Short Communication

Nuclear Localization of Catechol-O-Methyltransferase in Neoplastic and Nonneoplastic Mammary Epithelial Cells

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Catechol-O-methyltransferase (COMT) plays both a regulatory and protective role in catechol homeostasis. It contributes to the regulation of tissue levels of catecholamines and catecholestrogens (CEs) and, by blocking oxidative metabolism of catechols, prevents endogenous and exogenous catechols from becoming a source of potentially mutagenic electrophiles. Evidence implicating CEs in carcinogenesis, in particular in the hamster kidney model of estrogen-induced cancer, has focused attention on the protective role of COMT in estrogen target tissues. We have previously reported that treating hamsters with estrogens causes translocation of COMT to nuclei of epithelial cells in the renal cortex, the site of CE biosynthesis and where the cancers arise. This finding suggested that nuclear COMT may be a marker of a threat to the genome by catechols, including CEs. It is postulated that CEs play a role in the genesis of breast cancer by contributing to a state of chronic oxidative stress that is presumed to underlie the high incidence of this disease in the United States. Therefore, here we used immunocytochemistry to re-examine human breast parenchyma for nuclear COMT. In addition to confirming previous reports of cytoplasmic COMT in mammary epithelial cells, we identified nuclear COMT in foci of mammary epithelial cells in histologically normal breast tissue of virtually all control (macromastia) and cancer patients and in breast can-

cer cells. There was no correlation between tissue histology and the numbers of cells with nuclear COMT, the size of foci containing such cells, or intensity of nuclear COMT immunostaining. The focal nature of the phenomenon suggests that nuclear COMT does not serve a housekeeping function but that it reflects a protective response to an increased local catechol load, presumably of CEs and, as such, that it may be a characteristic of the population of women studied who share the same major risk factor for developing breast cancer, that of living in the industrialized West. (Am J Pathol 2000, 156:1841–1848)

A link between oxidative stress and carcinogenesis is well documented. Oxidative DNA damage is potentially mutagenic, and promoters used in two-stage models of carcinogenesis are characteristically generators of reactive oxygen species.^{1–5} The findings of Malins and co-workers⁶ indicating extensive oxidative DNA damage in breast tissue of women in the United States suggests that underlying the high rate of breast cancer in the industrialized West is a state of chronic oxidative stress. A mechanism by which primary estrogens (estrone and estradiol), hormones implicated in breast carcinogenesis, may contribute to such a state of oxidative stress has also been identified.^{7,8} The mechanism involves two steps of metabolic activation, first, aromatic hydroxylation of primary estrogens leading to the formation of 2- and 4-hydroxylated catechol estrogens (2- and 4-OH-CEs), followed by oxidation of the catecholestrogens (CEs) to their semiquinone and quinone derivatives, the quinone estrogens (QEs). The latter are electrophiles that can form potentially mutagenic DNA adducts.⁸ In addition, CEs can enter into redox cycling and, thereby, become a source of reactive oxygen species.⁷ Thus, estrogens may

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participate in carcinogenesis not only via their estrogen receptor-mediated actions as mitogens, but also by contributing to a pro-oxidant state via the CE/QE metabolic pathway.

Evidence implicating the CE/QE pathway of estrogen metabolism in carcinogenesis *in vivo* has been obtained principally from studies of estrogen-induced renal cancers in hamsters.^{9,10} These studies have led to the hypothesis that estrogens may contribute to oxidative stress when local production of CEs exceeds that which can be inactivated by phase II enzymes.

The phase II enzyme catechol-O-methyltransferase (COMT) is considered to play a critical role in the regulation of levels of endogenous catechols (catecholamines and CEs). In addition, COMT serves a protective role by blocking oxidative metabolism of endogenous and exogenous catechols and, thereby, the generation of potentially mutagenic electrophiles.^{11,12} The effects of estrogens on the expression of COMT in hamster kidney identified by us previously support this postulate.¹³ Administration of estrogen to hamsters induced COMT in epithelial cells of proximal tubules, the region of the kidney where the cancers arise. Moreover, it resulted in translocation of COMT to nuclei of these cells. Induction of nuclear COMT in hamster kidney by estrogens, first demonstrated by immunocytochemistry (ICC), was confirmed by immunoblot analysis of subcellular fractions prepared from hamster kidney cortex.¹³ Immunoblot analysis also identified nuclear COMT to be the soluble form of the enzyme (S-COMT). The study identified COMT to be a facultative nuclear enzyme and suggested that nuclear localization of COMT may serve as a marker of the threat to the genome posed by excess catechols. In the case of the hamster kidney the catechols responsible are presumably CEs, the putative mediators of actions of estrogens as complete carcinogens in this tissue.¹³

