vector Laboratories, Burlingame, CA, USA). Controls included cultures stained with either the primary or secondary antibodies alone to monitor the background staining. Cells were quantified as previously described (19,25,33–35) and viewed on Zeiss Axiovert 100 TV microscope (Carl Zeiss, Thornwood, NY, USA) using a 40X (11.3 NA) objective lens equipped with a laser scanning confocal attachment, LSM 410, (Carl Zeiss). To excite the Rhodamine secondary antibody, the fluorescent images were collected by an argon/krypton mixed gas laser (488 nm). Fluorescent images were collected in black and white and changed to red or green color by Photoshop software. Composite images were quantified by using Adobe Photoshop, version 5.0. Protein expression of the control and transformed cells was determined by a semi-quantitative estimation based on the relative staining intensity. This computer quatification gives the area and the intensity of the staining of the cells present in the culture dishes. The experiments were repeated with three similar passages. Standard errors of mean are shown in the different figures. The number of immunoreactive cells was counted in five randomly selected microscopy fields per sample (19,25,33–35). Statistical analysis was done with the ANOVA *F*-test (Randomized Block) and comparisons between groups with the Orthogonal-*t*-test with significance P value of < 0.05.

Isolation and Purification of Total RNA and mRNA

Total RNA was isolated from both the control MCF-10F and treated cells with TRIZOL reagent (Invitrogen Corp., Long Island, NY, USA). Each sample comprising 500 μg of total RNA was treated with 5 μL of DNAse I (10 units/μL) (Boehringer Mannheim, Indianapolis, IN, USA) for 60 min at 37°C. Then 10X Termination Mix (0.1 M EDTA, pH 8.0 and 1 mg/mL glycogen) (Clontech, Mountain View, CA, USA) was used to stop the reaction. Each sample was then purified following established procedure (36). Each purified RNA sample was measured by a spectrophotometer (the ratio of absorbance reading at 260 nm/280 nm greater than 1.8) and then electrophoresed on denaturing formaldehyde/agarose/ethidium bromide gel. Each sample of 500 μg of purified total RNA was then subjected to polyA$^+$ RNA analysis with the Oligotex mRNA Purification Kit (QIAGEN Inc., Valencia, CA, USA). PolyA$^+$ RNA was then purified following established procedure (36).

cDNA Expression Array

GE Array Q Series Human DNA cell cycle cDNA expression array membranes (SuperArray, Bethesda, MD, USA) are designed to profile gene expression of a panel of 96 key genes associated with cell cycle. The purified mRNAs were used for the synthesis of cDNA probes with Biotin-16-dUTP (Roche Pharmaceuticals, Indianapolis, IN, USA). Annealing mixture was prepared by mixing about 1.0-5.0 μg of mRNA with 3 μL of Buffer A (GE primer mix) (SuperArray) and the final volume was adjusted to 10 μL. The mixture was then incubated in a preheated thermal cycler at 70°C for 3 min, cooled to 42°C, and kept at that temperature for 2 min. Then 10 μL of RT cocktail was prepared by mixing 4 μL of 5X Buffer BN (for 50 μL 10X Buffer, add 1 μL of 1M DTT and 50 μL of 10X dNTP mix, 5 mM dATP, dCTP, dGTP, and 500 μM dTTP), 2 μL of Biotin-16-UTP, 2 μL of RNase free H_2O, 1 μL of RNase Inhibitor (Promega Corp., Madison, WI, USA) and 1 μL of MMLV Reverse Transcriptase (Promega Corp.). RT cocktail was then warmed at 42°C for 1 min and slowly mixed with 10 μL of pre-warmed annealing mixture. Incubation continued at 42°C for 90 min and then the labeled cDNA probe was denatured by heating at 94°C for 5 min, and quickly chilled on ice. cDNA probes were prepared from each of them and hybridized to the respective membranes. Experiments using the same mRNA preparation were repeated two or three times and measurable median-normalized expression values of each gene were compared to avoid false-positive signals (37).

