Hypoxia Promotes a Dedifferentiated Phenotype in Ductal Breast Carcinoma in Situ

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Abstract

In cultured neuroblastoma cells, hypoxia induces a dedifferentiated phenotype. We tested whether hypoxia-induced dedifferentiation also occurs in vivo in mammillary ductal carcinoma in situ with its well-defined lesions and distinct areas of necrosis. Ductal carcinoma in situ cells surrounding the central necrosis have high hypoxia inducible factor-1α protein levels, down-regulated estrogen receptor-α, and increased expression of the epithelial breast stem cell marker cytokeratin 19; lose their polarization; and acquire an increased nucleus/cytoplasm ratio, hallmarks of poor architectural and cellular differentiation. The hypoxia-induced changes were confirmed in cultured breast cancer cells. We propose that hypoxia-induced dedifferentiation is a mechanism that promotes tumor progression in breast cancer.

Introduction

Neovascularization in solid tumors is a prerequisite for their growth beyond the size of a few millimeters (1–3). This process is driven, at least in part, by angiogenic factors expressed in hypoxic tumor areas as a result of the hypoxia-dependent stabilization of two characterized transcription factors, HIF-1α and HIF-2α (4–6). Occurrence of hypoxia within a given tumor form is generally associated with a worse prognosis. This phenomenon is believed to be caused by a higher mutation rate and higher metastatic potential in hypoxic tumors, as well as a diminished response to radiation treatment and chemotherapy (7–9). We recently demonstrated that human neuroblastoma cells grown under hypoxic conditions lose their neuronal characteristics and develop a neural crest-like phenotype, changes that also occurred in vivo in necrotic areas of xenografted neuroblastoma tumors (10). In neuroblastoma, there is a well-established correlation between low expression levels of neuronal differentiation marker genes and highly malignant tumor behavior (11). On the basis of these two sets of observations, we hypothesized that hypoxia-induced dedifferentiation in neuroblastoma promotes a tumor phenotype with aggressive behavior (10). In this report we asked whether hypoxia-induced dedifferentiation is a general phenomenon that occurs in solid tumors other than neuroblastoma. As a first model we chose DCIS, a breast tumor characterized by well-defined tumor lesions with distinct areas of necrosis, lack of vascularization within the lesions, and focal stabilization of HIF-1α in the cells surrounding the central necrosis (Ref. 12; Fig. 1, A and B). We show that DCIS cells lose their polarization and that the nucleus/cytoplasm ratio increases with increasing proximity to the central necrosis and increasing HIF-1α protein expression. Parallel to these morphological changes, there was a down-regulation of ERα and an up-regulation of CK19, an epithelial breast stem cell marker (13). These changes in gene expression were also seen in hypoxia-treated breast cancer cell lines. We conclude that within the hypoxic region of DCIS, tumor cells acquire a less mature, dedifferentiated phenotype.

Materials and Methods

Tumor Material and Cultured Cells. We chose 19 cases of DCIS from among archived formalin-fixed, paraffin-embedded surgical breast tumor specimens at the Department of Pathology, Malmö University Hospital. Frequent symmetrical DCIS lesions with a central necrosis were the inclusion criteria. Seven cases represented pure DCIS, all with moderate to extensive central necrosis and nuclear grade III, whereas the remaining 12 were invasive ductal carcinomas, histological grade III, where the DCIS component had moderate to extensive central necrosis and nuclear grade III. The human breast cancer cell lines MCF7, CAMA, and T47D were maintained under standard conditions, and the hypoxic milieu was generated according to the method of Jögi et al. (10). Cells were exposed to hypoxic or normoxic conditions for 3 days. Cells for tissue arrays were fixed in 4% paraformaldehyde and embedded in paraffin. Tissue microarrays were constructed using Beecher Instrument (M-A Technologies, Silver Spring, MD).

Histology and Immunohistochemistry. H&E-stained sections were used for morphological grading of the DCIS lesions. The following monoclonal antibodies were used: anti-ERα (dilution, 1:200; DAKO A/S, Glostrup, Denmark), anti-CK19 (1:50; DAKO), Ki-67 (1:200; DAKO), and anti-HIF-1α (1:400; Abcam, Cambridge, United Kingdom). ERα, CK19, and Ki-67 immunohistochemistry was performed in a DAKO TechMate 500 after antigen retrieval by microwave treatment in citrate buffer (pH 6). HIF-1α immunohistochemistry was performed manually after antigen retrieval with DAKO Target Retrieval Solution. Immunoreactivity was visualized using the DAKO Catalyzed Signal Amplification System. All sections were counterstained with hematoxylin, and negative controls were obtained by omitting the primary antibody.

DNA fragmentation as a sign of apoptosis was visualized in tissue sections using TUNEL labeling according to the ApoAlert DNA Fragmentation Assay Kit User Manual (BD Biosciences Clontech, Palo Alto, CA). For cell culture arrays, cytoplasmic staining intensity of CK19 was graded as 0 < 1 < 2 < 3. For the nuclear antigens ERα and Ki-67, nuclear staining was scored as positive. To manually count cells, two fields were randomly chosen for each immunohistochemical staining and photographed at ×20 magnification. All cells within a field were counted (300–400 cells) and evaluated twice. Average intensity score was calculated for the controls and the hypoxic cells, and the relative change in staining intensity was shown statistically. Statistical analyses were performed using the Mann-Whitney test.