Discussions of COMT's role in physiology and pathophysiology have focused to date predominantly on the loss of reactivity or activity of catechols caused by O-methylation. There is, however, a growing body of evidence that at least in the case of CEs, O-methylation may generate metabolites with distinctive actions.¹⁴ Some of these, specifically the anti-angiogenic and anti-mitotic effects of 2,3-O-methyl-estradiol,¹⁴⁻¹⁸ suggest that O-methylation of CEs by COMT may be protective not only by preventing oxidative metabolism of CEs but also by generating metabolite(s) with tumor suppressor functions. The potential utility in cancer treatment of 2-meth-

ylated CEs and their synthetic analogs is now being explored by several groups.¹⁶⁻¹⁸

Evidence for the expression of COMT in mammary gland of both rodents and humans has been obtained previously by biochemical assays and by ICC and a role for the enzyme in local CE homeostasis in this tissue has been suggested.¹⁹⁻²¹ Nuclear localization of COMT, however, was not noted in any of these studies. In light of the association of nuclear COMT with estrogen-induced cancer in hamster kidney and the many observations implicating estrogens in breast carcinogenesis it was of interest to re-examine by ICC human breast parenchyma for nuclear COMT. We report here on the presence of nuclear COMT in foci of histologically normal epithelial cells in breast parenchyma from patients without as well as with breast cancer and also in breast cancer cells. The focal and apparently random distribution of cells with nuclear COMT suggests that nuclear COMT does not serve a housekeeping function but reflects a response to a localized threat posed to the genome by excess CEs.

Materials and Methods

Breast tissue was obtained from women undergoing reduction mammoplasty for macromastia or mastectomy for breast cancer. Tissue samples from reduction mammoplasty were obtained from lateral portion of the breast to minimize effects that pressure may have on the tissue. Specimens from breast cancer patients were obtained from sites distant from the cancers and, whenever available, also from the cancers. After removal of excess adipose tissue by blunt dissection, portions of breast parenchyma were flash-frozen (at -70°C in liquid freon) and stored at -80°C . Other portions were fixed in 10% neutral-buffered formalin and embedded in paraffin.

Cryostat sections and sections from paraffin-embedded tissue were cut at $8\ \mu\text{m}$ and processed for ICC using standard methodology (see below). Tissue sections from a total of 33 subjects were examined by ICC (19 from patients with macromastia, nine from patients with breast cancer, and five from subjects with extensive fibrocystic disease). The ages of the subjects ranged from 16 to 57 years. Use of the tissue for the purposes of this study was authorized by the Institutional Review Board for Use of Human Tissues.

ICC for COMT was carried out as described previously and with reagents from the same sources.¹³ Briefly, the rabbit polyclonal antibody developed against purified

Figure 1. Localization of COMT in nonneoplastic mammary epithelial cells. ICC was carried out as described in Methods with alkaline phosphatase as the reporter, 5-bromo-3-chloro-indolyl phosphate as the substrate, and nitroblue tetrazolium as the chromogen. Sections were not counterstained and visualized using Nomarski optics. **A** and **B**: Terminal lobular ductal unit (TDLU) at comparable stages of development (type I lobules) from two reduction mammoplasty specimens. Immunostaining in epithelial cells in **A**, from an 18-year-old patient is nuclear, whereas in **B**, from a 16-year-old patient, there is intense cytoplasmic immunostaining. **C**: Higher magnification of area in **B** indicated by arrows. **D**: Section from a mammoplasty specimen showing some epithelial cells with only cytoplasmic COMT whereas others show also intense nuclear COMT immunostaining. **E**: Higher magnification of epithelial cells showing some with intense cytoplasmic COMT and others with intense nuclear COMT. **F** and **G**: Interlobular ducts with nuclear and cytoplasmic localization of COMT, respectively. **H-J**: Localization of AlkP reaction product in various types of cysts. **H**: Apocrine cyst with nuclear COMT. **I**: Wall of a large cyst showing both nuclear and cytoplasmic immunostaining. **J**: A small cyst with cytoplasmic immunostaining and part of the wall of an adjacent cyst, indicated by arrow, with minimal immunostaining. **K**: TDLU from a mastectomy specimen from a patient 7 months pregnant with breast cancer. The dilated ducts containing secretory product are characteristic of changes associated with pregnancy (prelactational). The cancer cells from this patient showed much weaker immunostaining that was also cytoplasmic (not shown). Original magnification in **A**, **B**, **D**, **F**, **G**, **H**, and **I** is as indicated by black bar ($50\ \mu\text{m}$) in **A**. In **C** and **E** magnification indicated by black bars ($25\ \mu\text{m}$).

