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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% O_2) 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-20[14)] 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 progestin.[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

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hypoxic conditions, expressed less ER-α than negative tumors. To our knowledge, this is the first study suggesting that the hypoxic tumor microenvironment reduces hormone 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 previously.[15] In brief, this cell line was derived from a patient with recurrent breast cancer. Recurrent disease appeared during postoperatative 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) supplementation (estrogen-independent).[4, 5, 15] The ML-20 cell line is a transfectant of the MCF-7 cell line with a *lac*Z expression vector.[14] Its growth characteristics *in vitro* and *in vivo* are indistinguishable from those of the wild-type MCF-7 cell line.[16] This cell line is ER- and progesterone receptor (PgR)-positive and requires E2 supplementation for growth in intact female nude mice (estrogen-dependent).[14, 16]

Reagents E2 was purchased from Sigma Chemical Co. (St. Louis, Detroit, MI). ICI 182 780 and MPA were provided 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* experiments.[5] Because half of solid tumors have median oxygen concentrations less than 10 mmHg (approximately 1% O_2)[12] and a putative key factor of cellular hypoxic reaction, HIF-1α, is easily induced by a hypoxic condition of 1% O_2,[6] 1% O_2 was chosen as a hypoxic condition in the present study. Approximately 2×10^5 KPL-1 or ML-20 cells/well were inoculated into 12-well plates (Costar Corning, Inc., Corning, NY) and cultured in Dulbecco's modified essential medium supplemented with 5% FBS for 2 days. Then, the cells were washed twice with phosphate-buffered saline (PBS) and cultured for 2 or 4 days in the estrogen-deprived medium with 1 n*M* E2 or vehicle under normoxia or a hypoxic condition (1% O_2) using a CO_2/Multi-Gas Incubator APM-30D (Astec, Fukuoka). In a separate experiment, washed cells were cultured for 3 days in the estrogen-deprived medium with 0.1 μ*M* ICI 182 780, 1 μ*M* MPA or vehicle under normoxia or a hypoxic condition (1% O_2) using the same CO_2/Multi-Gas Incubator. After cell dispersion with 0.05% trypsin (Difco, Detroit, MI) and 0.02% EDTA in PBS for 10 min, the cell numbers were measured with a Coulter counter (Coulter Electronics, Ltd., Harpenden, UK). Triplicate wells were treated in each experiment. To clarify the effects of hypoxia on the mRNA expression levels of ER-α, PgR and VEGF in KPL-1 or ML-20 cells, semi-confluent cells in 6-well plates (Costar Corning, Inc.) were incubated with the estrogen-deprived medium plus 1 n*M* E2 for 48 h under normoxia or hypoxia. Collected cells were stored at −80°C until use.

ER-α and PgR measurement To clarify the effects of hypoxia on the protein expression levels of ER-α, semi-confluent cells in T-25 flasks (Costar Corning, Inc.) were incubated with the estrogen-deprived medium for 1, 2, 4 or 7 days under normoxia or hypoxia. In the re-oxygenation experiment, the cells exposed to hypoxia for 7 days were inoculated into other flasks and incubated under normoxia for 7 days. Then, the cells were inoculated into other flasks and incubated under normoxia for 7 days again. Culture medium was changed every other day. For the PgR assay, semi-confluent cells in T-25 flasks were incubated with the estrogen-deprived medium with 1 n*M* E2 for 2 days under normoxia or hypoxia. ER-α and PgR in the cell pellets of KPL-1 or ML-20 cells were measured by enzyme immunoassay using the ER-EIA and PgR-EIA kits (Dinabot, Inc., Tokyo) according to the manufacturer's recommendations.

VEGF secretion VEGF concentrations in the culture medium were measured with an enzyme-linked immunosorbent assay kit (Otsuka Assay Laboratory, Tokushima).[5] 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 concentration of VEGF was 0.2 pg/ml. Since the VEGF concentration in the fresh medium was undetectable, but then increased linearly for at least 2 days, VEGF secretion into the medium was defined as follows:

VEGF secretion per cell per 48 h
= Concentration of VEGF × Volume of medium / Mean cell number

mRNA expression levels of ER-α, PgR and VEGF Total cellular RNA was extracted using a TRIzol RNA extraction kit (Gibco BRL Life Technologies, Gaithersburg, MD). One microgram of total RNA and 1 μ*M* Oligo(dT) 18 primer in 12.5 μl of diethyl pyrocarbonate-treated water were heated to 70°C for 2 min followed by cooling on ice for 1 min. cDNA synthesis was initiated

