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Cell adhesion proteins altered by 17ß estradiol and parathion in breast epithelial cells

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Abstract. The association between breast cancer initiation and prolonged exposure to estrogen suggests that this hormone may also have an etiologic role in such a process. On the other hand, many studies have found an association between human cancer and exposure to agricultural pesticides such as parathion, an organophosphorous pesticide used in agriculture to control mosquito plagues. However, the key factors behind the initiation of breast cancer remain to be elucidated. The aim of this study was to determine the effect of 17ß estradiol (estrogen) and parathion on protein expression in cell transformation of human breast epithelial cells *in vitro*. Estrogen and parathion alone and in combination induced malignant transformation of an immortalized human breast epithelial cell line, MCF-I0F, as indicated by anchorage independency and invasive capabilities. The results indicate that a combination of estrogen and parathion increased the expression of related cell adhesion proteins such as Dvl, Notch, CD146 and ß catenin. In conclusion, it can be suggested that pesticides affect human breast cell adhesion changes indicative of transformation.

Introduction

Breast cancer is one of the most common diseases among women, in which environmental chemicals have been partially implicated (1-3). Breast cancer is a complex disease in which numerous genetic aberrations occur. Cellular and molecular changes that occur through the development of cancers can be mediated by a diversity of endogenous and environmental stimuli. Evidence indicates that breast cancer risk is associated with prolonged exposure to female ovarian hormones (4-7). Exposure to carcinogens plays an etiological role in the initiation of breast cancer. Organophosphorous compounds are of great interest because of the extensive use in agriculture, medicine and industry (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 affecting humans (12).

On the basis of the currently accepted view of breast cancer as a multistep process, it is possible that specific abnormalities may be required in the transformation from a normal to an invasive tumor cell. Some of these changes involve specific genetic loci that directly contribute to one or more attributes of transformation, i.e., unregulated proliferation and invasion, while other changes confer genetic instability that increases the possibility of acquiring subsequent, specific lesions relevant to tumorigenesis.

Cell transformation appears to involve an increase in cell division as well as an increased risk of genetic damage that induces activation and/or changes in the amplification of oncogenes and the loss or inactivation of tumor suppressor genes leading to regulation of signal transduction pathways and abnormal amplification of growth signals (13-16).

Little or no information is available on the identification of proteins altered in cell transformation by the effects of an endogenous substance such as estrogen and a pesticide such as parathion. There are cytoplasmic phosphoproteins that regulate cell proliferation, acting as a transducer molecule for developmental processes; furthermore *Dvl* is a component of the *Wnt* signaling pathways (17). It is a human homolog of the *Drosophila* dishevelled gene (*dsh*). The *Notch* genes are expressed in a variety of tissues suggesting that the genes are involved in multiple signaling pathways (18-21). Others are overexpressed or rearranged in human tumors such as the 280-330 kDa Notch protein (22). The *LIN-12/Notch* family of transmembrane receptors is believed to play a central role in development by regulating cell fate decisions. Others are membrane glycoproteins which function as a Ca^{2+}-independent cell adhesion molecule involved in heterophilic cell-cell interactions such as *CD146*, also known as Mel-*CAM, MUC18, A32* antigen, and *S-Endo-l* (23).

Cell-cell adhesion is mediated by the cadherin-catenin system, of which E-cadherin and ß catenin are important epithelial adhesion molecules in normal epithelium as a

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prerequisite for normal cell function and the preservation of tissue integrity (24). ß catenin as a component of a complex signal transduction pathway may serve as a common switch in central processes that regulate cellular proliferation and differentiation (25). The 92-kDa catenin is associated with the cytoplasmic portion of E-cadherin necessary for the function of E-cadherin as an adhesion molecule. It is a protein that binds to the highly conserved, intracellular cytoplasmic tail of E-cadherin (25,26). Loss of E-cadherin-ß catenin adhesion is an important step in the progression of many epithelial malignancies (27). *In vitro* model systems have been extensively used in the study of initiation and transformation in cancer (16,28-31). The human breast epithelial cell line MCF-I0F, spontaneously immortalized and derived from the breast tissue of a 36-year-old female, has the morphological characteristics of normal breast epithelial cell lines (16,32-35). Therefore an *in vitro* model system using the spontaneously immortalized normal MCF-10F human breast epithelial cell line provides a unique opportunity for studying proteins involved in breast cancer.