Differential Hybridization of cDNA Expression Array

Each array membrane was pre-wetted with 5 mL of deionized water and incubated at 60°C for 5 min. It was then replaced with 2 mL of pre-warm (60ºC) GEAprehyb solution (GEAhyb solution with a heat-denatured sheared salmon sperm DNA at a final concentration of 100 μg/mL) (SuperArray) and mixed gently for a few seconds. Pre-hybridization was continued at 60°C for 1 to 2 h with continuous gentle agitation. About 0.75 mL solution of GEAhyb was prepared by adding the entire volume of denatured cDNA probe onto GEAprehyb solution and kept at 60°C. Then GEAprehyb solution was replaced by GEAhyb solution and incubation continued overnight hybridization at 60°C with continuous gentle agitation. Subsequently, array membranes were washed twice in wash solution 1 (2× sodium chloride sodium citrate and 1% sodium dodecyl sulfate) at 60°C for 15 min each with gentle agitation and then twice with solution 2 (0.1× sodium chloride sodium citrate and 0.5% sodium dodecyl sulfate) at 60°C for 15 min each with gentle agitation. To assess the reproducibility of the hybridization array assays, pair-wise comparisons between array data sets for each cell line was tested by repeated hybridization and the mRNAs prepared in different lots were analyzed in scatter plots with multiple regressions like as previously described (37,38). In each case, expression levels of 95% of the genes had repeated values that were within 2.0 fold of each other (37).

Chemiluminescent Detection of cDNA Probes

After discarding the last wash, 2 mL of GEAblocking solution was added to each membrane and incubated for 40 min at room temperature with continuous agitation. Then binding buffer was prepared

by diluting alkaline phosphatase-conjugated streptavidin (AP) with 1× buffer F (SuperArray) in a 1:7500 dilution. GEAblocking solution was replaced by 2 mL of binding buffer and incubated for 10 min with continuous but gentle agitation. Then membrane was washed four times with 4 mL of 1× binding buffer F for 5 min in each washing and rinsed twice with 3 mL of rinsing buffer G (SuperArray). Then the membrane was covered with 1.0 mL of CDP-Star chemiluminescent substrate to incubate at room temperature for 2 to 5 min. It was then exposed to X-ray film (Kodak BioMax MS Film) (Kodak Corp.) with corresponding intensifying screen at room temperature for multiple exposures of 1–5 min.

Quantification of Array Hybridization

Quantification of hybridization signals on the expression array membranes were carried out by exposing the autoradiographic film in a densitometric scanner (model 300A; Molecular Dynamics, Sunnyvale, CA. USA). It was then estimated both with the ImageQuant (Molecular Dynamics) and ScanAnalyze program (Eisen Lab, Berkeley, CA, USA). Volume quantification was performed by calculating the volume under the surface created by a three-dimensional plot of pixel locations and pixel values as described (37,38). All raw signal intensities were corrected for background by subtracting the signal intensity of a negative control or blank. They also were normalized to that of a housekeeping gene. These corrected, normalized signals can then be used to estimate the relative abundance of particular transcripts. To delineate the potential signal interference between adjacent strong hybridization signals, equal-sized ellipses were drawn around each signal area (hybridization spots) by using the software ImageQuant/ScanAnalyze and each was then scanned separately and compared with housekeeping genes so the chances of interference between adjacent strong hybridization signals were minimized. Normalization of the expression levels of different housekeeping genes from multiple autoradiographic exposures between different hybridization experiments were done by taking the average signals of each of the housekeeping genes. Data from concentration spots was equal or higher than 2.5 fold versus control were used. Median background was subtracted, and signals that were minor to 2.5 fold below were considered too low to accurately measure and were omitted from the analysis. Signals for each individual gene were also normalized to the geometric mean of the expression level of that gene across the set of membranes being compared. Mean signals were calculated from quadruplicate measurable spots, or if three of the four spots were measurable. Then the change folds indicated whether a gene exhibits increased, decreased, or unchanged expression. They are based on statistical criteria (38).

RESULTS

The present study shows the typical morphological phenotypes induced either by parathion, an environmental agent, alone or in combination with estrogen in human breast epithelial cells. Origin and phenotypic characteristics of cells used in these studies are shown in Figure 1A. The parental MCF-10F cell line was treated with E2, parathion, and a combination of E2 with parathion for 20 passages in culture. Anchorage independent growth and invasive capabilities were analyzed in control and treated cells. MCF-10F cells did not form colonies in agar (Figure 1B) and colony-forming efficiency scored 21 days after plating in agar fluctuated from 1 to 3% in the presence of parathion and combination of E2 and parathion, respectively. Frequency of colonies upon treatment with parathion and E2 plus parathion-treated cells is shown in Figures 1C and D, respectively.