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-polymerase 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 internal control gene (β-actin) and one of the target genes in a single reaction, multiplex PCR was carried out. The ratios of the primer-sets between the target gene and the control gene are also shown in Table I. These ratios and the numbers of PCR cycles were chosen to amplify both products logarithmically. Each PCR reaction contained 1/100 cDNA, the indicated concentrations of primers of each target gene and the control gene, 200 μM deoxynucleotide triphosphates, 10 m*M* Tris-HCl (pH 8.8), 2.5 m*M* $MgCl_2$, 50 m*M* KCl, 0.08% Nonidet P40, and 1 unit of recombinant *Thermus aquaticus* DNA polymerase (MBI Fermentas, 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, Mortlake, 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.[17] 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. Franking 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 peroxidase; 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

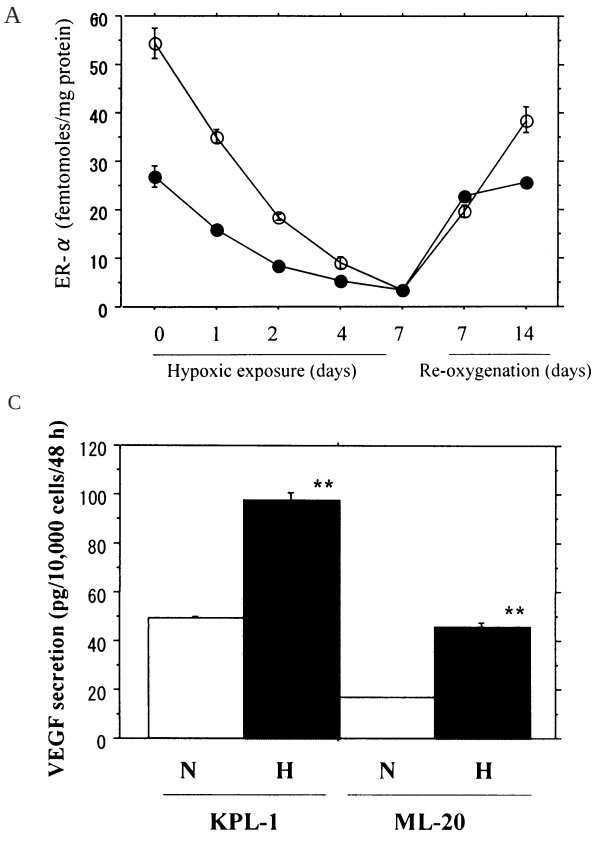
Target genes	Primer sequences		Annealing temperature (°C)	Primer ratio	No. of PCR cycles	Expected size (bp)
	Forward	Reverse				
ER-α	5′-agacatgagagctgccaacc-3′	5′-gccaggcacattctagaagg-3′	59	0.3/2.0	35	299
PgR	5′-agttgtgagagcactggatgc-3′	5′-gatctgccacatggtaaggc-3′	56	0.4/2.0	35	475
VEGF	5′-gcagaatcatcacgaagtgg-3′	5′-gcatggtgatgttggactcc-3′	58	1.0/2.0	38	212
β-actin	5′-tgacggggtcacccacactgtgcccatcta-3′	5′-ctagaagcatttgcggtggacgatggaggg-3′	NA[a)]	NA	NA	661

a) Not assessible.

RESULTS

Hypoxia down-regulates ER-α expression but up-regulates VEGF expression The hypoxic condition (1% O_2) 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.[19, 20] 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 n*M* 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 n*M* 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-