protein was used at a dilution of 1:2,500. The signal from the biotinylated secondary antibody was amplified using Vectastain ABC reagent (Vector, Burlingame, CA) with alkaline phosphatase as the reporter, 5-bromo-4-chloro-3-indolyl phosphate as substrate and 4-nitroblue tetrazolium (Sigma Chemical Co., St. Louis, MO) as the chromogen. Endogenous alkaline phosphatase was inhibited by preincubating sections with 0.2 N HCl for 5 minutes. Sections incubated with only the secondary antibody served as controls.

Results

Representative photomicrographs of immunoreactive COMT in nonneoplastic breast parenchyma are presented in Figure 1 and in breast cancers in Figure 2. In agreement with previous reports immunoreactive COMT with cytoplasmic localization was seen uniformly in mammary epithelial cells throughout the mammary gland, as well as in breast cancer cells.²⁰ There were no consistent or obvious differences in the intensity of staining as a function of the stage of differentiation of the terminal ductal lobular units (type I, II, or III lobules)²² or the histological characteristics of the cancers. Immunostaining of both intra- and interlobular stromal cells was always of a much lower intensity than that of epithelial cells in the same tissue sections. Cytoplasmic COMT immunostaining was also weaker in some epithelial cells within involuting terminal ductal lobular units and in the attenuated layer of epithelial cells lining some cysts (Figure 1J).

Nuclear COMT was seen in some mammary epithelial cells in breast parenchyma from virtually all patients whether without or with cancer, as well as in breast cancer cells (Figures 1 and 2). In contrast to cytoplasmic COMT, epithelial cells with nuclear COMT were seen only in some regions of individual sections. The size and number of foci of cells with nuclear COMT varied greatly; in some subjects they encompassed large portions of mammary gland within a section whereas in others nuclear COMT was restricted to a few scattered cells intermingled with cells showing only cytoplasmic COMT (Figure 1). In tissue sections from cancers nuclear COMT was present in some cases in the majority of cancer cells whereas in others there were only a few such cells scattered among cells with only cytoplasmic COMT (Figure 2). Nuclear COMT was most prominent in cells in which immunostaining in the cytoplasm was relatively weak compared to that in the nucleus. Control sections incu-

Figure 3. Controls for immunocytochemistry of COMT in human breast parenchyma: adjacent sections from a breast cancer incubated either with both primary and secondary antibodies (original magnification, $\times 40$, **A** and **C**, top panels) or with secondary antibody only (original magnification, $\times 400$, **B** and **D**, bottom panels). There is intense immunostaining that is primarily cytoplasmic in **A** and both cytoplasmic and nuclear in **C**, but none in **B** and **D**. Arrows in **A** and **C** indicate areas shown at higher magnification below in controls.

bated with secondary antibody only were consistently negative (Figure 3). As in hamster kidney, nuclear immunoreactivity for COMT could be visualized only in sections from tissue fixed in formalin and embedded in paraffin, reflecting a need for retrieval of COMT antigen associated with nuclear proteins.¹³

The presence of nuclear COMT in noncancerous breast parenchyma could not be correlated with patient's age (Figure 1, **A** and **B**) or any histological characteristics, including those associated with an increased risk for developing breast cancer, ie, epithelial hyperplasia without or with atypia.^{23,24} Nor was there any consistent association between the histological type of cancer and nuclear COMT. It was absent in some highly undifferentiated cancers but prominent in others.