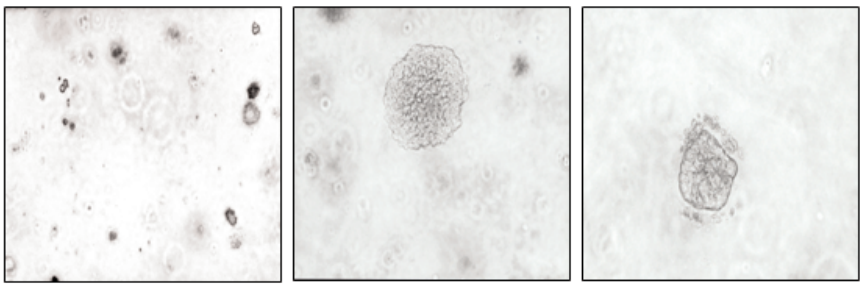
Invasive characteristics of control and treated-MCF-10F cells were scored 20 h after plating onto matrigel basement membranes using modified Boyden's chambers. The results are presented in the form of histogram bars with standard error (SE) of the mean (Figure 1E). Values represent the invasiveness characteristics of control, E2, parathion, and a combination of E2 and parathion. Parathion-treated cells were able to develop colonies after 20 passages with an efficiency of 25%. The colony forming ability of cells was slightly increased (35%) in cells treated with a combination of estrogen and parathion as compared with cells with parathion alone (25%). Control MCF-10F cell line and E2-treated cells did not show any invasive capabilities and there was no significant difference among these two groups. Addition of parathion to the growth medium significantly ($P < 0.05$) enhanced the invasive phenotype of cells in comparison to control and E2. E2 combined with parathion had greater and statistically significant ($P < 0.05$) invasive capability than control MCF-10F and E2-treated cells. However, no significant difference was observed between parathion alone and E2 combined with parathion.

Expression of a few proteins that are frequently associated with signaling pathways (EGFR, c-Kit, Trio, Rac 3) next was analyzed to determine whether the alterations of protein expression correlate with cell transformation induced by E2 and parathion. Results indicated that parathion either alone or in combination with E2 significantly ($P < 0.05$) increased the expression of EGFR, c-Kit, Trio, and Rac 3 proteins compared with control MCF-10F and E2-treated cells. Treatment with either pesticide alone or in combination with E2 elevated the expression of EGFR by 1.7 fold in comparison to control and E2-treated cells. However, combined treatment induced approximately a 3.0-fold increase in c-Kit protein expression in comparison to control and a 2.0-fold increase in comparison to E2-treated cells. Parathion treatment alone induced 1.8-fold and 1.3-fold increases in Trio protein expression in comparison to control and E2-treated cells, respectively. However, E2 combined with parathion induced roughly 1.5- to 2.0-fold increase in Trio protein expression in comparison to control and E2-treated cells. Parathion alone induced 2.8-fold and 1.6-fold in-

A

Origin and phenotypic characteristics of cell lines

Cell lines	Origin	AIA	IA
MCF-10F	MCF-10F parental cells	-	-
E2	MCF-10F treated with 17 β estradiol	-	-
Parathion	MCF-10F treated with parathion	+	+
E2 + parathion	MCF-10F treated with combination	+	+



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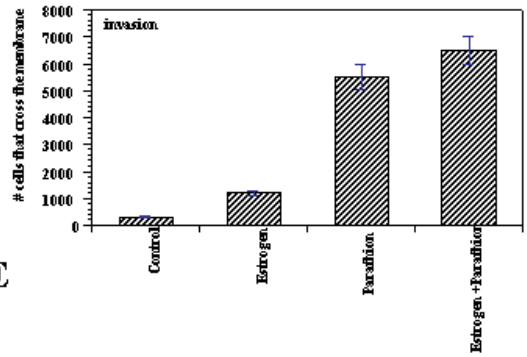

E

Figure 1. (A) The parental MCF-10F cell line was treated with 17β-estradiol (E2) 10^{-8} M, parathion (100 ng/mL) and combination of E2 plus parathion during 20 passages. AIA: Anchorage Independence Assay. IA: Invasion Assay. Positive signs (+): referred to the lack of anchorage independent growth and invasiveness. Negative signs (–): referred to the lack of both characteristics. (B) MCF-10F cell line did not form colonies in agar. (C) Colony formed by parathion-treated cells. (D) Colony formed by combination of E2- and parathion-treated cells. Phase contrast microscopy (4X). (E) Invasive characteristics of control and treated-MCF-10F cell line were scored 20 h after plating onto matrigel basement membranes using modified Boyden's chambers constructed with multi-well cell culture plates and cell culture inserts. Values represent the invasive characteristics of MCF-10F, E2, parathion, and a combination of E2- and parathion-treated cells obtained by scoring the number of cells that crossed the filters.

creases in Rac 3 protein expression in comparison to control and E2-treated cells, respectively. However, both substances combined induced roughly 3.0× and 1.7× Rac 3 protein expression in comparison to control and E2-treated cells, respectively. No significant difference in protein expression was observed between the group of control and E2-treated cells and that of parathion- or E2-plus-parathion-treated cells. Immunofluorescent analysis of these proteins and their quantitative assessment are shown in Figures 2 (A-D). The staining of protein expression among individual cells was very intensively uniform in all the groups.