Materials and methods

Cells. MCF-10F cells were cultured with DMEM/F-12 (1:1) medium supplemented with antibiotics [100 U/ml penicillin, 100 μg/ml streptomycin, 2.5 μg/ml amphotericin B (all from Life Technologies, Grand Island, NY)] and 10 μg/ml and 5% equine serum (Biofluids, Rockville, MD), 0.5 μg/ml hydrocortisone (Sigma, St. Louis, MO) and 0.02 μg/ml epidermal growth factor (Collaborative Research, Bedford, MA) were added (16,35). The experiments used MCF-10F cells as a control, MCF-l0F cells continually treated with estrogen at 10^{-8} M (Sigma-Aldrich, St. Louis), MCF-10F cells treated continuously with parathion (100 ng/ ml) (parathion-ethyl) (Sigma-Aldrich), and MCF-10F cells treated with a combination of both.

Protein expression. Exponentially growing cells were plated on a glass chamber slide (Nunc Inc., Naperville, IL) as previously described (16,35) at a density of $1x10^4$ cells in 1 ml of medium. The experiments were repeated three times with similar passages. Primary antibodies to Dvl, Notch, CD146 and ß catenin protein (all from Biotechnology Inc., Santa Cruz, CA) were used at a 1:500 dilution overnight at 4˚C. The secondary antibody used was Rhodamine-conjugated secondary antibody (Jackson ImmunoResearch Lab., West Grove, PA) at a 1:1000 dilution. Slides were mounted with Vectashield mounting media (Vector Laboratories, Burlingame, CA). Cells were quantified as previously described (16,35) and viewed on a Zeiss Axiovert 100 TV microscope (Carl Zeiss, Thornwood, NY) using a x40 11.3 NA objective lens equipped with a laser scanning confocal attachment (LSM 410; Carl Zeiss). To excite the fluorescent secondary antibody fluorescent images were collected by argon/krypton mixed gas laser (488 nm). Fluorescent images were collected in black and white and changed to green color by Photoshop. Composite images were generated using the Adobe Photoshop, 5.5 program. A semi-quantitative estimation based on the relative staining intensity of protein expression by the control and transformed cells was determined. The computer program gives the area and the intensity of the staining of the cells present in the culture dishes. Standard errors of the mean are given. The number of immunoreactive cells (30 cells/ field) was counted in 5 randomly selected microscopy fields per sample. Again, standard errors of the mean are shown. Statistical analysis was performed with the F-test (randomized block) and comparisons were made between groups with the Bonferroni t-test with significance at a P-value of <0.05.

Table I. Origin and phenotypic characteristics of cells.

Treatment	Origin	AIA	IA
None	MCF-10F parental cells	-	-
Estrogen	MCF-10F treated with 17ß estradiol	-	-
Parathion	MCF-10F treated with parathion	+	+
E + Parathion	MCF-10F treated with combination	+	+

The parental MCF-10F cell line was treated with 17ß estradiol (E) 10^{-8} M, parathion (100 ng/ml) and a combination of E plus parathion. AIA, Anchorage Independence Assay; IA, invasion assay. +, Anchorage-independent growth and invasiveness characteristics; -, lack of both characteristics. Invasive characteristics of control and treated MCF-10F cells were scored 20 h after plating onto matrigel basement membranes using Boyden's chambers.

Results

The normal growth characteristics and non-invasive capabilities of the MCF-10F cell line have been previously demonstrated (16,34,35). The independent growth characteristics and invasive capabilities of the parathion- and parathion plus estrogen-treated cells were previously shown, thus the control cells did not form any colony. The origin and phenotypic characteristics of cells used in this study are shown in Table I. The expression of several proteins as markers of cell transformation frequently associated with breast cancer was determined in these cells in the present study. Dvl (Fig. lA), Notch (Fig. 1B), CD146 (Fig. 1C) and ß catenin (Fig. 1D) protein expression was examined in the control MCF-10F cells, and those treated with estrogen, parathion and a combination of both. The quantification of results is represented on the left side of the Fig. 1 with histograms showing the standard error. Results of the immunofluorescent imaging of stained cells showed that protein expression was significantly ($P<0.05$) greater in cells treated with parathion alone and a combination of estrogen and parathion than in control and estrogen-treated cells. Immunofluorescent staining of protein expression indicated that the staining intensity among individual cells was fairly uniform in the population of all the groups. The right side of Fig. 1 shows the immunofluorescent staining of protein by confocal microscopy of Dvl (Fig. 1A), Notch (Fig. 1B), CD146 (Fig. 1C) and ß catenin (Fig. 1D) protein expression in control MCF-10F cells and those treated with estrogen, parathion and a combination of both. The CD146 and ß catenin protein expression in the membranes was significantly ($P<0.05$) greater in cells treated with parathion alone and estrogen combined with parathion in comparison to control and estrogen-treated cells. However, CD146 protein expression was significantly ($P<0.05$) greater in the combination-treated

