Hypoxia Reduces Hormone Responsiveness of Human Breast Cancer Cells

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Resistance to hormonal therapy frequently occurs following successful treatment in breast cancer. The mechanism responsible for this acquired resistance is still unknown. It has been suggested that a hypoxic tumor microenvironment promotes malignant progression of cancer, i.e., hypoxia may promote estrogen-independent growth (a more malignant phenotype) of breast cancer. To clarify this hypothesis, the effects of hypoxia on the growth responses to hormonal agents and the expression levels of estrogen receptor (ER)-α and progesterone receptor (PgR) were investigated in two human breast cancer cell lines, ML-20 and KPL-1. The expression level of ER-α was significantly decreased by hypoxia (1% O2) in a time-dependent manner in both cell lines. Hypoxia also significantly reduced the growth-promoting effect of estradiol (E2) and the growth-inhibitory effects of an antiestrogen,ICI 182,780, and a progestin, medroxyprogesterone acetate, in both cell lines. In addition, hypoxia markedly suppressed the induction of PgR mRNA and protein by E2 in both cell lines. To clarify further the effect of hypoxia on ER-α expression, the expression levels of hypoxia-inducible factor-1α (HIF-1α), a marker of hypoxia and ER-α were immunohistochemically examined in 36 breast cancer specimens. ER-α expression (both its proportion and intensity) was significantly lower in nuclear HIF-1α-positive tumors than in negative tumors. These findings indicate that hypoxia down-regulates ER-α expression as well as ER-α function in breast cancer cells. These processes may lead to an acquired resistance to hormonal therapy in breast cancer.

Key words: Hypoxia — Breast cancer — Estrogen receptor — Hypoxia-inducible factor-1α — Hormone resistance

Although hormonal therapy is effective for the treatment of most patients with estrogen receptor (ER)-positive breast cancer, resistance to hormonal agents frequently occurs following successful treatment. There are several hypotheses to explain the mechanisms of this acquired resistance, but the major mechanism is still unknown.1) Our previous studies suggested that the overexpression and/or dysregulation of angiogenic factors, such as fibroblast growth factor (FGF)-4 and vascular endothelial growth factor (VEGF), may cause resistance to hormonal agents in ER-positive human breast cancer cells in female athymic nude mice.2–5) These findings suggest that paracrine effects mediated by angiogenic factors may play an important role in the hormone responsiveness of breast cancer cells in vivo.

Recent studies have suggested that the hypoxic tumor microenvironment induces the hypoxia-inducible factor (HIF)-1 signaling pathway and promotes angiogenesis and glycolysis, both of which are essential for tumor cells to survive under hypoxic conditions.6) It has been reported that HIF-1α expression, which is a critical element of the HIF-1 signaling, is detected in two-thirds of common cancers but not in the corresponding normal tissues.7,8) Several studies have suggested a positive correlation between hypoxia or HIF-1α expression and malignant progression of cancer, such as the acceleration of histological grade, invasion and metastasis.9–13) These findings suggest that adaptation to hypoxia in the tumor microenvironment may cause malignant progression of tumor cells in an epigenetic manner.

Therefore, we hypothesized that adaptation processes to hypoxia, such as up-regulation of angiogenic factors, may cause acquired resistance (a more malignant phenotype) to hormonal agents in breast cancer. To clarify this hypothesis, two ER-positive human breast cancer cell lines, ML-2014) and KPL-1,15) were used in the present study. The ML-20 cell line is a subclone of the MCF-7 cell line, estrogen-sensitive in vitro and estrogen-dependent in vivo.14) In contrast, the KPL-1 cell line originates from breast cancer resistant to two hormonal agents, antiestrogen and progesterin.15) This cell line is estrogen-sensitive in vitro but estrogen-independent in vivo.4,5) The findings of the present study showed that hypoxia significantly down-regulated ER-α expression and its function of breast cancer cells in a time-dependent manner. Moreover, hypoxia apparently reduced the growth responses to hormonal agents in both cell lines. In addition, according to our immunohistochemical (IHC) analysis, nuclear HIF-1α positive breast tumors, which are likely to survive under
hypoxic conditions, expressed less ER-α than negative

tumors. To our knowledge, this is the first study suggest-
ing that the hypoxic tumor microenvironment reduces hor-
mone responsiveness of breast cancer cells and may lead
to an acquired resistance to hormonal agents in breast
cancer.