To calculate the N/C ratio, the most symmetrical DCIS lesion with a central necrosis was chosen in each of the 19 tumor sections and photographed at ×40 magnification. The cytoplasmic and nuclear areas of cells from the basal membrane to the central necrosis were measured in two different places within each DCIS lesion, using the Image-Pro Plus 4.0 image analyzing software. The number of cell layers varied between 4 and 10 as indicated in Fig. 1D. The average N/C ratio was then calculated for each tumor. Statistical analyses were performed using the Mann-Whitney test. Two tumors were excluded: one attributable to lack of suitable lesions and one because irregular cell morphology made it impossible to determine the N/C ratio.

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3 The abbreviations used are: HIF, hypoxia inducible factor; DCIS, ductal carcinoma in situ; ER, estrogen receptor; CK, cytokeratin; TUNEL, terminal deoxynucleotidyl transferase (TdT)-mediated nick end labeling; N/C, nuclear/cytoplasm.
Western Blot and Flow Cytometry Analyses. Western blot analyses were performed as described previously (10) with the same antibodies used in the immunohistochemical studies. The Western blot analyses were performed six times with anti-ER and four times with anti-CK19 antibodies, and the same results were obtained. Flow cytometry analyses were performed five times as described previously (14).

Results and Discussion

Nineteen tumors containing DCIS with necrosis were first analyzed for local variation in HIF-1α expression within the DCIS lesions. Immunohistochemistry revealed a gradient in HIF-1α staining, with the strongest signal in the cell layers surrounding the central necrosis (Fig. 1, A and B), findings in agreement with published data (12). The staining intensity and number of positive cells varied between lesions. The fact that the strongest HIF-1α signal was found close to areas of necrosis suggested that this staining defines a hypoxic zone of tumor cells. Although most of the studied DCISs were poorly differentiated (nuclear grade III), we observed DCIS lesions with a higher differentiation state in 7 of 19 cases, i.e., there was cellular polarization with radial orientation of the apex of the cells toward an intercellular lumen. Interestingly, these features were lost with growing proximity to the central necrosis (Fig. 1C). Furthermore, there was a statistically significant increase in the N/C ratio with growing distance from the basal membrane (Fig. 1D). We conclude that by traditional histopathological criteria, cells under hypoxic conditions acquire a morphology usually associated with a more malignant breast cancer phenotype (15).

We next tested whether the changes in cell morphology in the hypoxic zone were accompanied by alterations in the expression of genes normally associated with the differentiation status of breast tumors. The ER expression in breast cancer cells correlates positively to the degree of cellular differentiation (16): cancers with high levels of ER have a better prognosis than those with low levels or no receptor expression. Of the 19 tested tumors, 8 stained positive for ERα, with the staining confined to the nuclei of the tumor cells (Fig. 2, A and B). Although staining intensity could vary between DCIS lesions, all but the innermost cell layers were ERα positive, i.e., the 2–6 cell layers in closest proximity to the necrosis were ERα negative (Fig. 2, A and B). The changes in nuclear morphology and ERα expression suggested that cells closer to the necrotic area had acquired...
Hypoxic zone (Fig. 2, sions, but the staining intensity was greatly enhanced within the carcinoma (19, 20). We found CK19 expression throughout the le-
expression correlates with a high grade of malignancy in DCIS as well as in invasive breast cancer (12).

Our observation that hypoxia induces dedifferentiation and HIF-1α expression in DCIS cells suggests that it is dedifferentiation that drives the hypoxia-associated promotion of the aggressive phenotype in breast cancer, neuroblastoma, and possibly other cancer forms. Mammary epithelial differentiation involves stepwise acquisition of a CK expression pattern specific for the luminal and the myoepithelial cells, respectively. During the early weeks of gestation, the two cell types develop from CK19-positive cells (17). CK19, a marker of the luminal phenotype in the adult gland, is therefore also one of the earliest markers of mammary gland development, and mammary multipotent progenitor cells in the adult organ are found among the CK19-positive luminal cells (13). Thus, the up-regulation of CK19 and down-regulation of ERα, together with the loss of morphological differentiation features in the hypoxic zone of DCIS, strongly suggest that hypoxia promotes the development of immature tumor cells. The hypoxia-induced down-regulation of ERα expression in DCIS (Fig. 2, A and B) and in invasive mammary carcinoma4 has potential clinical relevance and suggests a reason that some ERα-positive tumors become resistant to antiestrogen treatment. This in turn suggests that increasing oxygen tension in these tumors might enhance the therapeutic effect of antiestrogens. In addition, progression to infiltrative carcinoma is associated with suppression of myoepithelial cell-specific intermediate filament expression and up-regulation of CK19 (19, 20).

DCIS, if left untreated, becomes invasive, and because hypoxic DCIS cells acquire an immature, CK19-positive phenotype, increased invasiveness could be another consequence of the development of hypoxic regions in breast cancer. It remains to be explained how centrally located hypoxic cells could physically become invasive. It is noteworthy that the number of viable cell layers within a tumor duct varies and that the hypoxic zone can be separated from the basal membrane by only one or two cell layers. Taking into account that tumor growth is a dynamic process, one might envisage a situation in which the hypoxic zone extends to the basal membrane and the hypoxic cells break down basal membrane proteins and become invasive as the result of an acquired aggressive phenotype. Thus, apart from the already known effects of hypoxia on tumor behavior, such as decreased vulnerability to cytotoxic drugs and ionizing radiation (7), increased metastatic potential (8), and increased mutation frequency (9), the data presented here support dedifferentiation as an additional consequence of hypoxia and a novel mechanism for tumor progression in hypoxic breast cancer lesions (Fig. 4).

References


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