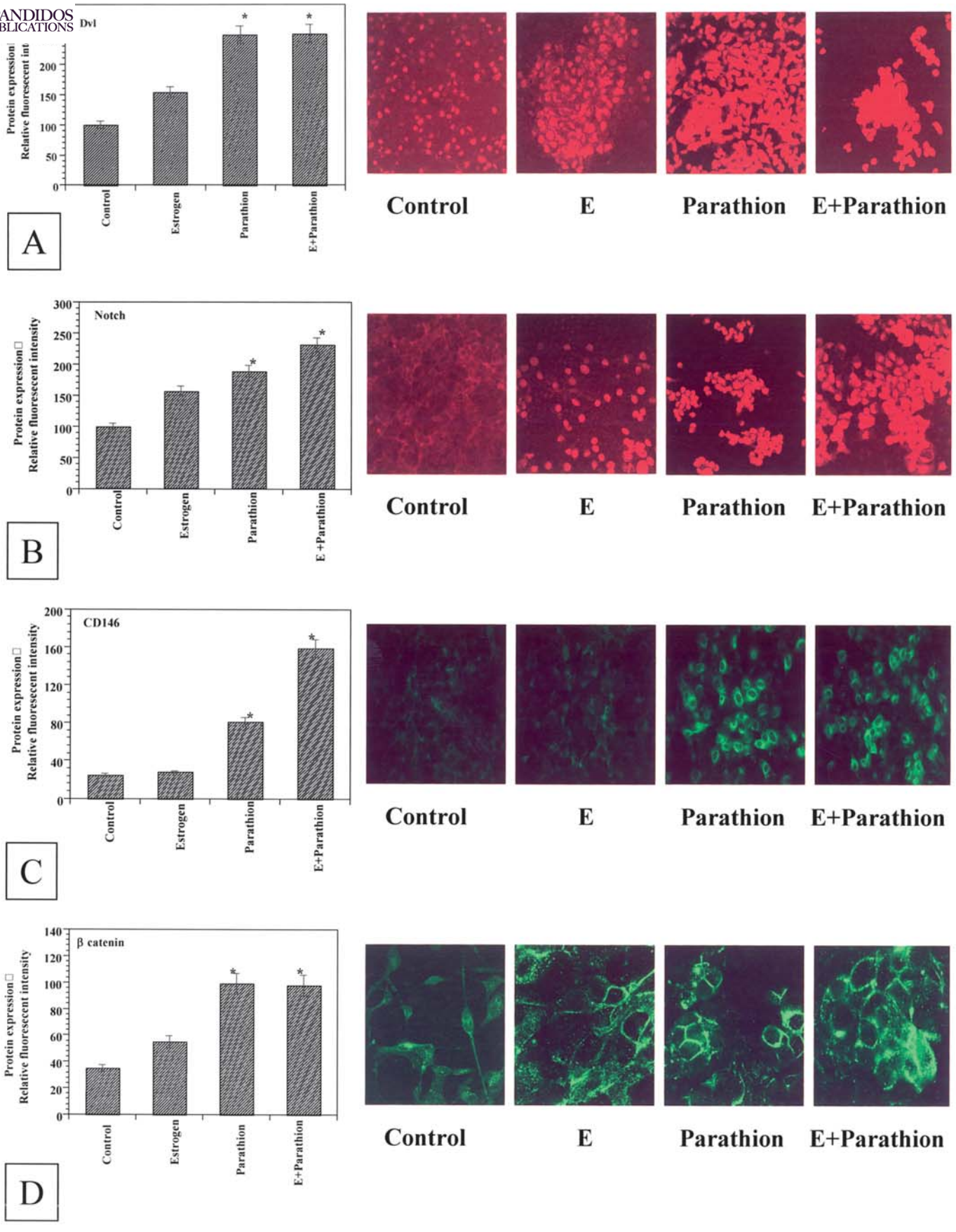

Figure 1. Protein expression of cells was determined by immunofluorescent staining and quantified using confocal microscopy and a computer program, which gives the area and the intensity of the staining as described in the text. The primary antibody used was mouse monoclonal antibody (Biotechnology Inc., Santa Cruz, CA). On the left side bars represent the average and standard error of Dvl (A), Notch (B), CD146 (C) and ß catenin (D) protein expression in MCF-10F cells not treated or treated with estrogen, parathion, or a combination of estrogen and parathion. On the right side, representative immunofluorescence staining of Dvl (A), Notch (B), CD146 (C) and ß catenin (D) protein expression in MCF-10F cells not treated or treated with estrogen, parathion, or a combination of estrogen and parathion.

cells in comparison to those treated with parathion alone. These results showed that the presence of parathion induced the majority of changes related to cell adhesion.

