Determination of the Expression of COX-2 and Aromatase Protein in Parent and Tamoxifen-resistant Subline of Human Breast Cancer T47D Cells

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Abstract: Genetic differences among tumor cells of breast cancer patients are the main reasons for therapeutic failure. COX-2 and aromatase proteins have been determined to be important in breast cancers as potential targets for prevention and can also be a part of the therapeutic regimen of these cancers. Consequently, we decided to determine the expression of these proteins in human breast cancer T47D cells and its established tamoxifen resistant subline, namely T47D/TAMR-6 cells. Immunocytochemical technique was employed using primary antibodies for each protein followed by visualization of results with LSAB2 detection kit under the microscope. Our data showed that expression of COX-2 was relatively the same in parent and resistant T47D cells in the presence and absence of Tamoxifen (1 μM). Unlike tamoxifen, celecoxib could dramatically decrease the expression level of COX-2 in both cell types. Aromatase protein expression seems to be absent or is expressed at very low levels in both cell types and under all experimental conditions. Our data indicates no significant difference between studied cell types with respect to expression of aromatase and COX-2 enzymes. Therefore, these data suggest different possible outcomes for specific inhibitors of these enzymes alone or in combination with tamoxifen in the therapeutic regimen of breast cancer patients.

Key words: Breast cancer, COX-2, aromatase, T47D cells, tamoxifen

INTRODUCTION

Breast cancer is the most common cancer among women throughout the world with more than one million individuals being diagnosed with breast cancer each year. It is responsible for 18% of all women cancers which are more prevalent in western countries (Pharoah et al., 1998; McPherson et al., 2000). Breast cancer is sporadic and hereditary. According to some reports, breast cancer can be inherited separately from individuals. These features include early onset of disease, prevalence of significant involvement of bilateral breast cancer, being associated with other malignancies such as ovarian cancer, prostate and colon in the same person or other family members and also disease transmission to the next generation (Fitzgibbons et al., 2000). Cancer is a multi-factorial and complex disease that involves genetic and environmental factors. Unfortunately, despite significant scientific advances, breast cancer continues to have high mortality rate among cancers (Wild et al., 2002).

Genetic differences of tumor cells formed in patients, gene variation in the incidence and progression of the disease and also response to existing therapies are the reasons for major causes of treatment failure in which type of cancer. Approximately one-third of all breast cancers and two-third of postmenopausal breast cancer is hormone dependant which requires estrogen for tumor growth (Brueggeheimer et al., 2005) and aromatase, cytochrome P450 enzyme complex, catalyzes estrogen biosynthesis (Brueggeheimer et al., 1999). Cyclooxygenase (COX) is an enzyme responsible for converting arachidonic acid to prostaglandins (Ruan et al., 2009; Anwar et al., 2010). Studies have shown that COX-2 is present in breast tumors but are not detectable in normal human breast tissue (Singh et al., 2006).

COX inhibitory drugs such as indomethacin, piroxicam and sulindac have shown an inhibitory effect on the growth of colon and mammary tumors in rodent models of chemical carcinogenesis (Narisawa et al., 1993; Fulton, 1984). Genes producing COX-2 and aromatase are considered to play a part in breast cancer. Protein inactivating agents can be used as therapy by targeting the over expression of these proteins in breast cancer cells (Brodie et al., 2001; Miller, 2004; Miller et al., 1990;

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Lipton et al., 1992; Lu et al., 1996; Zhao et al., 1996). COX-2 may be over expressed by inflammatory disease and certain cancers which could play a role in the disease process. Drugs inhibiting COX-2 activity are effective in prevention of colon cancer (Sheng et al., 1997). Therefore, preventing the activity of this enzyme should help to prevent cancer progression.

The enzyme aromatase is involved in the production of estrogen which is found in a number of patient’s breast cancers. This enzyme plays a role in proliferation of estrogen-dependent breast cancer and so specific aromatase inhibitors can be helpful in the treatment of these patients (Buzdar et al., 1998; Cepa et al., 2008; Gershonovich et al., 1998; Liszwan et al., 2008).

Therefore, by taking into consideration the importance and prevalence of breast cancer in women and the high mortality rate in patients, we evaluated the expression of COX-2 and aromatase enzymes in T47D breast cancer cell line and its subgroups resistant to tamoxifen (T47D/TAMR-6) by immunochemistry.

