The Potential Role of Reactive Oxygen Species in Mammary Neoplasm

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Abstract: The current study investigated the presence of oxidative stress markers (8-OHdG and HNE) in breast carcinoma in females from Saudi Arabia, and to examine the growth modulatory effects of HNE (induce cell growth inhibition) by measuring the expression of Ki-67 protein (cell proliferation antigen). Furthermore, to record if there is any significant prognostic indicator of these markers. In order to estimate the level of oxidative damage in breast cancer, 8-hydroxy-2'-deoxyguanosine (8-OHdG) and 4-hydroxy-2-nonenal (HNE) expressions were investigated in breast carcinoma and benign lesions of breast, and were estimated by semi-quantitative immunohistochemical method. The observations of the present study showed that the level of expressions of 8-OHdG and HNE were significantly higher than those in controls. Reduction of oxidative stress is thought to be a very important measure for primary prevention of breast carcinoma.

Key words: Reactive oxygen species, oxidative stress, breast cancer, 8-hydroxy-2'-deoxyguanosine, 8-OHdG, 4-hydroxy-2-nonenal, HNE, immunohistochemistry.

INTRODUCTION

Breast cancer continues to be a major cause of morbidity and mortality throughout the world (Ferlay et al 2007). While it had once been presumed that the incidence of breast cancer in Saudi Arabia was low, more recent data has indicated the contrary. Not only breast cancer is a significant disease in the Kingdom, as elsewhere in the world, but its pattern is very disturbing as it affects mainly young premenopausal women with advanced stage (Amr et al 1995, Chiedozi et al 2003). The exact cause of breast cancer is essentially speculative, but the evidence suggests that it could be mediated through oxidative stress and reactive oxygen species (ROS) and consequently structural modifications in DNA nucleotide bases that affect template-directed DNA synthesis (Franco et al 2008). Damage by reactive oxygen species (ROS) occur in vitro despite the presence of multiple antioxidant defense and repair system. Such damage is thought to make a significant contribution to the development of breast malignancies (Brown & Bicknell 2001). It is getting clear that relatively low load of oxidative stress promotes cellular proliferation. Cancer cells are usually exposed to more oxidative stress than normal cells but it appears that the stress is not strong enough to cause apoptosis or necrosis (Brown & Bicknell 2001, Burdon 1995). Furthermore, estrogen mediated oxidative DNA damage in mammary glandular epithelium has been suggested in progression of breast neoplasia (Mobley and Brueggemeier 2004).

One of the most abundant adducts and subtle oxidative modification in DNA is the oxidative base lesion 8-hydroxy-2'-deoxyguanosine (8-OHdG). The expression of 8-OHdG in DNA is considered a potentially important factor in carcinogenesis because 8-OHdG is known to cause GC® TA transversions, causing mispairing and ultimately producing multiple amino acid substitutions (Karihtala et al 2006, Moriya 1993, Cheng et al 1992, Shibutani et al 1991, Breimer 1990).

The other oxidative stress marker is 4-hydroxy-2-nonenal (HNE), which is an α,β-unsaturated aldehyde that is generated during oxidation of membrane polyunsaturated fatty acids and is a major component of lipid...
peroxidation products (Poli et al 2008, Esterbauer et al 1991), and may be the most reliable index of free radical-induced lipid peroxidation (Poli et al 2008, Toyokuni 1999). HNE reacts with protein molecules and forms stable HNE-modified protein adducts, inducing the functional insufficiency of target proteins. HNE inhibits DNA and protein synthesis (Cerbone et al 2007, Poot et al 1988). It is also reported that a physiological concentration of HNE shows growth modulatory effects (Cerbone et al 2007, Zarkovic 2003, Zarkovic et al 1993), implying that HNE is not only a toxic product of lipid peroxidation, but also a physiological growth-regulating factor as well (induce cell growth inhibition) (Poli et al 2008, Cerbone et al 2007). Recently, increasing evidence suggests that HNE is causally involved in many of the pathophysiological effects associated with oxidative stress in cells and tissues (Karihtala et al 2006). The results of Hu et al (2002) also suggest that 4-HNE may be an important etiological agent for human cancers that have a mutation at codon 249 of the p53 gene.

The aims of the current study are to investigate the presence of oxidative stress markers (8-OHdG and HNE) in breast carcinoma in females from Saudi Arabia, and to examine the growth modulatory effects of HNE (induce cell growth inhibition) by measuring the expression of Ki-67 protein (cell proliferation antigen). Furthermore, to record if there is any significant prognostic indicator of these markers.

