Immunohistochemical Characterization of Antioxidant Enzymes in Human Breast Cancer

Patricia A THOMAS, Dilek OYKUTLU, Bel POU, Denise TYLER, Larry W OBERLEY, Robert A ROBINSON, Julia C LENEL

Department of Pathology and the Radiation Research Laboratory; University of Iowa Hospitals and Clinics, Iowa City, USA

Intrinsic antioxidant enzymes (AE) are essential for protection against potential cellular damage by free radicals (FRs), which affect a variety of biological processes. The levels or activities of AEs can be abnormal in human malignancies in general, and FR production is a possible mechanism of estrogen related carcinogenesis specifically. However, the role of AEs in breast cancer remains unclear. Immunodetectable AEs were characterized in 95 node negative cancers using rabbit polyclonal antibodies. Results were correlated with established and experimental biomarkers of breast cancer. AEs were greater than benign differentiated epithelium in more than 40% and lower in 10-14% of tumors. Patterns of staining were enzyme and tumor pattern specific. Increased immunodetectable AE was associated with large, poorly differentiated tumors, and younger age. Catalase correlated with nuclear grade and disease related death (p<0.05), and highlighted tumor microvasculature. Additional work in this area may further elucidate the role of AEs in breast cancer growth and progression. (Pathology Oncology Research Vol 3, No 4, 278-286, 1997)

Key words: Breast cancer, antioxidant enzymes, immunohistochemistry

Introduction

Free radicals (FR) and reactive oxygen species (ROS) comprise a diverse group of substances which are generated during normal oxidative metabolism. FRs and ROS also exist in a wide variety of environmental agents or as readily formed derivatives of these agents. While free radicals facilitate a number of important biological processes, they can also exert deleterious effects. Intrinsic protective mechanisms exist to prevent potential damage. These cellular defenses include small molecular weight antioxidants or free radical scavengers, such as vitamin E, carotene, and selenium, as well as complex enzyme systems which occupy characteristic subcellular compartments. Antioxidant enzymes (AEs) include: Manganese superoxide dismutase (MnSOD) predominantly mitochondrial; Copper and Zinc SOD (CuZnSOD) cytosolic and nuclear; and Catalase (CAT), a peroxosomal and cytoplasmic enzyme. The proper balance between these free radical scavengers and free radical production is essential for the health and survival of the organism.

Elaboration of free radicals has been implicated in carcinogenesis, in general, and specifically, with regard to human breast cancer, free radical formation has been postulated as a putative mechanism of hormonal carcinogenesis caused by estrogens. Estrogen dependent DNA damage by free radicals can be blocked by the addition of AEs in breast cancer cell lines, supporting an anticarcinogenic role for these cellular enzymes. There is conflicting observational evidence from a few clinical trials suggesting that increased intake of the small molecular weight antioxidants provides protection against cancer. Malignant human tumors have been reported to have abnormal levels of AEs compared to the benign differentiated cellular counterparts from which they derive. Several studies show lower levels of antioxidant enzymes
in tumors, while a few describe increased antioxidant enzymes in some tumors compared to their benign tissues of origin. Recently, Li et al have demonstrated that overexpression of MnSOD suppresses the malignant phenotype in the human breast carcinoma MCF-7 cell line, further suggesting a role in malignancy.

Immunohistochemical identification of proteins or enzymes offers the advantages over many of these prior studies, which used tissue homogenates: immunostaining allows direct visualization and semiquantitation of AE in tumor and its corresponding benign differentiated epithelium. We herein characterize antioxidant enzymes in invasive, lymph node negative breast cancer by immunohistochemistry, compared to the corresponding adjacent benign mammary epithelium, and biomarkers of breast cancer.

**Materials and Methods**

102 consecutive lymph node negative breast cancers having at least 3 years of clinical follow up and no initial treatment were obtained through The University of Iowa Hospitals and Clinics Tumor Registry. Clinical data was obtained or validated by chart review (DO). The size of the tumor, tumor type, disease free interval, and survival status were recorded. Estrogen and progesterone receptor status was obtained from patients charts (quantitative immunoassay results when possible were performed). Both estrogen and progesterone receptor assays were performed individually using the dextran-coated charcoal method. Four micron (4 μ) sections of formalin-fixed paraffin-embedded tissue were used for immunoperoxidase staining.