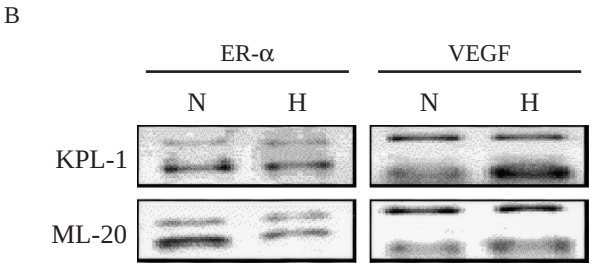


Fig. 1. Effects of hypoxia on the protein and mRNA expression levels of ER-α and VEGF. A, hypoxia (1% O_2) time-dependently decreased ER-α protein levels in KPL-1 (●) and ML-20 (○) cells. Re-oxygenation to normoxia time-dependently restored ER-α protein levels in both cell lines. Both cell lines were incubated with the estrogen-deprived medium for 1, 2, 4 or 7 days under hypoxia using a CO_2/Multi-Gas Incubator. The cells exposed to hypoxia for 7 days were inoculated into other flasks and incubated under normoxia for 7 days. Then, the cells were inoculated into other flasks and again incubated under normoxia for 7 days. ER-α of collected cells at each time point was measured with an enzyme immunoassay kit. B, hypoxia (1% O_2) decreased the ER-α mRNA expression in ML-20 cells, but not in KPL-1 cells. In contrast, hypoxia increased the VEGF mRNA expression in both cell lines. Both cell lines were incubated with the estrogen-deprived medium for 2 days under normoxia (N) or hypoxia (H) using a CO_2/Multi-Gas Incubator. Expression levels of ER-α and VEGF 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 the PCR products for each target gene. C, hypoxia (1% O_2) significantly increased the VEGF secretion into medium from both cell lines. Both cell lines were incubated with the estrogen-deprived medium for 2 days under normoxia (N) or hypoxia (H) using a CO_2/Multi-Gas Incubator. The VEGF concentration of the collected medium was measured using an enzyme-linked immunosorbent assay kit. The VEGF secretion per cell per 48 h was calculated as described in "Materials and Methods." Values are the means. Bars, SE. **, $P<0.01$.

ever, hypoxia significantly inhibited this growth stimulation (treated versus control ratio: 1.26 on day 2 and 0.94 on day 4 for KPL-1 cells; 1.17 on day 2 and 1.29 on day 4 for ML-20 cells; Fig. 3).

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.[7, 8] Therefore, nuclear HIF-1α positive tumors are likely to survive hypoxic con-

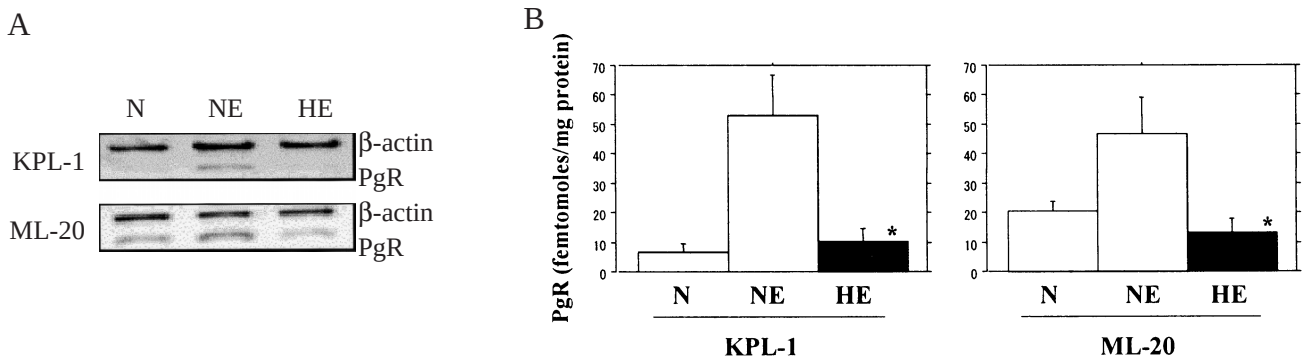


Fig. 2. Effects of hypoxia on the protein and mRNA expression levels of PgR induced by E2. A, PgR mRNA expression induced by E2 was significantly decreased by hypoxia (1% O_2) in both cell lines. Both cell lines were incubated with the estrogen-deprived medium plus or minus 1 nM E2 for 2 days under normoxia or hypoxia using a CO_2/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% O_2) 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 CO_2/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).

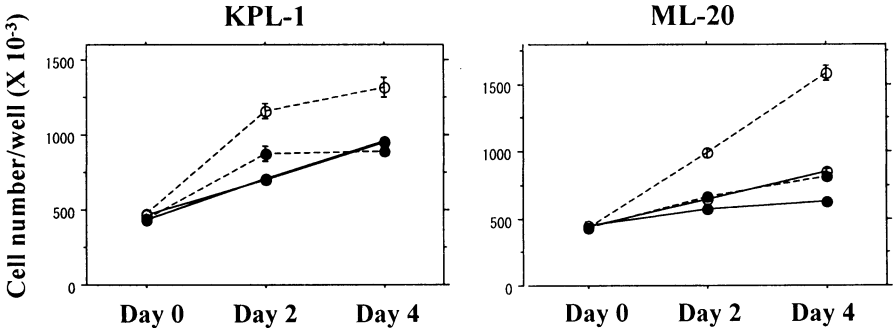


Fig. 3. Effects of hypoxia on the growth-promoting effect of E2. Both cell lines were cultured for 2 or 4 days in the estrogen-deprived medium with 1 nM E2 or vehicle under normoxia or a hypoxic condition (1% O_2) using a CO_2/Multi-Gas Incubator. The cell numbers were measured with a Coulter counter. 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.