**MATERIAL AND METHODS**

**Human Breast Tissues:**
Forty five cases of breast tissues (invasive ductal carcinoma, ductal carcinoma in situ, atypical ductal proliferation, and from normal breast tissue removed for benign lesions) were randomly and retrospectively selected from the files of the Academic Department of Pathology, King Khalid University Hospital, Riyadh, Kingdom of Saudi Arabia. The total number of samples comprising benign (n = 9) and malignant (n = 36) tissues. Specimens obtained at surgery were routinely fixed in 10 % neutral formalin and embedded in paraffin. The clinical stage was determined according to the international TNM staging system. The histological grade of tumors was also determined according to the WHO criteria as follows: grade I as well differentiated, grade II as moderately differentiated, and grade III as poorly differentiated and grade IV as undifferentiated tumors. Information on histopathological characteristics, age, smoking and receptor status of the samples was obtained through the pathology reports.

**Immunohistochemistry:**
Immunohistochemical staining for 8-hydroxy-2'-deoxyguanosine (8-OHdG), 4-hydroxy-2-nonenal (HNE) and Ki-67 was performed according to the standard avidin-biotin-peroxidase complex method (Hsu et al 1981) except that autoclaving procedure was used for antigen retrieval pretreatment. Serial 4 mm thick sections were cut and dewaxed in xylene and rehydrated in a graded ethanol series. The sections were immersed in 3 % hydrogen peroxide in methanol for 15 minutes to block endogenous peroxidase activity, and rinsed in running water. After that, sections were immersed in citrate buffer PH 6.0 for autclave for 10 min at 120 C, The sections then were washed three times in PBS. Prior to immunohistochemical staining, the sections were first incubated with 10 % goat serum for 30 minutes. The sections then incubated with primary monoclonal antibodies against 8-hydroxy-2'-deoxyguanosine (N45.1), 4-hydroxy-2-nonenal (HNE), and Ki-67 (dilution 1/1000 with 0.01 BSA+10MPBS+ 0.1% azide) overnight. Then the sections were washed in PBS three times for 5 min and incubated with biotinylated secondary antibodies (dilution 1/300 with 0.01 BSA+10MPBS+ 0.1% azide) for 40 min. Then the sections were washed in PBS three times for 5 min and ABC complex was applied for 50 min. The sections were washed as before and incubated with alkaline phosphatase substrate for 3-5 min followed by washing in distilled water and then dehydrated and coverslipped.

**Evaluation of Score:**
The slides were examined and markers expression was scored, both the extent and intensity of immunopositivity were considered. The intensity of positivity was scored as follows: 0 as negative, 1 as weak, 2 as moderate, 3 as strong. The extent of positivity was scored as follows: 0 = no stained cells, 1 =<25 %, 2 = 25-50 %, 3=50-75 %, and 4 =<75 % of the target cells in the respective lesions. The final score was determined by multiplying the intensity of positivity and the extent of positivity scores, yielding a range from 0 to 12. Scores 9-12 were defined as strong staining pattern (+++), 6-8 as moderate staining pattern (++), and 1-4 as markedly weak or negative expression (+).
RESULTS AND DISCUSSIONS

Results:

Intensity and distribution of 8-OHdG immunostaining in the tumor and the surrounding non-tumorous tissues were studied. The staining was predominantly confined to nuclei (figure 1). Nuclei of normal breast epithelial cells showed weaker immunostaining than those of carcinoma cells. Primary breast carcinoma revealed stronger immuno-reactivity than the corresponding non-tumorous epithelial cells and the benign lesions (table 1). There was no association between the differentiation state of carcinoma and the intensity of immunostaining. Interestingly, cells of all the cases of benign lesion used in the present study revealed weaker staining than carcinoma cells and were almost in the same level of staining intensity as non-tumorous epithelial cells (figure 2).

![Fig. 1: Expression profile of 8-OHdG in breast carcinoma (x40)](image1)

![Fig. 2: Expression profile of 8-OHdG in benign tissue of breast (x40)](image2)

Immunostaining of HNE-modified proteins was observed in the cytoplasm (figure 3). The majority of cases (27 cases of 36) of primary breast carcinoma showed strong immuno-reactivity. In contrast, the corresponding non-tumorous epithelial cells were weakly or negatively stained in 22 cases of 36 (table 1). More precisely, transformed cells of invasive tumors showed slightly stronger immunostaining than the transformed cells of in situ tumors, whereas there was no local difference in immunostaining in the carcinoma cells. There was no significant correlation between immunostaining intensity and either age, clinical stage, or differentiation of carcinoma.
Fig. 3: Expression profile of HNE in breast carcinoma (x40)