**Histologic grade and nuclear grade**

Histologic grade and nuclear grade were determined in all cases by consensus opinion by two pathologists using histologic criteria previously described by Elston and Ellis. Nuclear grade criteria were derived from the modified Scarff Bloom-Richardson method. For both histologic and nuclear grade a scale of 1 to 3 was used; grade 1 corresponding to well differentiated or low nuclear grade tumors, grade 2 to moderately differentiated or intermediate nuclear grade, and grade 3 to poorly differentiated or high nuclear grade tumors.

**Her-2/neu Fluorescent In Situ Hybridization**

HER-2/neu amplification was determined using the Oncor® INFORM HER-2/neu (ERBB2) Gene Amplification Detection System (for experimental purposes only). The kit contains a biotinylated DNA probe and 4′,6-diamidino-2-phenylindole (DAPI) nuclear stain.

Briefly, the methodology is as follows: Thin sections (4 μ) of formalin-fixed, paraffin-embedded breast tissue are deparaffinized and then pretreated chemically and enzymatically to remove proteins that block DNA access; the DNA in the sections is converted from double-stranded to single-stranded by denaturation at 75°C using a mixture of the 20x Saline Sodium Citrate (SSC) and formamide; biotinylated DNA probe, complementary to HER-2/neu (ERBB2) gene sequences, is then applied and the slide is subsequently incubated under conditions favorable to the annealing of the probe DNA sequences and the genomic DNA sequences. The unannealed probe is washed off using a mixture of the 20x SSC and formamide. Hybridized probe is detected using fluorescein labeled avidin. A DAPI nuclear counterstain is employed.

**Antibody preparation**

Rabbit polyclonal antisera comprised of anti-human kidney MnSOD, anti-bovine liver CuZnSOD, and anti-bovine liver CAT was provided by LWO and prepared as previously described. The specificity of these antibodies has also been previously characterized.

**Immunohistochemical staining and scoring**

Immunohistochemical staining was accomplished using overnight incubation at 4–5°C and a labeled streptavidin-biotin peroxidase (LSABII) detection system (Dako Corp Santa Barbara, CA). The presence of staining or distribution (D) (percentage of cells staining) and intensity (I), was evaluated for each enzyme in both tumor cells and the adjacent normal mammary epithelium. Cases were also scored from 0 to 3 for distribution of staining for each enzyme: 0 = no cells; 1 = <10% of cells; 2 = 10 to 50% of cells; 3 = >50% of the cells. Cases were scored from 0 to 3 for intensity of staining: 0 = absent or not detectable; 1 = faint; 2 = moderate; and 3 = intense. Kidney was used as a known positive control. Negative controls were accomplished by omission of primary antibody. Bovine serum albumin (BSA) was used in buffer washes to diminish background staining. Immunohistochemical evaluation was performed, without knowledge of other prognostic factors or outcome, by 3 observers. The most common or the mean of the 3 observers was used as the value in the final analyses.

**Statistical methods**

Results were used for statistical purposes only when there was both tumor and adjacent benign, differentiated epithelium on the same immunostained slide. The number of qualified cases of statistical analyses, therefore, varied from the total number of cases (i.e. 95 immunostained) for each enzyme. All analyses were performed with SAS version 6–10 software.
Table 1. Percentage of Tumors Positive for Antioxidant Enzyme

<table>
<thead>
<tr>
<th>Enzyme</th>
<th># Tumors Evaluated</th>
<th># Tumors Positive</th>
<th>% Tumors Positive</th>
</tr>
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<tbody>
<tr>
<td>MnSOD</td>
<td>90</td>
<td>76</td>
<td>84%</td>
</tr>
<tr>
<td>CuZnSOD</td>
<td>89</td>
<td>88</td>
<td>96%</td>
</tr>
<tr>
<td>CAT</td>
<td>92</td>
<td>62</td>
<td>67%</td>
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* Numbers of tumors or cases (out of 95 total) for which representative invasive tumor was present on immunostained sections