Discussion

As demonstrated here, nuclear COMT can be seen in both breast cancer cells and in histologically normal mammary epithelial cells in women without as well as with breast cancer. The focal and apparently random distribution of epithelial cells with nuclear COMT suggests that nuclear COMT does not serve a housekeeping function,

Figure 2. Localization of immunoreactive COMT in five breast cancers. Immunocytochemistry was carried out as described under Methods with alkaline phosphatase (AlkP) as the reporter, 5-bromo-4-chloro-3-indolyl phosphate as the substrate, and nitroblue tetrazolium as the chromogen. Sections were not counterstained and were visualized using Nomarski optics. **A, B, C, D,** and **E** show cancers in which immunostaining is predominantly cytoplasmic. In **A** cytoplasmic immunostaining is intense and there is no evidence of nuclear COMT. **B:** From the same cancer as **A**, shows a terminal lobular ductal unit being invaded by cancer cells: COMT is cytoplasmic in both the ductal epithelial and in breast cancer cells. However, immunostaining in the cancer cells is much more intense than in ductal epithelial cells. **C:** From another patient, shows some cancer cells with intense cytoplasmic immunostaining, some with intense nuclear immunostaining (indicated by arrows) and a few appear to have both intense nuclear and cytoplasmic COMT. **D:** Cancer cells with only weak immunostaining that is predominantly cytoplasmic. **E:** Higher magnification of part of **D** (outlined). **F-I:** Shown are cancers in which immunostaining is predominantly nuclear. **F:** A cancer with intense nuclear immunostaining in the majority of cells. **G:** Higher magnification of part of **F** indicated by arrows. A cell with apparently only cytoplasmic COMT among the many with prominent nuclear COMT is indicated by an arrow. **H** and **I:** Sections from another mastectomy specimen. **H:** Cancer cells with intense nuclear immunostaining interspersed with islands of cells apparently devoid of COMT immunostaining. At higher magnification, in **I**, the areas of apparently devoid immunostaining, such as seen in **H**, can be seen to be made up of cells with only minimal amount of reaction product in the rim of cytoplasm surrounding large nuclei devoid of reaction product. Original magnification, $\times 400$ (**A-D**, **F**, and **H**) and $\times 1000$ (**E**, **G**, and **I**).

but is induced in response to an excess catechol load. This notion is supported by the numerous observations of the focal nature of responses to even low doses of potential carcinogens administered to experimental animals.²⁵ We postulate that in mammary epithelial cells, as in kidneys of estrogen-treated hamsters, CEs are the inducers of nuclear COMT.

The notion that CEs are the principal substrates in mammary epithelial cells for COMT is supported by several lines of evidence. Oxidation of primary estrogens to CEs by human breast parenchyma *in vitro* has been demonstrated, forms of P450 that can catalyze the reaction have been shown to be expressed in this tissue and significant concentrations of CE have been reported to be present in breast cyst fluid.^{26–28} Substrates for CE synthases are also likely to be available in ample amounts in breast parenchyma of both pre- and postmenopausal women, because estrone and estradiol are maintained in this tissue at a high level by deconjugation and aromatization *in situ* of circulating conjugated and C19 steroid precursors, respectively.^{29–31}

Functions of COMT in mammary epithelial cells are likely to include modulation of levels of CEs generated *in situ* presumably for physiological purposes. Several scenarios could lead to CE formation in breast parenchyma and, secondarily, to the induction of nuclear COMT. These include induction of CE synthases (P450s and peroxidases) that can catalyze 2- and 4-hydroxylation of primary estrogens^{9,32–34} and increased availability of primary estrogen that are substrates for CE synthases. Information on concentrations of CEs and their metabolites in breast parenchyma needed to test these postulates await the development of analytical methods with the requisite sensitivity.

In renal tubular epithelial cells of hamsters, in contrast to breast epithelial cells, the principal substrate for COMT under normal physiological conditions is likely to be dopamine, which is produced in these cells for the purpose of regulating sodium excretion.³⁵ CEs are postulated to become major substrates for COMT in these cells only after the administration of doses of estrogens known to cause renal cancers in this species and only then did nuclear COMT become prominent.¹³ Induction of nuclear COMT in hamster kidney was evident by 2 weeks after initiation of the treatment with estrogens (the earliest time point examined), many months before the appearance of the cancers.¹³

In interpreting the finding presented here it is necessary to consider the characteristics of the tissue donor population. For noncancerous tissue we had to rely on women with macromastia. Whether and how such tissues may differ from the norm is not known. More importantly, however, all of the tissues used were obtained from subjects sharing the same significant risk factor for breast cancer, that of living in an industrialized Western country. It is estimated that one in eight women living in the United States can expect to be diagnosed with breast cancer in her lifetime.³⁶ Environment is recognized to be the major risk factor for breast cancer in this population. Its importance is underscored by the rise in breast cancer incidence in immigrants to the United States from countries

such as Japan where the incidence, although rising, is still only one-sixth of that in the United States.^{37–39}

The finding of a pattern of extensive oxidative DNA damage in breast parenchyma of women in the United States suggests that underlying the high breast cancer incidence in this population is a state of chronic oxidative stress.⁴⁰ Diverse factors could contribute to this state. The findings presented here support the notion that one such factor could be electrophiles generated as a consequence of oxidative metabolism of CEs. To determine whether this interpretation is correct will require examining breast tissue from subjects from a population with a significantly lower breast cancer incidence than that characteristic of our study population.