MATERIALS AND METHODS

Cell lines The KPL-1 cell line was established in our
laboratory and its characterization was described previ-
ously. In brief, this cell line was derived from a patient
with recurrent breast cancer. Recurrent disease appeared
during postoperative chemo-endocrine therapy including
tamoxifen and was resistant to a second-line hormonal
agent, medroxyprogesterone acetate (MPA). This cell line
expressed a wild-type ER-α, but not ER-β. This cell line is
tumorigenic in intact female nude mice and transplanted
tumors grow rapidly without estradiol (E2) supplemen-
tation (estrogen-independent). The ML-20 cell line is a
transfectant of the MCF-7 cell line with a lacZ expression
vector. Its growth characteristics in vitro and in vivo are
indistinguishable from those of the wild-type MCF-7 cell
line. This cell line is ER- and progesterone receptor
(PgR)-positive and requires E2 supplementation for growth
in intact female nude mice (estrogen-dependent).

Reagents E2 was purchased from Sigma Chemical Co.
(St. Louis, Detroit, MI). ICI 182 780 and MPA were pro-
vided by Zeneca Pharmaceuticals (Macclesfield, UK) and
Kyowa Hakko Kogyo Co., Ltd. (Tokyo), respectively. These
hormonal agents were dissolved with 100% ethanol
and added to the medium at a final ethanol concentration
of 0.1%.

In vitro experiments To reduce endogenous estrogenic
activity, phenol red-free RPMI-1640 medium (Gibco BRL,
Bethesda, MD) supplemented with 5% dextran-coated
charcoal-stripped fetal bovine serum (FBS) (Hyclone, UT)
estrogen-deprived medium) was used for in vitro experi-
ments. Because half of solid tumors have median oxygen
concentrations less than 10 mmHg (approximately 1%
O2) and a putative key factor of cellular hypoxic reac-
tion, HIF-1α, is easily induced by a hypoxic condition of
1% O2, 1% O2 was chosen as a hypoxic condition in the
Corning, Inc., Corning, NY) and cultured in Dulbecco
cells/well were inoculated into 12-well plates (Costar
Bas, Tokyo) according to the manufacturer’s recommenda-
tions.

VEGF secretion VEGF concentrations in the culture
medium were measured with an enzyme-linked immuno-
носorbent assay kit (Otsuka Assay Laboratory, Toku-
shima). Briefly, a mouse anti-human VEGF monoclonal
antibody was used as the first antibody and a rabbit anti-
human VEGF polyclonal antibody labeled with alkaline
phosphatase as the second antibody. After removal of the
unbound second antibody, horseradish peroxidase solution
was added. As the standard, 20–10 000 pg/ml of human
recombinant VEGF was used. The minimal detectable
dephrase concentration of VEGF was 0.2 pg/ml. Since the VEGF
congestion in the fresh medium was undetectable, but
then increased linearly for at least 2 days, VEGF secretion
into the medium was defined as follows:

\[ \text{VEGF secretion per cell per 48 h} = \frac{\text{Concentration of VEGF} \times \text{Volume of medium}}{\text{Mean cell number}} \]

mRNA expression levels of ER-α, PgR and VEGF

Total cellular RNA was extracted using a TRIsol RNA
extraction kit (Gibco BRL Life Technologies, Gaithers-
burg, MD). One microgram of total RNA and 1 μM
Oligo(dT) 18 primer in 12.5 μl of diethyl pyrocarbinate-
treated water were heated to 70°C for 2 min followed by
cooling on ice for 1 min. cDNA synthesis was initiated