The means of the immunostaining scores (intensity and distribution of cytoplasmic immunostaining) for each enzyme, for which there was both tumor and benign adjacent epithelium, were determined. The differences between the means for the tumor staining versus the benign epithelium staining were also determined for each enzyme. For statistical analysis, t-tests of comparison were performed on the differences between the mean values of tumor staining and benign epithelial staining for all cases, and for cases limited to invasive ductal, not otherwise specified (NOS). Levels of staining by age and the potential of effect of age on tumor staining results was determined by dichotomizing the patients by age, i.e. < 50 and > 50 years (restricted to cases with invasive ductal carcinomas, not otherwise specified). Two-way ANOVAs were run for these analyses.

Standard table and loxproportional hazards methods were used to examine risk of early recurrence, and disease related death associated with AE immunostaining.

The distribution and intensity of staining for each enzyme and the following prognostic factors were also compared to the following prognostic factors: tumor size, type, histologic grade, nuclear grade, c-erb-B2 amplification, disease free interval, and overall survival.

Results

There were a total of 95 lymph node negative cases which had representative invasive cancer remaining in the tissue block after sectioning and immunostaining. The patients' ages range from 24-90 years of age (mean = 58.3). Tumor sizes ranged from 0.3 to 7 cm (mean = 2.3 cm). No patient in this study received adjuvant therapy. The histologic types of the tumors examined included 77 invasive ductal carcinoma not otherwise specified (NOS), 6 invasive lobular cancers, 2 mixed ductal and lobular and 10 tumors of special types (i.e., medullary, tubular, colloid). The histologic grades were: 28/78 (36%) grade 1, 38/78 (49%) grade 2, and 12/78 (15%) grade 3. Nuclear grades were: 18% – grade 1, 50% – grade 2, 32% – grade 3. Quantitative estrogen receptor (ER) and progesterone receptor (PR) results were available in 75 cases: 56% of tumors were ER positive and 55% were PR positive. The percentage of tumors showing positive antioxidant enzyme immunostaining were: 84% for MnSOD, 96% for CuZnSOD, and 67% for catalase (Table 1). Patterns of immunostaining are illustrated in Figures 1-4. Well differentiated tumors (Figure 4) and its benign, differentiated ductal epithelial cells of origin (not shown) tended to show coarse perinuclear and cytoplasm granular staining of luminal surfaces or cells with MnSOD, with variable intensity. Staining was prominent at the luminal surface of tumor cells in tumors with gland formation (Figure 4), this luminal staining was also seen in benign, non-proliferative differentiated extralobular ducts and terminal lobular or ductular units (Figure 1a). Myoepithelial cells of differentiated ducts were usually negative while those in the terminal lobular unit were occasionally positive (Figure 1a). Positive immunostained stroma or basement membrane could often be observed at the tumor interface. Tumors exhibiting poor gland formation showed intense MnSOD staining in cells located at the periphery of tumor cell nests, the centers of solid tumor nests showed less MnSOD staining (Figure 1b and Figure 1c). Staining patterns of CuZnSOD and CAT ranged from variable granular cytoplasmic to focal membranous staining. In general, CuZnSOD immunostaining intensity was less than that of the other enzymes in all cases. CuZn often accentuated a rim of peripheral cytoplasm in many tumor cells (Figure 2). Diffuse staining of the fibrous stroma was observed for catalase in many cases, and CuZnSOD in fewer, particularly when the tumor had a marked desmoplastic stromal response. In those cases, the desmoplastic stroma appeared to stain more strongly than the normal stromal component of the breast. CuZnSOD highlighted just the basement membrane and connective tissue immediately adjacent to the benign epithelium, epithelial stromal interface (for tumor nests) more specifically than the other enzymes and CAT stained the tumor microvasculature. When an intraductal component was present, there was often prominent periductal immunostaining of small vessels by CAT. The in situ component was often negative or only weakly positive for all three enzymes, except when that component had high nuclear grade or necrosis. Proliferative ductal or lobular epithelium showed more positive cells and higher intensity of staining than non-proliferative and involuted epithelium.