Since completion of this study Tenhunen and co-workers⁴¹ have reported their findings on the localization of COMT in breast cancers from 32 women and in histologically normal breast tissue from the same patients in a cohort of Finnish women. Using ICC these investigators did observe nuclear COMT but only in an unspecified number of invasive ductal breast cancers. There is no obvious explanation for the differences in findings. They could reflect a difference in study population. This is of special interest in light of differences in the composition diet of women in the United States and Finland and the effect that this may have on estrogen metabolism.^{42,43}

An opportunity to test the hypothesis that COMT plays a protective role in breast carcinogenesis in the United States is provided by a well-characterized COMT polymorphism. This polymorphism is responsible for reduced catalytic activity of the enzyme in ~25% of Caucasians.¹² Several epidemiological studies have been initiated to test the hypothesis that the low-activity COMT genotype is associated with an increased breast cancer risk. Overall, the findings from three of the four case control studies reported to date are consistent with the hypothesis.^{44–46} A significant positive association between breast cancer risk and low-activity COMT genotype was identified in three of these studies. However, the findings differ with respect to the age group in which this association is evident (ie, whether in pre- or postmenopausal women or both). In the fourth study no association was found between low-activity COMT genotype and breast cancer risk.⁴⁷ These differences in findings may be resolved by larger prospective studies.

Properties of COMT differ not only as a function of the genotype. The soluble (S-COMT) and membrane bound (M-COMT) forms of the enzyme, generated from alternative start sites of the COMT transcript, also differ in their catalytic properties.^{48–50} The affinity of S-COMT for 2- and 4-OH-CEs is one order of magnitude higher than that for catecholamines.⁴⁸ The additional 50 amino acids at the N-terminus of M-COMT increase the affinity of the enzyme for catecholamines by an order of magnitude without, however, affecting its affinity for 2-OH-CEs.⁴⁹ Whether S-COMT and M-COMT differ in their catalytic properties with respect to 4-OH-CEs has yet to be determined. Nuclear COMT in kidneys of estrogen-treated hamsters and in transfected cells has been identified to be S-COMT.^{13,51} It is, therefore, likely that S-COMT is also the form identified by ICC in nuclei of mammary epithelial

cells. It will be of interest to re-examine the catalytic efficiency of the two forms of COMT specifically for 4-OH-CEs because of evidence suggesting that they play a greater role in carcinogenesis than 2-OH-CEs, in particular, as potential mutagens.⁸

With respect to the mechanism of translocation of COMT to the nucleus it is of interest that human COMT sequence contains a classical nuclear localization signal (NLS), as well as a second presumptive NLS (KKGK).¹³ Translocation of S-COMT to the nucleus can occur, however, in the absence of a NLS. Nuclear S-COMT was seen in hamster kidney although hamster COMT sequence does not contain a NLS¹³ and eliminating the NLS from human COMT by site-directed mutagenesis did not prevent entry of recombinant S-COMT into nuclei of transfected mammalian cells.⁵¹ Conversely, M-COMT has not yet been found in the nucleus although it possesses the same NLS as S-COMT. How the expression of the two forms is regulated and what factors lead to the induction of nuclear S-COMT remains to be determined.

In summary, the study establishes the presence of nuclear COMT in histologically normal human mammary epithelial cells as well as in breast cancer cells. The focal and apparently random distribution of cells with nuclear COMT suggests that in these cells, as in epithelial cells of proximal convoluted tubules of kidneys of estrogen treated hamsters, translocation of COMT is a biomarker for an increased catechol load, presumably of CE. The finding is consistent with the postulate that oxidative metabolism of CE contributes to the state of chronic oxidative stress that is presumed to exist in breast tissue of the donor population and hence, to the high incidence of breast cancer in this population.⁴⁰

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