Figure 3 shows the representative images and the quantification of fluorescence intensity for Rho-A and mutant p53 protein expression in the form of a histogram with error bars representing the standard error of the mean. E2 combined with parathion induced 5.0-fold increases in Rho-A protein as compared with control cells and 2.0-fold increases in comparison to E2-treated cells (Figure 3A). Such expression was significantly ($P < 0.05$) greater in parathion alone and E2-plus-parathion-treated cells in comparison to control and E2-treated cells, respectively. However, there was no significant difference in Rho-A expression between cells treated either with parathion alone or in combination with E2. Quantification of the immunofluorescent intensity revealed a significant increase ($P < 0.05$) in Rho-A protein expression in E2-treated cells in comparison to control. E2 combined with parathion elevated the mutant p53 expression by 2.7 and 2.9 fold in comparison to control and E2-treated cells, respectively (Figure 3B). Such expression was significantly ($P < 0.05$) greater in parathion alone and E2-plus-parathion-treated cells in comparison to control and E2-treated cells, respectively. However, there was no significant difference in cells treated with either parathion alone or in combination. Further the difference was also not observed between control and E2-treated cells.

To determine whether the malignant transformation induced by parathion or its combined treatment with E2 is accompanied by alterations in gene expression, an analysis was carried out using the commercially available cDNA arrays comprising cell cycle regulatory genes. Gene expression in MCF-10F was analyzed in comparison to E2-, parathion-, and E2-plus-parathion-treated cells. Re-