Enzyme immunostaining was greater in tumor cells compared to benign adjacent epithelium (of appropriate differentiation) in greater than 40% of the tumors analyzed for all three enzymes as determined by the difference in the mean value for intensity and distribution of tumor immunostaining versus the mean values for intensity and distribution of adjacent benign epithelium staining for each enzyme (Table 2 and Figure 5). Differentiated medi-
Figure 1a–c. Rare cytoplasmic MnSOD immunostaining and prominent accentuation of basement membrane or epithelial-stromal interface (2–3+ intensity) in a terminal ductule and lobule of benign epithelium. Rare myoepithelial cells appeared to show cytoplasmic staining. Note stromal and inflammatory cells. Immunoperoxidase, 100x. (a) A different case showing staining of the epithelial stromal interface of infiltrating malignant epithelium. Immunoperoxidase 100x; (c) Coarsely granular cytoplasmic MnSOD immunostaining, (3+ intensity), can be seen in this moderately differentiated ductal cancer. Immunoperoxidase; 200x.
Figure 2. CuZnSOD (2–3+ intensity) immunostaining of breast carcinoma. Immunoperoxidase, 200x. Note the accentuation seen at the periphery of the cytoplasm in many cells characteristic of CuZn immunostaining.

Figure 3. Characteristic immunostaining patterns for catalase in this moderately differentiated breast cancer. Immunoperoxidase, 200x. Note 3+ "membranous" and 2-3+ intensity cytoplasmic granular staining.
um sized ducts, or extralobular ducts, were used for comparison to ductal and special type cancers; terminal ductular units or lobular acini were used for lobular carcinomas. The next most frequent result was for tumor immunostaining to be equal to that of the benign adjacent epithelium. In less than 10–14% of cases, tumor stained less than the benign adjacent epithelium.

The difference between the paired means, where each pair was the difference between the staining value for the tumor minus the staining value for the adjacent benign tissue, with the exception of CuZnSOD, were significant at a level of p < 0.05 (Figure 5).

When only invasive ductal carcinomas were considered (all other types excluded) and divided into 2 groups by age, women ≤ 50, and patients > 50 years of age. ANOVA analyses revealed that the mean staining values for all enzymes (staining intensity and distribution or percentage of cells stained) were greater for patients who were 50 years old or younger (data not shown). This affect of age was significant at p < 0.05 for MnSOD intensity (p = 0.0269), CuZnSOD (p = 0.0160) distribution of staining only. There was no interaction between age (dichotomized) and tumor immunostaining.

Comparison of the difference of the mean staining values (tumor versus benign cells) with prognostic markers including ER, PR, histologic grade, nuclear grade and outcome revealed that CAT immunostaining correlated with both nuclear grade and outcome. Specifically, the distribution or percentage of positive CAT staining tumor cells correlated in a direct linear fashion with nuclear grade (correlation coefficient r = 0.293, p < 0.05) and inversely with disease free or time to relapse (correlation coefficient r = −0.213, p < 0.05). The intensity of CAT immunostaining also inversely correlated with the disease free interval (r = −0.232, p < 0.05). Tumors without detectable immunostaining tended to be low nuclear grade and small tumors, but this trend did not reach

Table 2. Comparison of Invasive Tumors with Benign Differentiated Adjacent Epithelium

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>% of Tumors &gt; Benign</th>
<th>% of Tumors = Benign</th>
<th>% of Tumors &lt; Benign</th>
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<tbody>
<tr>
<td>MnSOD</td>
<td>33/71 (44%)</td>
<td>35/71 (4%)</td>
<td>3/71 (4%)</td>
</tr>
<tr>
<td>CuZnSOD</td>
<td>28/69 (41%)</td>
<td>37/69 (54%)</td>
<td>4/69 (6%)</td>
</tr>
<tr>
<td>CAT</td>
<td>34/75 (45%)</td>
<td>38/75 (51%)</td>
<td>3/75 (4%)</td>
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</table>