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with 200 units of recombinant Molony murine leukemia virus reverse transcriptase (CLONTECH Laboratories, Inc., Palo Alto, CA), and the reaction was allowed to proceed at 42°C for 1 h. The reaction was terminated by heating at 94°C for 5 min. The cDNA was dissolved to a final volume of 100 µl by adding 80 µl of diethyl pyrocarbonate-treated water and then frozen at −30°C until use. Oligonucleotide primers for the reverse transcriptase-polym-merase chain reaction (RT-PCR) were designed using a published sequence of each target gene and synthesized by the solid-phase triester method. The primers and conditions used and the expected sizes from the reported cDNA sequence are shown in Table I. To amplify both the inter-
gene and the control gene, 200

\[
\text{MgCl}_2, 10 \text{ mM Tris-} \text{HCl (pH 8.8), 2.5 mM MgCl}_2, 10 \text{ mM KCl, 0.08% Nonidet P40, and 1 unit of recombi-}
\]

natrix. Vilnius, Lithuania) in a final volume of 20 µl. After initial denaturation at 94°C for 4 min, various cycles of denaturation (at 94°C for 15 s), annealing (at various temperatures as shown in Table I for 15 s), and extension (at 72°C for 30 s) were performed on a DNA Thermal Cycler 2400 (PC-960G Microplate Gradient Thermal Cycler, Mortlak, Australia). The final extension was performed for 5 min. After visualization of the PCR products on 1.2% agarose gel stained with ethidium bromide, gel images were obtained using the FAS-II UV-image analyzer (Toyobo Co., Ltd., Tokyo), and the densities of the products were quantified using Quantity One version 2.5 (PDI, Inc., Huntington Station, NY). The gel images are shown in inverted presentation. The relative expression levels were calculated as the density of the product of the respective target genes divided by that of the control gene. Reproducibility was confirmed in at least three separate experiments.

**Immunohistochemistry** A total of 36 breast cancer specimens (formalin-fixed and paraffin-embedded blocks) were randomly selected and obtained from the Department of Pathology, Kawasaki Medical School. They contained 28 invasive ductal carcinomas and 8 intraductal carcinomas. Serial 5 µm sections were prepared, and one was conventionally stained with hematoxylin-eosin solution. Framing sections were dewaxed with xylene and hydrated with PBS. Then, the IHC assay was performed as follows: retrieving the antigen by autoclaving (121°C, 5 min); blocking endogenous peroxidase with 0.1% sodium azide and 0.3% hydrogen peroxide; blocking nonspecific protein binding with 10% ovalbumin; and binding with primary mouse monoclonal antibody ER1D5 (Immunotech, Marseille, Cedex, France) at a dilution of 1:500 or with primary mouse monoclonal antibody H1α67 (Novus Biologicals, Littleton, CO) at a dilution of 1:1000 at 4°C overnight. Control experiments were performed by substituting normal mouse serum for the primary antibody. The reaction was visualized with a Histofine kit (Nichirei, Tokyo). The sections were counterstained with hematoxylin. One pathologist (T. M.) evaluated the immunohistochemistry. The IHC results for HIF-1α protein were classified as follows: −, no nuclear staining; +, nuclear staining with or without cytoplasmic staining. For ER-α analysis, the IHC scoring system was applied. In brief, a proportion score was assigned which represented the estimated proportion of positive-staining tumor cells (0, none; 1, less than 1%; 2, 1–10%; 3, 10% to one-third; 4, one-third to two-thirds; and 5, over two-thirds). An intensity score was assigned which represented the average intensity of positive tumor cells (0, none; 1, weak; 2, intermediate; and 3, strong). The proportion and intensity scores were then added to obtain a total score, which ranged from 0 to 8.18

**Statistical analysis** All values are expressed as the mean±SE. ANOVA analysis with a StatView computer software (ATMS Co., Tokyo) was used to compare the differences in cell number, PgR concentrations, secretion of VEGF and IHC score for ER-α between two groups. A two-sided P value less than 0.05 was considered to be statistically significant.