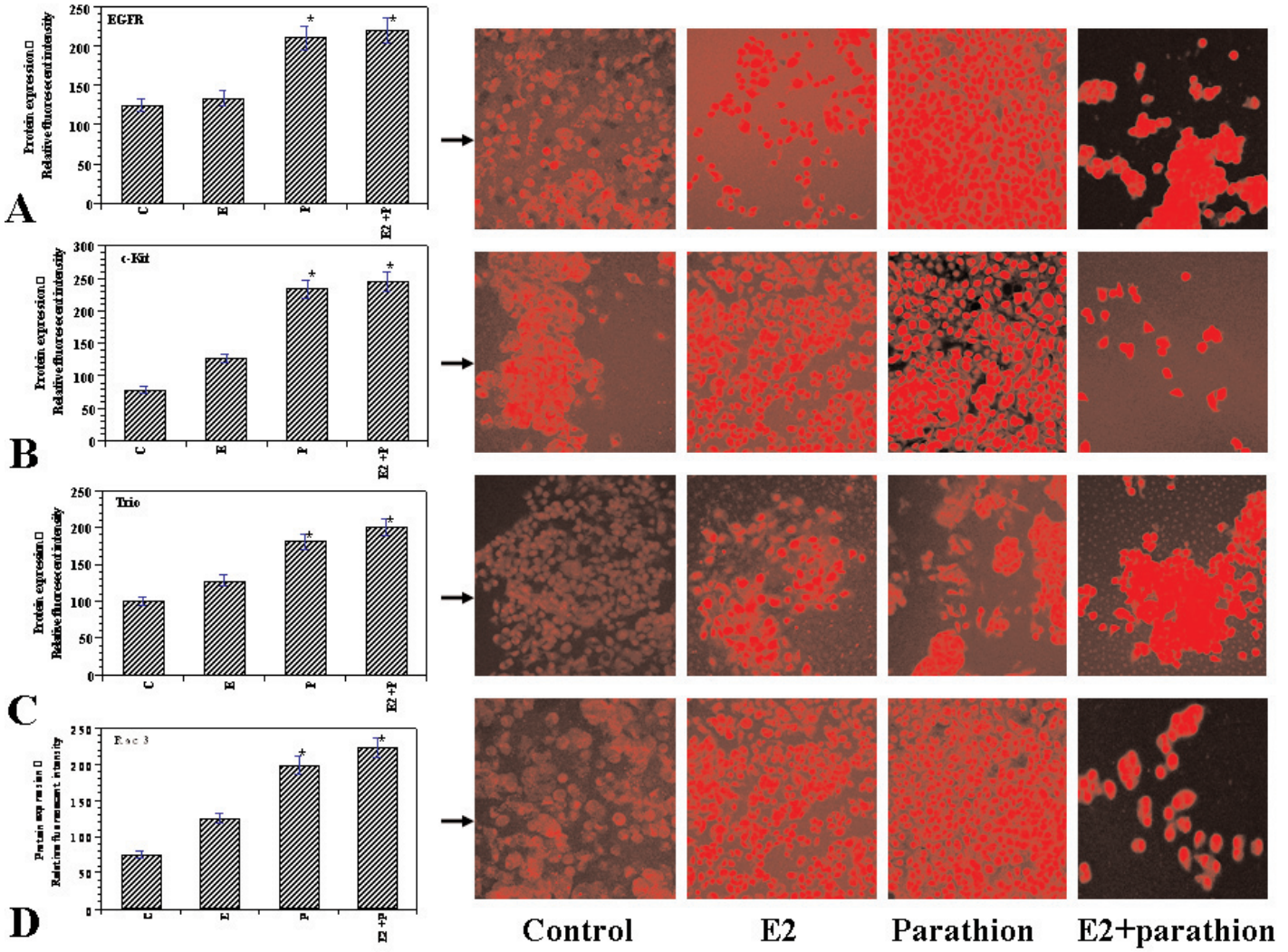

Figure 2. Bars represent the average and standard error of (A) EGFR, (B) c-Kit, (C) Trio and (D) Rac 3 of MCF-10F, E2, parathion and a combination of both. Representative immunofluorescence- stained images of (A) EGFR, (B) c-Kit, (C) Trio and (D) Rac 3 protein expression of MCF-10F, E2, parathion and a combination of both. Protein expression was determined by immunofluorescent staining and quantified using confocal microscopy and a computer program, which gives the area and the intensity of the staining as described in the text. The primary antibodies used were: rabbit polyclonal for EGFR, c-Kit and Trio and goat polyclonal for Rac 3 antibody (Biotechnology Inc., Santa Cruz, CA, USA).

sults indicated that differential transcriptional response was observed in 22 genes out of a total of 96 genes in the array. The results of gene expression arrays are shown in Table 1. The genes that showed differential expression, included Cyclin A2 (CCNA2) which was down-regulated with E2, parathion and combination of both; Cyclin C (CCNC), CDC6 and CDKNIA (p21) which were only up-regulated more than 3.0 fold with parathion in comparison to controls, Cyclin D3 (CCND3) which was up-regulated more than 4.0 fold with E2 and parathion. The CDKN2C (p18) which is associated with cell cycle checkpoint regulation was down-regulated 3.0 fold with both E2 and parathion. The CKSIB corresponding to genes affecting G2 phase and G2/M transition was down-regulated 3.0 fold with both E2 and parathion. The mini-chromosome maintenance deficient known as MCM6 (Mis5) was up-regulated by 4.0 fold in the presence of E2 and E2 plus parathion in comparison to the expression of control cells, whereas the MDM2 was elevated by 2.5 fold with the combined treatment of E2 and parathion.