* Includes only the positive immunostained cases for each enzyme (see Table 1) for which both invasive tumor and adjacent epithelium was present on immunostained sections; therefore, denominators vary and are not necessarily equal to 95.
Figure 5. The frequencies of differences in mean values for tumor staining (intensity and distribution) versus benign differentiated epithelium for each antioxidant enzyme. Mnl stain = mean intensity for MnSOD immunostaining. MnD stain = mean distribution for MnSOD immunostaining. CuZnl stain = mean intensity for CuZnSOD immunostaining. CuZnD stain = mean distribution for CuZnSOD immunostaining. Cat I stain = mean intensity for catalase immunostaining. Cat D stain = mean distribution for catalase immunostaining. Tumor-Benign = differences in the mean immunostaining values obtained or the mean immunostaining values across the tumors versus the mean immunostaining values of the corresponding, appropriate benign differentiated epithelium. Frequency = the equency or percentage of tumors staining less than equal to, or greater than, benign epithelium.

statistical significance. Positive immunostaining of tumor cells for antioxidant enzymes did not show statistically significant correlations with tumor type or hormonal status.

Discussion

Free radicals (FRs) are critical mediators of many important biological processes, including inflammation, mitosis, cell differentiation, and apoptosis. Steady state levels of intracellular free radicals are of concern since the persistence or overabundance of reactive oxygen species (ROS) can result in profound deleterious effects in cells or organisms, including carcinogenesis and cell death. Intrinsic antioxidant systems exist to protect the host from such potential damage.

Study of these free radical scavengers may not only further elucidate their role in the pathobiology of breast cancer, but also promises to offer vital clues for future preventive and therapeutic approaches to breast cancer.

Further, there is growing observational evidence from clinical trials supporting a preventive role for antioxidants in general, namely increased intake of small molecular weight antioxidants such as 13-carotene and vitamin E. The relationship of endogenous AE regulation and carcinogenesis is currently being actively investigated. The implications for AE in both prevention and treatment (e.g.,...
direct effects and interaction with radiotherapy or chemotherapy) has led to this intense interest. Antioxidant enzymes are lower in content and activity in transitional cell carcinoma, and some malignant renal tumors. In contrast, malignant ovarian epithelial tumors, lung, colon, gastric and brain malignancies have been reported to have higher levels of the AEs as measured by mRNA levels, biochemically or immunohistochemically compared to benign counterparts. AEs are abnormal in breast cancers and have been reported as low in some studies, relative to differentiated benign breast tissue, and high in others. One study, looking at enzymatic activities, reported significant overlap between tumor and benign epithelium for MnSOD levels.

Decreased antioxidant enzyme activity was observed in some breast cancers compared to metabolically active benign tissues. Enzyme activities were compared, however, to enzyme activities of predominantly non-mammary tissues or breast tissue from other patients. Further, studies reporting abnormal antioxidant enzyme activities of breast cancers which use tissue homogenates inevitably contain tumor and a variable amount of non tumor tissue, e.g., stroma, blood vessels, inflammatory cells. Assuch, results may not accurately reflect antioxidant enzyme levels in breast cancer cells versus benign breast epithelium. Increased mRNA levels in studies without corresponding protein studies do not establish an increase in actual AE protein. Immunohistochemistry on the other hand allows direct visualization of tumor immunostaining for each enzyme and as well as the cellular compartment involved. Immediate comparison of adjacent benign tissue from the same patient, same breast, with identical tissue processing offers obvious advantages over other approaches. The ability to use archival paraffin embedded tissues for which outcome data and other prognostic information can be readily available is an additional advantage with regard to postulating the pathobiologic significance of AEs in breast cancer.