MATERIALS AND METHODS

Cell line and culture conditions: The human breast cancer T47D cell line was purchased from the National Cell Bank of Iran (Pasteur Institute of Iran, Tehran, Iran). The subline of the tamoxifen-resistant T47D human breast cancer cell line was established and kept in our lab as described in continues. Cells were kept in RPMI-1640 culture medium supplemented with 10% fetal bovine serum, 100 µ g/mL streptomycin, and 100 µ g/mL penicillin in a 5% carbon dioxide (CO₂) incubator at 37°C.

Cytotoxicity assay: T47D cells were seeded in two 24-well plates (4×10⁴ cells/well) in the absence (Blank) and presence of tamoxifen (1×10⁻⁶-1×10⁻⁵ M) for 48 h and 1 week in the described medium. The cell number in each group was then measured using the trypan blue dye exclusion method.

Tamoxifen-resistant subline: Based on the results of the cytotoxicity assay, the tamoxifen-resistant subline was isolated by continuous exposure of T47D cells to tamoxifen at concentrations starting from 1×10⁻⁶ M and increasing in a stepwise manner to 1×10⁻⁵ M within 6 months. Cells that were capable of sustained growth in medium containing 1×10⁻⁶ M of TAM were considered to be resistant to TAM and are referred to T47D/TAMR-6 cells. Cell viability was determined after each step using trypan blue dye exclusion method.

Growth determination of parent and TAM resistant T47D cells: T47D and T47D/TAMR-6 cells were seeded in 24-well plates at 5×10⁴ cells/well in RPMI 1640 culture medium and incubated in 5% CO₂ at 37°C incubator. After washing with PBS, the cells were trypsinized and then counted using the trypan blue dye exclusion method every 48 h for 6 days. Doubling time for each cell population was then determined from its growth curve, in which each point is the average determination of 4 wells in three independent experiments.

Immunocytochemical analysis of COX-2 and aromatase protein expression: The parent T47D and TAM-resistant cells were seeded in 4-well chamber slides in RPMI 1640 for 48 h. The cells were then fixed in methanol/aceton (9/1) at -20°C. Endogenous peroxidase activity and nonspecific binding sites were blocked by incubating fixed cells in 3% hydrogen peroxide in methanol for 30 min and 5% BSA for 60 min, respectively. Cells were then incubated overnight at 4°C with COX-2 and aromatase monoclonal antibody. The primary antibody was used at a dilution of 1/50. The results were visualized using the streptavidine-biotin immunoperoxidase detection kit (LSAB2, Dako Corporation, Denmark) and AEC chromogen based on the manufacturer’s instruction with necessary modifications. Finally, cells were counterstained with Meyer’s hematoxyline, mounted and studied under light microscope. A section in which incubation with the primary antibody was omitted was used as negative control. Stained cells were then classified into 5 categories based on the nuclear expression of COX-2 and aromatase.

Statistical analysis: SIGMASTAT™ (Jandel Software, San Raphael, CA) was used to perform statistical analysis of data. The student’s t-test was used to examine the differences among treatments. Mean differences with p-values less than 0.05 were considered to be significant.

RESULTS

Cytotoxicity determination of T47D cells in different concentrations of tamoxifen: Unlike 48 h, after a week of cell exposure to 1×10⁻⁶ and 1×10⁻⁵ M to tamoxifen, significant decrease (24.4% and 52.2%, respectively) in cell growth was seen compared to control RPMI (Fig. 1). No significant differences between control RPMI (C) and Blank (B) were observed in cell proliferation (0.5% v/v EtOH, used as co-solvent of tamoxifen) (Fig. 1).

Doubling time determination of parent and TAM resistant T47D cells: Doubling time of T47D/TAMR-6 cells significantly increased (44.1%, p<0.001) compared
Fig. 1: Cytotoxicity of tamoxifen concentrations on T47D cells. The T47D cells were seeded in two 24-well plates (4×10^4 cells/well) in the absence (Blank) and presence of TAM (1×10^{-8} - 1×10^{-6} M) for 48 h and 1 week. The number in each group was determined using trypan blue dye exclusion method. Data are presented as Mean±SE of the average determination of 4 wells in three independent experiments. *Indicates significant differences compared with C (p<0.05), **indicates significant differences compared with C (p<0.001).