DISCUSSION

Epidemiological evidences on the relationship between chemical pesticides

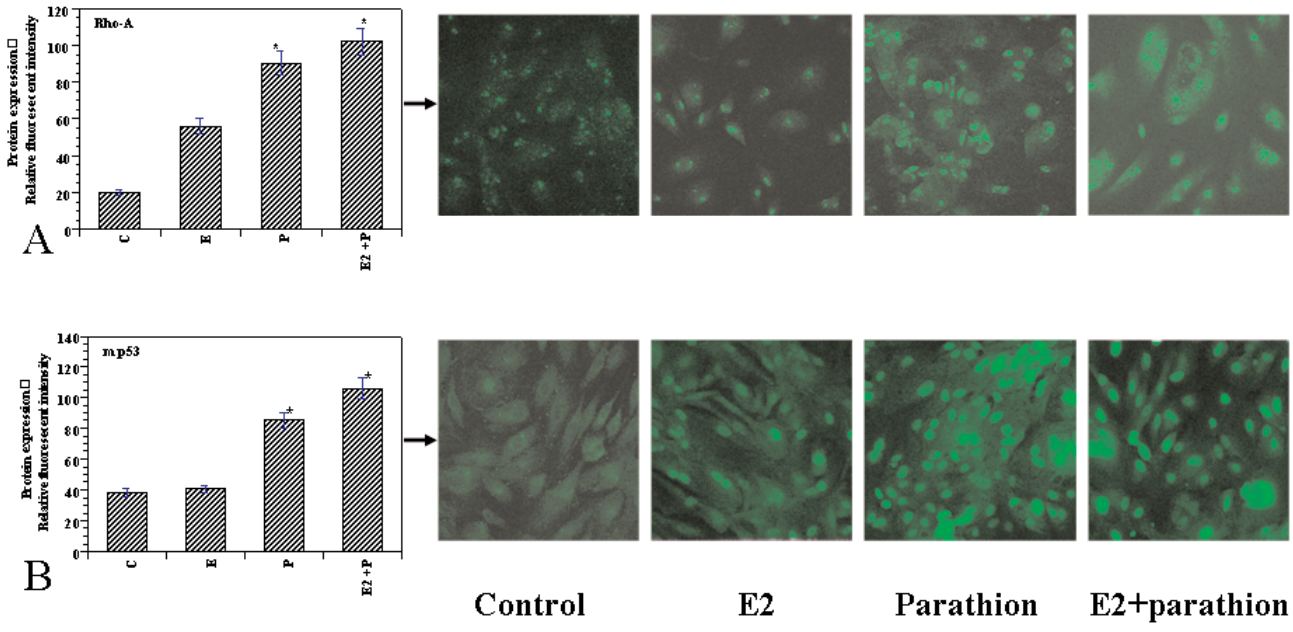

Figure 3. Bars represent the average and standard error of (A) Rho-A and (B) mutant p53 protein expression of MCF-10F, E2, parathion and combination of both. Representative immunofluorescence stained images of analyzing (A) Rho-A and (B) mutant p53 and protein expression of MCF-10F, E2, parathion and combination of both. The primary antibodies used were mouse monoclonal antibody (Biotechnology Inc.).

and cancer incidence have been previously reviewed (39). The organophosphorous compounds are causally linked with non-Hodgkin's lymphoma and leukemia. In animal studies, many pesticides are proven to be carcinogenic (for example, organochlorines, creosote, and sulfallate) while others (organochlorines DDT, chlordane, and lindane) are tumor promoters. Some contaminants in commercial pesticide formulation also may pose a carcinogenic risk. In humans, insecticides used occupationally have been classified as carcinogens by the International Agency for Research on Cancer. However, human data are limited due to restricted number of studies that have evaluated individual pesticides. Organophosphorous pesticides are currently used to help in boosting agricultural production. However, these pesticides pose serious threats to multiple organisms, including humans.

Epidemiological and experimental evidences also implicate estrogens in the etiology of breast cancer. Human populations, however, are exposed to mixtures

Table 1. Differentially expressed genes in estrogen and parathion-treated cells

Array Location	Gene Name (Symbol) Function	MCF-10F[a]	E2[bcd]	Parathion[bcd]	E2+ parathion[bcd]
8	Cyclin A2 (CCN1, CCNA2) Regulation of cell cycle	–	↓	↓	↓
11	Cyclin C (CCNC) Regulation of cell cycle	–	–	↑	–
14	Cyclin D3 (CCND3) Regulation of cell cycle	–	↑	↑	–
29	CDC18L/HsCDC18(CDC6) Regulation of cell cycle	–	–	↑	–
36	CAP20/CDKN1 (CDKN1A) Regulation of cell cycle	–	–	↑	–
41	INK4C/p18 (CDKN2C) Cell cycle arrest	–	↓	↓	–
44	CKS1/PNAS-16 (CKS1B) G2&G2M regulation	–	↓	↓	–
67	MCG40308/Mis5 (MCM6) S phase & DNA replication	–	↑	–	↑
69	Hdm2 (MDM2)	–	–	–	↑

[a]Both up regulated and down regulated of gene expression over 2.0 to 5.0 fold alterations are taken into consideration.
[b]Up arrow indicates the up-regulated expression of that gene with respect to control MCF-10F.
[c]Down arrow indicates the down-regulated expression of that gene with respect to control MCF-10F.
[d](–) Indicates no change in expression of that gene with respect to control MCF-10F.

of estrogenic or estrogen-like agents and it is therefore necessary to consider the impact of combined effects of pesticides and estrogens. In vivo and in vitro experimental models are needed to understand the effects of these compounds in breast carcinogenesis. A rat mammary tumor model induced by eserine and the pesticides parathion and malathion, possibly through acetyl cholinesterase inhibition (40) and by 17β-estradiol has already been established (41). Identification of factors involved in cell proliferation and transformation has been facilitated by studies using various human in vitro cell systems.