Table 1: Staining pattern of malignant and non-malignant tissues of breast

<table>
<thead>
<tr>
<th></th>
<th>Staining pattern</th>
<th>Total number of cases</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>+++++</td>
<td>++</td>
</tr>
<tr>
<td>8-OHdG</td>
<td>13 (36%)</td>
<td>20 (56%)</td>
</tr>
<tr>
<td>Transformed epithelium</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adjacent benign epithelium</td>
<td>0</td>
<td>13 (36%)</td>
</tr>
<tr>
<td>Benign Lesions</td>
<td>0</td>
<td>4 (44%)</td>
</tr>
<tr>
<td>NHE</td>
<td>27 (75%)</td>
<td>9 (25%)</td>
</tr>
<tr>
<td>Transformed epithelium</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adjacent benign epithelium</td>
<td>1 (3%)</td>
<td>13 (36%)</td>
</tr>
<tr>
<td>Benign Lesions</td>
<td>0</td>
<td>4 (44%)</td>
</tr>
</tbody>
</table>

(++++) strong staining pattern; (+++) moderate staining pattern; (+) markedly weak or negative expression

Discussion:

Increased rates of radical production and persistent oxidative stress are characteristic features of carcinoma cells, both in vivo and in vitro, caused principally by enhanced glycolytic metabolism, macrophage infiltration to tumors and ROS generation during the reperfusion phase following hypoxia in the defective tumour vascular system (Marnett 2000). Although certain ROS-derived metabolites, such as H2O2, HNE and NO, play a role as essential intracellular messengers, they are also thought to be significant mediators of every step of carcinogenesis.

In the present study, the extent of oxidative DNA damage was assessed by immuno-semiquantitating the surrogate markers 8-OHdG and HNE in the genome of histologically different human breast tissues to investigate the prognostic relevance and the role of these promutagenic oxidative lesions in the etiology of breast cancer. This study further provides important supportive evidence that significant and readily marked differences exist in the oxidized base lesion profile between normal, benign and malignant human breast tissues.

Higher level of expression and stronger immunostaining of two kinds of oxidatively modified products were consistently observed in more than 95% of tumor cells of each case of breast carcinoma. The level of expression and staining intensity were lower and lesser in benign lesions and normal breast tissues. Consistent with other reports (Karihtala et al 2006, Musarrat et al 1996) the results of the present study have clearly shown oxidative metabolism differences between benign and malignant human breast tissues.

The data indicate the presence of a substantially higher level of 8-OHdG in DNA from malignant breast tissues, whereas, the normal and benign tissues were estimated to contain lower level of 8-OHdG. These results are in agreement with previously reported results (Malins and Haimanot 1991). However, in that study, the reported values were based on a limited number of tissue samples and the control used was a commercially available calf thymus DNA rather than the DNA from actual normal breast tissue. Nevertheless, the overall magnitude of 8-OHdG for cancerous and normal breast tissues, identified in the two studies, clearly supports the occurrence of elevated oxidative stress in breast cancer.

The present study examined the association of ROS and cellular proliferation based on the hypothesis that sufficient levels of “persistent oxidative stress” may stimulate cellular proliferation (Toyokuni et al 1995). Notably, practically all cancer cells were uniformly exposed to oxidative stress, whereas only a fraction was
positive for ki-67. The current study of ki-67 and oxidative stress showed a proportional association between the levels of oxidative stress in cancer cells, as indicated by 8-OHdG, and stimulation of growth, whereas the levels of HNE-modified proteins were maintained at a constant high level (Karihtala et al. 2006). Considering the strong cytotoxicity of HNE (Esterbauer et al. 1991), the results of the present study suggest that tumor cells have a high capacity to adjust oxidative stress to a level sufficient to stimulate tumor proliferation but not for apoptosis or necrosis. Although there are several reports that HNE inhibits cellular proliferation (Poli et al. 2008, Cerbone et al. 2007, Uchida 2003), only cancer cells with a capacity to dispose of HNE or lipid peroxide can accelerate proliferation (Uchida 2003, Grune et al. 1994). The observations of the current research, together with reports of other investigators, suggest that the accumulation of HNE-modified proteins will inhibit proliferation of cancer cells.

In conclusion, the outcomes of the present study demonstrate that all breast tumor cells are exposed to more oxidative stress than the surrounding non-tumorous epithelial cells. Oxidative stress in cancer cells appears to be maintained at high levels that promote cellular proliferation but insufficient to cause neither apoptosis nor necrosis and reduction of oxidative stress is thought to be a very important measure for primary prevention of breast carcinoma. Oxidative metabolism in benign breast lesion is different from that in breast carcinoma cells. Further understanding of tumor biology from the standpoint of reactive oxygen species may be helpful for establishing a new strategy for cancer prevention and treatment.

ACKNOWLEDGMENT

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