We demonstrated that antioxidant enzymes (AEs) were immunohistochemically detectable in the majority of the breast cancers we tested. Immunodetectable AEs showed significant overlap in staining intensity and distribution with the benign differentiated epithelium from which it derived, i.e., medium sized ducts and extralobular ducts or ductules for ductal carcinomas, and lobular acini or terminal ductular units for lobular carcinoma. When staining values of tumors differed compared to benign adjacent mammary epithelium, they were most often increased.

These observations are supported by a recent study which analyzed a small group of human breast cancers and found had elevated MnSOD mRNA and NTnSOD enzymatic activity compared to normal breast tissue, suggesting increased expression of antioxidant enzyme genes in breast carcinoma. Further, tumor necrosis factor (TNF), a multifunctional polypeptide cytokine associated with cachexia and tumor toxicity in experimental animals, has been shown to induce MnSOD mRNA. The role of increased or inducible AE levels is not clear at present. Care should be taken in future studies to standardize or agree on what constitutes appropriate "control" tissues or cell populations. The cyclic physiologic changes in breast microanatomy and function which occur with menses or the terminal differentiation and metabolic activity of lactation were not tested. Our choice of benign control, i.e., adjacent tissue, did offer the advantage of being from the same patient and subjected to the same fixation and processing conditions as the tumor.

Our findings of CAT enhancement of tumor microvasculature and the correlation of increased CAT immunostaining with poor prognostic factors are provocative. It is possible that tumors take advantage of host enzymes, i.e., increased AEs in microvasculature potentially protect blood supply for tumor growth, for their own protection or progression. Conversely, inducible levels of AEs in tumor vasculature may provide protection to the host against high levels of FRs and ROS produced by the tumor. Damage to vascular endothelium is thought to play an important role in metastasis and such endothelial injury has been shown to be produced by FRs in melanoma cell lines. The implications of CAT staining with respect to angiogenesis, tumor progression, and metastasis are intriguing. While mean MnSOD immunostaining levels showed similar trends as the other AEs, the pattern of staining was characteristic of this particular AE. Positivity was often restricted to the edges or outside layers of cells in the invading tumor nests. In other words, all the positive cells were at the edges while the center of tumor nests, especially large non-gland forming groups, were very often entirely negative. This trend has been observed in prostate cancers as well (unpublished data). Interestingly, in prostatic adenocarcinoma immunodetectable MnSOD is absent in in situ lesions, appearing only in invasive lesions (unpublished data). We did not specifically look at sLu breast cancers in this study. However, when an in situ component was present, ductal or lobular, it tended to stain with low intensity if at all. Exceptions included those non-invasive with high grade nuclei or features of comedo ductal carcinoma in situ. Coarsely granular staining isconsistent with MnSOD's known mitochondrial localization. The CuZnSOD pattern of CuZnSOD immunostaining was also interesting, often showing a very striking "zonal" preference at the periphery of the tumor cells' cytoplasm. The pattern of staining was often "smooth" as opposed to granular, suggesting a cytosolic yet zonally restructured location. Granular cytoplasmic staining, consistent with organelle associated AE protein, and nuclear staining were observed.

Further studies are necessary to elucidate the biological functions of antioxidant enzymes in breast cancer. It is not
clear what role elevated AEs play in tumor cells or whether it is a host specific or tumor specific phenomenon. In vitro studies demonstrating tumor suppressor activity in MCF-7 cells transfected with MnSOD\textsuperscript{9} and abolition of DNA damage by increased catalase\textsuperscript{10,11,12} suggests that increased AEs are host protective. The functional integrity or activity of elevated AEs needs to be specifically addressed. Detailed characterization of different staining profiles of human lactiferous ducts, segmental ducts, subsegmental, extralobular and intralobular ducts compared to malignant ductal cells from the same patient would be useful. The best differentiated benign controls for lobular cancer may not be that of benign adjacent lobules, rather lactation is the endpoint of differentiation for that compartment. Analysis of cytokines in relationship to CAT immunostaining might provide very exciting information about breast cancer growth and progression. Hopefully, this pathogenetic information can be translated quickly into breast cancer prevention and nonsurgical treatment strategies.

References

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