### Table I. Primer Sequences, Conditions and Product Sizes for the Multiplex RT-PCR

| Target gene | Primer sequences | Annealing temperature (°C) | Primer ratio | No. of PCR cycles | Expected size (bp) |
|-------------|------------------|---------------------------|--------------|------------------|-------------------|
| ER-α        | 5'-agacagtgagagctccaacc-3' | 59 | 0.3/2.0 | 35 | 299 |
| PgR         | 5'-aggtgtagacagctgtgc-3' | 56 | 0.4/2.0 | 35 | 475 |
| VEGF        | 5'-gcacagctcagcaagtg-3' | 56 | 0.4/2.0 | 35 | 475 |
| β-actin     | 5'-acaggggtcccccaactgtgca-3' | 58 | 1.0/2.0 | 38 | 212 |

a) Not assessible.
RESULTS

Hypoxia down-regulates ER-α expression but up-regulates VEGF expression

The hypoxic condition (1% O₂) significantly decreased the protein expression level of ER-α in a time-dependent manner in both cell lines (Fig. 1A). The ER-α concentration was less than 5 femtomols/mg protein after the 7-day exposure to hypoxia in both cell lines. The decline of the ER-α expression level appeared to be more pronounced in ML-20 cells than in KPL-1 cells. Re-oxygenation to normoxia time-dependently restored the ER-α expression level in both cell lines (Fig. 1A). Stable ER-α expression levels of both cell lines cultured under normoxia were confirmed under similar culture conditions (data not shown). The mRNA expression level of ER-α was slightly decreased by the 48-h exposure to hypoxia in ML-20 cells, but not in KPL-1 cells (Fig. 1B). In contrast, both protein and mRNA expression levels of VEGF were up-regulated by the 48-h exposure to hypoxia in both cell lines (Fig. 1, B and C). It was reported that hypoxia stimulates the HIF-1 signaling pathway and transactivates the expression of VEGF in normal and malignant cells. However, this is the first study describing hypoxia time-dependently decreasing the expression levels of ER-α in human breast cancer cells.

Hypoxia inhibits PgR induction and growth stimulation by E2

To clarify the effects of hypoxia on ER-α function, the induction of PgR expression and the growth stimulation by E2 were studied. In both cell lines, 1 nM E2 significantly induced both mRNA and protein expression levels of PgR under normoxia. However, hypoxia markedly inhibited these inductions by E2 (Fig. 2). In addition, in both cell lines, 1 nM E2 significantly stimulated cell growth under normoxia (treated versus control ratio: 1.64 on day 2 and 1.37 on day 4 for KPL-1 cells; 1.73 on day 2 and 1.87 on day 4 for ML-20 cells). How-
Hypoxia reduces growth-inhibitory effects of antiestrogen and progestin

To clarify the influences of hypoxia on the growth response to hormonal agents, the growth-inhibitory effects of 0.1 µM ICI 182 780 and 1 µM MPA were studied. In both cell lines, each agent significantly inhibited the cell growth under normoxia (treated versus control ratio: 0.80 for ICI 182 780 and 0.62 for MPA in KPL-1 cells; 0.63 for ICI 182 780 and 0.77 for MPA in ML-20 cells). However, hypoxia clearly ameliorated these growth-inhibitory effects (treated versus control ratio: 0.91 for ICI 182 780 and 0.97 for MPA in KPL-1 cells; 0.90 for ICI 182 780 and 1.15 for MPA in ML-20 cells; Fig. 4).