The present work was undertaken with the purpose of evaluating the factors that modulate the transformation in vitro by the effect of estrogen and parathion of human breast epithelial cells. Previous studies have shown that four carcinogens, 7, 12, dimethylbenz(α)anthracene (DMBA), benzo(α)pyrene (BP), methyl-N-nitro-nitrosoguanidine (MNNG), and N-methyl-N-nitrosourea (NMU) were unable to transform primary human breast epithelial cell cultures (42, 43). Normal human breast epithelial cells were maintained in vitro senesce after ten to twenty passages. It was found that ten out of twenty samples obtained from the breast tissue of women who underwent reduction mammoplasty expressed increased survival efficiency only in agar methocel after carcinogen treatment when compared with control cells (43). The DMBA and BP require metabolic activation, whereas NMU and MNNG are directly acting carcinogens. DMBA, BP, and MNNG were dissolved in DMSO and NMU in 0.9% NaCl containing 0.3% acetic acid. The carcinogens were adjusted to a final concentration of 1.0 μg/mL. These results indicated that primary cultures grown in 1.05 mM Ca^{+2} underwent terminal differentiation after a certain number of population doublings, whereas such cells grew in 0.04 mM Ca^{+2} for many passages. When cells were treated with the four carcinogens, only one sample in a total of 20 slightly increased the number of population doublings over the control (42). The immortalized normal human breast epithelial cell line, MCF-10F derived from such cells was named sample 130 (31). This cell line showed similar effects. The same ten samples showed increased survival efficiency in agar methocel in comparison to non-treated cells.

The immortalized MCF-10F cell line treated with the same chemical carcinogens induced expression of malignant phenotypes and point mutations in codons 12 and 61 of c-Ha-ras oncogene were found in treated cells. MCF-10F cells transfected with the mutated c-Ha-ras gene exhibited the same malignant phenotypes shown by carcinogens mentioned above and induced tumorigenesis in SCID mice. It was concluded that both chemical carcinogens and mutated c-Ha-ras gene induced malignant transformation of immortalized breast epithelial cells, suggesting that the critical point in the transformation pathway is previous immortalization of the cells (26,42,43).

The present study shows that both parathion alone and its combinations with E2 were capable of altering cell proliferation and inducing transformation of MCF-10F cell line. The first noticeable sign was morphological changes that occurred at or around the twentieth passage of cells after treatment, which may well represent an early step of transformation. The increase in cell proliferation rate indicated by a shorter doubling time, anchorage independent growth, and in vitro invasive capability suggests a very aggressive phenotype. Treatment of parathion alone or its combination with E2 exhibited significant invasive capabilities as compared with control cells. The chemoinvasion, or the ability of transformed cells to cross basement membranes in vitro, has been correlated with the malignant characteristics of cells (19,22–26). Therefore, this study shows that estrogen and parathion did not have a synergistic effect. The lack of demonstrable synergism between the two substances does not rule out the possibility that synergism exists. Numerous investigations involving hypophysectomy and ovariectomy have indicated that prolactin and estrogen are the key hormones in mammary tumorigenesis in DMBA-induced rat mammary carcinoma in vivo (44). However, previous in vitro studies showed that prolactin in combination with 17β-estradiol failed to induce DNA synthesis of such carcinomas (data not shown). Others (44) have also demonstrated that estrogen blocked the prolactin stimulatory effect on DNA synthesis. Thus, it is possible that estrogen may act through other mechanisms (i.e., via the pituitary).

To assess cell transformation, the expression of several proteins frequently associated with signaling pathways was determined in pesticide- and estrogen-treated cells in comparison to control MCF-10F. The present results showed an increase in EGFR protein expression in cells treated with parathion alone and with the combination of E2 and parathion in comparison to control and E2-treated cells alone. This is an important finding if we consider that growth factors and their receptors are included in the group of proteins which are functionally related to cell proliferation. Furthermore EGFR has been found at abnormally high levels on the surface of many types of cancer cells (45,46).