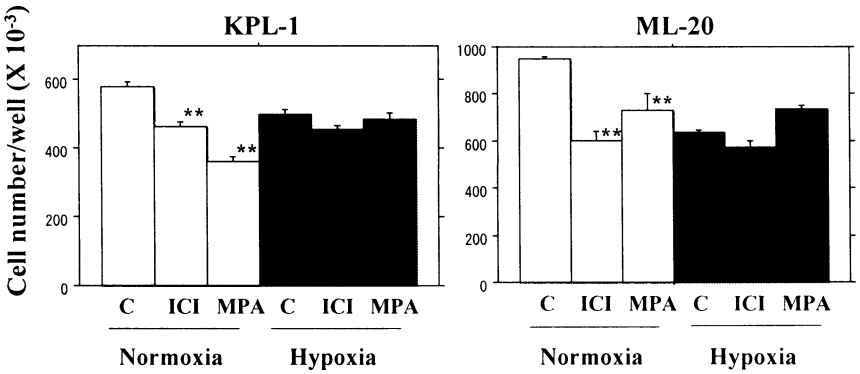


Fig. 4. Effects of hypoxia on the growth-inhibitory effects of ICI 182 780 and MPA. Both cell lines were cultured for 3 days in the estrogen-deprived medium with 0.1 μM ICI 182 780 (ICI), 1 μM MPA or vehicle (C) under normoxia or a hypoxic conditions (1% O_2) using a CO_2/Multi-Gas Incubator. The cell numbers were measured with a Coulter counter. Triplicate wells were treated in each experiment. Values are the means. Bars, SE. **, $P<0.01$ (in comparison with the controls).

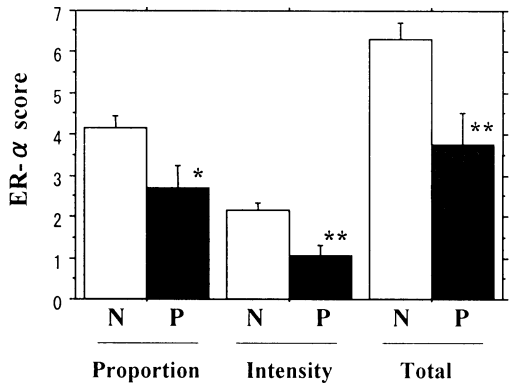


Fig. 5. Inverse relationship between the nuclear HIF-1α positivity and IHC ER-α scores in breast cancer tissues. A total of 36 breast cancer specimens were stained by the immunoperoxidase procedure using the respective monoclonal antibodies as the first antibody. The HIF-1α positivity was classified as follows: −, no nuclear staining (N); +, nuclear staining with or without cytoplasmic staining (P). For ER-α analysis, the IHC scoring system (18) was applied as described in "Materials and Methods." Values are the means. Bars, SE. *, $P<0.05$; **, $P<0.01$.

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.[1] 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-