Expressions of HIF-1α and ER-α in breast cancer

HIF-1α immunostaining was heterogeneous primarily within the nucleus in breast cancer tissues. Cytoplasmic staining was occasionally observed in tumor cells. No nuclear or cytoplasmic HIF-1α staining was observed in flanking normal tissue. Within tumors, HIF-1α-positive cells were most dense at the periphery of necrotic regions. Nuclear HIF-1α expression is well known to be induced by a hypoxic microenvironment. Therefore, nuclear HIF-1α positive tumors are likely to survive hypoxic con-

Fig. 2. Effects of hypoxia on the protein and mRNA expression levels of PgR induced by E2. A, PgR mRNA expression induced by E2 was significantly decreased by hypoxia (1% O₂) in both cell lines. Both cell lines were incubated with the estrogen-deprived medium plus or minus 1 nM E2 for 2 days under normoxia or hypoxia using a CO₂/Multi-Gas Incubator. Expression levels of PgR were measured using a multiplex reverse transcription-PCR method. The upper bands show PCR products for β-actin (internal control) and the lower bands show PCR products for PgR. B, PgR protein expression induced by E2 was significantly decreased by hypoxia (1% O₂) in both cell lines. Both cell lines were incubated with the estrogen-deprived medium plus or minus 1 nM E2 for 2 days under normoxia or hypoxia using a CO₂/Multi-Gas Incubator. PgR of collected cells was measured with an enzyme immunoassay kit. N, under normoxia without E2; NE, under normoxia with E2; and HE under hypoxia with E2. Values are the means. Bars, SE. *, P<0.05 (NE versus HE).

Fig. 3. Effects of hypoxia on the growth-promoting effect of E2. Both cell lines were cultured for 2 or 4 days in the estrogen-deprived medium with 1 nM E2 or vehicle under normoxia or a hypoxic condition (1% O₂) using a CO₂/Multi-Gas Incubator. The cell numbers were measured with a Coulter counter. Triplicate wells were treated in each experiment. Values are the means. Bars, SE. ——, under normoxia without E2; –—–, under normoxia with E2; ——–, under hypoxia without E2; ——–, under hypoxia with E2. Because growth curves of KPL-1 cells under normoxia and hypoxia without E2 are identical, open circles (under normoxia without E2) can not be seen.
ditions. To clarify the relationship between hypoxia and ER-α expression in vivo, the IHC score for ER-α was compared between nuclear HIF-1α-positive and negative tumors. HIF-1α-positive tumors expressed a significantly lower level of ER-α than negative tumors (4.2±1.3 for negative tumors and 2.7±2.2 for positive tumors in the proportion score, \( P=0.018 \); 2.2±0.8 and 1.1±0.9 in the intensity score, \( P<0.001 \); and 6.3±1.8 and 3.8±3.1 in the total score, \( P=0.004 \), Fig. 5). Representative microphotographs are shown in Fig. 6.

**DISCUSSION**

The experimental findings in the present study suggest that a hypoxic tumor microenvironment may down-regulate both ER-α expression and function in hormone-responsive breast cancer cells and reduce their responsiveness to hormonal agents. If so, hypoxia may play a key role in the development of acquired resistance to hormonal agents.

This hypothesis is consistent with other studies as follows: 1) At the early stage of breast cancer, most intraductal breast cancer cells express a high level of ER-α but comedo-type intraductal breast cancer cells, which exist close to the necrotic area, express a low level of ER-α or none.\(^{21}\) 2) Larger breast tumors, in which most tumor cells survive hypoxia, tend to be ER-negative.\(^{22}\) 3) Breast tumors expressing a high level of VEGF, in which VEGF is presumably up-regulated by hypoxia, tend to be ER-negative and resistant to antiestrogen therapy.\(^{23}\) 4) Some hormonal agents have been reported to have anti-angiogenic activity and to induce tumor hypoxia.\(^{24-26}\) It is conceivable that a subclone resistant to apoptosis under hypoxia as well as hormonal agents may selectively survive in hormone-responsive breast cancer. 5) Almost all recurrent breast cancers eventually become resistant to hormonal agents.\(^{21}\) It is likely that a ubiquitous phenomenon, such as the hypoxic tumor microenvironment, causes this resistance.