Results also showed increase in c-Kit, Trio, and Rac 3 protein expression in cells treated with parathion alone and with the combination of E2 and parathion in comparison to either control or E2-treated cells alone. Activating mutations in many genes are oncogenic and serve to liberate cancer cells from normal homeostatic mechanisms, allowing self-sufficient proliferation (46,47). The *c-Kit* is a proto-oncogene identified as a member of the receptor tyrosine kinase family. Signaling pathways related to cell proliferation often begin with the activation of a receptor tyrosine kinase by a growth factor, although activation of some G-protein–coupled receptors can also activate certain branches of this signaling pathway (48,49). Trio is a protein localized in the nuclei (50). One of the Trio

domains exhibits Rac-specific activity whereas the other exhibits Rho-specific activity (51). Depending on the proteins subsequently recruited by the activated receptor, several downstream signaling pathways also may be activated. These proteins regulate many fundamental processes in all eukaryotic cells such as growth, vesicle traffic, and cytoskeletal organizations. Rac is a GTP-binding protein belonging to Ras super family that regulates the same processes (48,52–56). Both Rho and Ras are expressed in a wide range of cell types (52–56). The level of Rho-A protein expression was largely enhanced in the E2, parathion, and combination of E2 and parathion-treated cells in comparison to control. A previous study (52) has addressed the question of a putative relevance of Rho proteins in tumor progression by analyzing their expression on protein level in breast tumors and they found that the level of Rho-A protein was increased in all breast tumor samples as compared with normal tissues (53).

The mutant p53 protein expression was greater in parathion alone and in the combination of E2 and parathion-treated cells in comparison to control and E2-treated cells. It is known that *p53* is a tumor suppressor gene in normal cells and, when it is inactivated, cells may undergo uncontrolled cell proliferation (57–60). Then an over-expression of oncoproteins, such as mutant p53, is important in the initiation in cancer of human breast epithelial cells by several carcinogens as DMBA and BP (42). The MCF-10F cell line exposed to double doses of α particle radiation and simultaneously treated with E2 showed a very complex pattern of allelic imbalance during the process of cell transformation affecting *p53* gene at chromosome 17p13.1 (61). Several studies have shown that the expression of mutant p53 increases as breast cancer progress from early in situ to advanced metastatic lesions (58–60).

The protein expression of estrogen and parathion-treated breast epithelial cells did not show a synergistic effect, suggesting that estrogen follows a different pathway in relation to the proteins analyzed. Further work is in process to understand the pathways involved in the process of breast carcinogenesis induced by estrogens.

A second approach was to investigate the relationship between cell transformation and the regulatory proteins that are typically involved in the regulation of cell cycle. It is known that genetic changes that are relevant to tumor progression often involve the accumulation of multiple abnormalities in individual cells. It has been suggested that early loss of cell cycle control in the presence of a mitogenic stimulus allow a cell to continue dividing. When a cell cycle becomes deregulated, it can lead to aberrant cell proliferation resulting in cancer. Such uncontrolled proliferation occurred in the presence of parathion, leading to a high level of genomic instability. In this study, an array-based approach was used to monitor the expression of genes that are particularly involved in the regulation of cell cycle. Results indicated that, among the 96 genes of human cell cycle array, 22 genes were altered either by E2, parathion, or the combination of both. Among those genes involved in the regulation of the cell cycle were: Cyclin A2, which was down-regulated with E2, parathion, and the combination of both; Cyclin C, CDC6, and CDKNIA, which were only up-regulated 3.0 fold with parathion in comparison to controls; Cyclin D3, which was up-regulated with E2 and parathion. The CDKN2C associated with cell cycle checkpoint and cell cycle arrest was down-regulated with E2 and parathion. The CKSIB corresponding to genes affecting G2 phase and G2/M transition was down-regulated with E2 and parathion. The present results indicated that MCM6 was up-regulated in the presence of E2 and parathion plus E2 in comparison to the control value. The MCM6 labeling index has been correlated previously with proliferative activity and tumor grade in chondrosarcomas (62). The MDM2 that seems to promote degradation of p53 (63) and contribute to its oncogenic activity was up-regulated with the combination of E2 and parathion.

In summary, effects of estrogen and parathion, an organophosphorous pesticide, were evaluated on cell transformation of human breast epithelial cells in vitro determined by phenotypic characteristics, protein expressions, and differentially expressed cell cycle regulatory genes. Results of the present study demonstrated that parathion alone or in combination with estrogen influenced phenotypic transformation of previous immortalized human breast epithelial cells through alterations in the expression cell cycle regulatory genes.

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