Fig. 2: Doubling time determination of T47D and TAMR-6 cells. The T47D and T47D/TAMR-6 cells were seeded in 24-well plates at 5×10^4 cells/well in RPMI 1640 culture medium. Cells were counted using trypan blue dye exclusion method every 48 h for 6 days. Data are presented as Mean±SE of determination of 4 wells in 3 independent experiments. ***Indicates significant difference compared with C (p<0.0001)

with parental T47D cells which indicate the slow proliferation pattern of TAM-resistant cells (Fig. 2).

Fig. 3: Growth determination of tamoxifen concentrations on parent and TAM resistant T47D cells. The T47D cells were seeded in 24-well plates (4×10^4 cells/well) in presence of TAM (1×10^{-8} - 1×10^{-6} M) for 1 week. The number in each group was determined using trypan blue dye exclusion method. Data are presented as Mean±SE of the average determination of 4 wells in three independent experiments. *Indicates significant differences compared with C (p<0.01).

Growth determination of parent and TAM resistant T47D cells in different concentrations of TAM: As shown in Fig. 3, both growth rate of the parent and TAM resistant T47D cells decreased by increasing concentrations of TAM. Growth of TAM resistant T47D cells at 1×10^{-6} M concentration of TAM showed significant differences (16.6%) compared with parent T47D cells (p<0.01).

Determination of COX-2 protein in parent and TAM resistant T47D cells by immunocytochemistry: Cell count and intensity of red dye related to COX-2 protein was observed to some extent increase the TAM resistant T47D cells compared with parent T47D cells. However a significant decrease in COX-2 expression in both cell types, particularly in parent T47D cells was observed in the presence of Celecoxib (1 uM) (Fig. 4).

Determination of aromatase protein in parent and TAM resistant T47D cells by immunocytochemistry: Both parent and TAM resistant T47D cells showed little response after staining with anti-aromatase antibody which indicates negligible or no expression of this enzyme in T47D breast cancer cells (Fig. 5).
DISCUSSION

Breast cancer is common worldwide and has different prevalence in different parts of the world so that some regions such as North America and Western Europe have been classified as high risk regions and other regions of the world such as China and Japan have been classified as low risk regions (Martin and Weber, 2000). Investigation of breast cancer prevalence and its contributing factors is worthwhile in each population. Studies have shown the role of COX-2 and aromatase enzymes in incidence and progression of breast cancer (Cepa et al., 2008; Liszwan et al., 2008; Gershonovich et al., 1998). Currently using drugs to inhibit the activity of these enzymes in high risk populations can be considered to be a method of prevention and treatment for breast cancer. Present results show that these two cytoplasmic enzymes are expressed with entirely different values in parent and TAM resistant T47D breast cancer cells as seen by the very low expression of aromatase but significantly high expression of COX-2 in both cells. Decreased expression of COX-2 in both cells by celecoxib, a COX-2 inhibitor, is also of interest. The very low or lack of expression of aromatase in our study has also been reported in previous studies which is related to different types of tumors and genetic structure (Brodie et al., 2001; Miller, 2004; Miller et al., 1990; Lipton et al., 1992). In one of these studies, 102 breast tumor samples were used to evaluate the presence of aromatase enzyme by immunohistochemistry, only 58 samples were positive (Brodie et al., 2001). This study did not show any relationship between aromatase and COX-2 expression, however, some studies have shown a direct correlation between expression of these enzymes (Brueggemeier et al., 1999; Brodie et al., 2001). This issue
can be linked to the genetic nature of tumor cells, testing methods and the materials and reagents that are used. Significant differences in the expression of these enzymes are of importance; their different expression shows the need for further consideration to carry out clinical studies using inhibitors of these enzymes in the prevention and treatment of breast cancer. On the other hand, no significant difference in the expression of these enzymes in parent and TAM resistant T47D cells suggests the same probability of usefulness of inhibitors of these enzymes in patients. Furthermore, using aromatase inhibitors combined with tamoxifen which is established for preventing and treatment of breast cancer cannot be predicted to be very useful. This point has been already demonstrated in administration of letrozol combined with tamoxifen (Cepa et al., 2008, Buzdar et al., 1998; Lisztwan et al., 2008, Gershonovich et al., 1998; Lu et al., 1999).

CONCLUSION

Present results suggest that treating breast cancer should be individually-based considering the tumor levels of markers including COX-2 and aromatase protein for choosing the best chemotherapeutic regimen.

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REFERENCES