However, there are some unresolved questions as follows: 1) The action mechanism(s) responsible for the down-regulation of ER-α by hypoxia is still unknown. Our preliminary experiment revealed that hypoxia did not induce constitutive hypermethylation of the ER-α promoter region. In addition, treatments of hypoxic cells with actinomycin D or cycloheximide did not reduce the down-
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regulation of ER-α (unpublished data). 2) Both growth stimulation and PgR induction by E2 were significantly inhibited by hypoxia in ER-positive breast cancer cells. This effect may be mainly due to the down-regulation of ER-α. However, the decrease in PgR expression appears to be more pronounced than that in ER-α expression. Some other mechanisms, such as a direct inhibition of ER-α transactivation of the PgR gene by hypoxia, may participate in the rapid decrease of PgR expression. 3) The long-term effects of hypoxia on ER-positive breast cancer cells are still unknown. Our preliminary experiment revealed that both KPL-1 and ML-20 breast cancer cells can survive hypoxic conditions (1% O2) for over 6 months. Both cell lines exposed to hypoxia for a long time expressed a lower level of ER-α, but a high level of VEGF. In addition, subcutaneous inoculation of KPL-1 cells cultured under hypoxia produced more rapidly growing tumors than did KPL-1 cells cultured under normoxia in female nude mice. However, this was not the case for ML-20 cells (unpublished data). It is difficult to clarify whether hypoxia induces clonal selection of hormone-resistant cells in vivo as well as in vitro. However, it is interesting to note that our IHC study revealed a lower ER-α expression in nuclear HIF-1α-positive breast tumors. Nuclear HIF-1α overexpression is rapidly induced by hypoxia. Thus, it is likely that nuclear HIF-1α-positive breast cancer cells can survive a more hypoxic environment than negative cells. A negative relationship between HIF-1α and ER-α expressions strongly suggests that ER-α expression is down-regulated by hypoxia in breast cancer tissues. In contrast, another IHC analysis using a different antibody against HIF-1α has revealed a positive relationship between HIF-1α and ER-α expressions in breast cancer tissues. However, the expression levels of ER-α were not quantitatively

Fig. 6. Representative microphotographs of IHC analysis for HIF-1α and ER-α. Breast cancer specimens were stained by the immunoperoxidase procedure using the respective monoclonal antibodies as the primary antibody. A, nuclear HIF-1α-positive cells were most dense at the periphery of a necrotic region in a breast tumor (original, ×160). B, most tumor cells were weakly positive for ER-α staining in the same part of the tumor (original, ×160). C, all tumor cells were negative for HIF-1α staining in a breast tumor (original, ×160). D, all tumor cells were strongly positive for ER-α staining in the same part of the tumor (original, ×160).
analyzed in that study. Moreover, a high proliferation was suggested to correlate with an increased expression of ER-α in the study. It is unlikely that rapidly growing breast cancer cells express an increased level of ER-α. Further analysis is needed to clarify the relationship between HIF-1α and ER-α expressions in breast cancer tissues. 4) No clear difference was observed between estrogen-independent KPL-1 cells and estrogen-dependent ML-20 cells in various responses to hypoxia in vitro explored in this study. Our previous findings suggested that KPL-1 cells exhibit paradoxical hormonal responses in vivo, but not in vitro.4,5,15 It may be important to explore the responses of KPL-1 transplanted tumors to hypoxia. Changes induced by hypoxia in the expression levels of paracrine effectors may be different between KPL-1 and ML-20 cells. Further analysis of the interaction between the tumor cells and stromal components is necessary for understanding the overall effects of the hypoxic microenvironment on in vivo behaviors of tumors.

Accordingly, it could be hypothesized that the hypoxic tumor microenvironment, which is induced by hormonal agents, may play a key role in the development of acquired resistance to hormonal agents in breast cancer. Although this hypothesis still requires full testing, it is conceivable that the combined administration of hypoxia-selective cytotoxic agents, such as bioreductive anticancer agents,27 with hormonal agents might be clinically effective for the delay or prevention of occurrence of resistance to hormonal agents in breast cancer patients. Since hormonal therapy is generally less toxic than cytotoxic chemotherapy and provides a better quality of life, the prolongation of the antiproliferative effect of hormonal agents is highly desirable for patients with advanced breast cancer.

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