Discussion

Experimental studies have also demonstrated that mammary cancer is a hormone-dependent (4) and multi-step process that can be induced by a variety of compounds and mechanisms (5). Identification of factors involved in cell proliferation and transformation has been facilitated by studies using various human epithelial cell lines. Since there is little or no information available on estrogen- and pesticide-induced breast cancer, an *in vitro* breast transformation model provides a unique opportunity for studying breast carcinogenesis.

In vitro systems based upon the use of the MCF-10F cell line have been used to detect sensitivity to carcinogens as both chemical, e.g., 7, 12, dimethylbenz (a) anthracene (DMBA), benzo (a) pyrene (BP) (34), and environmental, e.g., ionizing radiation (16). Estrogen and parathion induced anchorage-independent growth and invasiveness (40). We have previously shown that estrogen was necessary during the carcinogenic process by the effect of LET radiation (16). Based on the data obtained from several transformed cell lines that represent the various stages of the transformation process, a conclusion can be made regarding these kinds of cells. Similar phenotypic properties of growth rate, anchorage-independent growth and invasive characteristics have also been reported during the process of transformation by chemical carcinogens (34) and environmental factors, e.g., ionizing radiation (16). The chemoinvasion or the ability of transformed cells to cross basement membranes *in vitro* correlated with the malignant characteristics of the cells.

A great deal of progress has been made in understanding the pathogenesis of breast cancer, allowing the development of useful tumor markers. We have also previously (41) shown that estrogen and the organophosphorous compound parathion were capable of altering cell proliferation and inducing transformation in an immortalized normal human breast epithelial cell line. Dvl protein expression was increased in the transformed MCF-10F cells in the presence of estrogen and parathion, suggesting that Dvl may regulate cell proliferation. It has been shown to be involved in controlled proliferation and migration of vascular endothelial cells (17). It has been suggested to act as a transducer molecule for developmental processes and it is a component of the *Wnt* signaling pathway. Notch protein expression was also increased in the presence of estrogen and parathion, suggesting that these genes are involved in multiple signaling pathways (18-21). The Notch proteins are also overexpressed or rearranged in human tumors (22) and are believed to play a role in regulating cell fate decisions.

The cell adhesion analyzed by CD146 protein expressed in the membranes was greater in cells treated with parathion alone and estrogen combined with parathion in comparison to control and estrogen-treated cells. However, CD146 protein expression was greater in the combination-treated cells in comparison to those treated with parathion alone. The present study indicated that CD146 protein expression was increased in the transformed MCF-10F cells treated with estrogen and parathion. Other authors also found CD146 expression in malignant neoplasms such as melanomas and breast tumors (23) by using immunohistochemistry with CD146-specific antibodies. CD146 has been suggested to play an important role in tumor progression. The complex interactions between CD146-expressing cells and their microenvironment are in need of further investigation in order to elucidate the functions of this molecule in biology and in pathological states. The specific expression pattern of CD146 may be useful in the differential diagnosis of certain lesions including melanomas and malignant tumors (23). CD146 is a cell adhesion molecule and its biological functions and role as a diagnostic marker in pathology are now being recognized.

The cell adhesion was also analyzed by ß catenin protein expressed in the membranes that was greater in cells treated with parathion alone and estrogen combined with parathion in comparison to control and estrogen-treated cells. The function of the cadherin-catenin system in cell adhesion as well as intracellular signaling appears to be subjected to multi-factorial control by a variety of different mechanisms. ß catenin had a similar reaction in the presence of parathion alone and combined with estrogen in comparison to the control and in the presence of estrogen. However, it seems that estrogens did not play a role in this pesticide-induced model mediated by the cadherin-catenin complex since both substances had equal effect. This complex may modulate or initiate signaling events implicated in differentiation and growth control. Previously, malignant transformation of human bronchial epithelial cells with the tobacco-specific nitrosamine, 4-(methylnitrosamino)-I-(3-pyridyl)-1-butatone showed increased ß catenin protein expression (36). Studies have indicated that the E-cadherin-catenin complex is the target of many growth factor and hormone-dependent signaling pathways which regulate its function and expression (25). Other authors have found that catenin, especially when it is expressed in the cytoplasm, seems to be a very sensitive prognostic marker with the E-cadherin complex in invasive breast cancer (37-39). It may have invasive capabilities since a possible role of the E-cadherin/α-catenin complex in modulating cell-cell and cell-matrix adhesive properties of invasive colon carcinoma cells has been reported (25).

In summary, results showed that the presence of parathion induced the majority of changes related to cell adhesion. This study clearly demonstrated that an organophosphorous pesticide such as parathion can influence the phenotypic transformation of human breast cells.

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