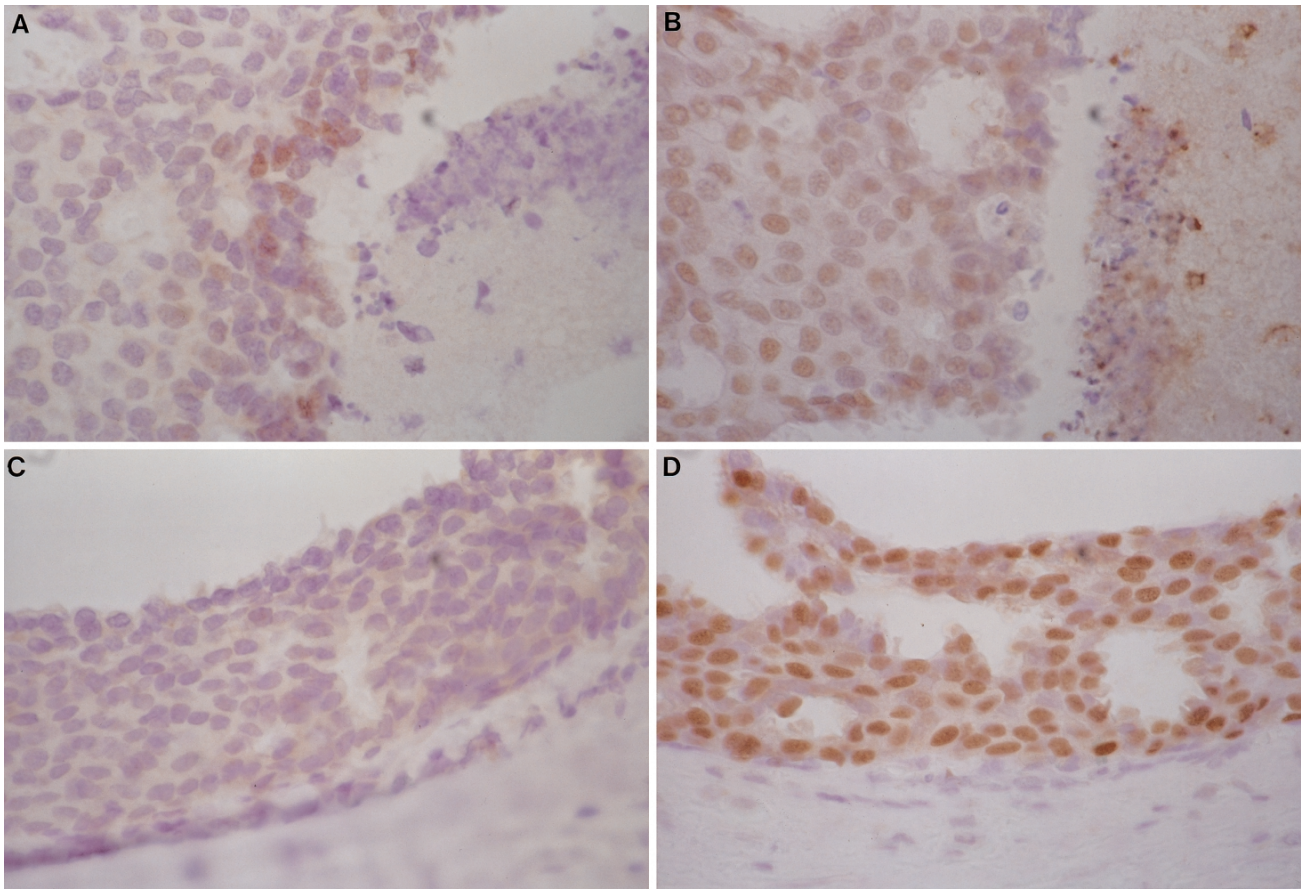


Fig. 6. Representative microphotographs of IHC analysis for HIF-1α and ER-α. Breast cancer specimens were stained by the immunoperoxidase procedure using the respective monoclonal antibodies as the primary antibody. A, nuclear HIF-1α-positive cells were most dense at the periphery of a necrotic region 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).

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% O_2) 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.[6] 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.[13] However, the expression levels of ER-α were not quantitatively

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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REFERENCES

1) Katzenellenbogen, B. S., Montano, M. M., Ekena, K., Herman, M. E. and McInerney, E. M. William L. McGuire Memorial Lecture. Antiestrogens: mechanisms of action and resistance in breast cancer. *Breast Cancer Res. Treat.*, **44**, 23–38 (1997).
2) McLeskey, S. W., Kurebayashi, J., Honig, S. F., Zwiebel, J., Lippman, M. E., Dickson, R. B. and Kern, F. G. Fibroblast growth factor 4 transfection of MCF-7 cells produces cell lines that are tumorigenic and metastatic in ovariectomized or tamoxifen-treated athymic nude mice. *Cancer Res.*, **53**, 2168–2177 (1993).
3) McLeskey, S. W., Zhang, L., Kharbanda, S., Kurebayashi, J., Lippman, M. E., Dickson, R. B. and Kern, F. G. Fibroblast growth factor overexpressing breast carcinoma cells as models of angiogenesis and metastasis. *Breast Cancer Res. Treat.*, **39**, 103–117 (1996).
4) Kurebayashi, J., Otsuki, T., Yamamoto, S., Kurosumi, M., Nakata, T., Akinaga, S. and Sonoo, H. A pure antiestrogen, ICI 182,780, stimulates the growth of tamoxifen-resistant KPL-1 human breast cancer cells *in vivo* but not *in vitro*. *Oncology*, **55** (Suppl. 1), 23–34 (1998).
5) Kurebayashi, J., Kunisue, H., Yamamoto, S., Kurosumi, M., Otsuki, T. and Sonoo, H. Paradoxical hormone responses of KPL-1 breast cancer cells *in vivo*: a significant role of angiogenesis in tumor growth. *Oncology*, **59**, 158–165 (2000).
6) Semenza, G. L. Hypoxia, clonal selection, and the role of HIF-1 in tumor progression. *Crit. Rev. Biochem. Mol. Biol.*, **35**, 71–103 (2000).
7) Zhong, H., De Marzo, A. M., Laughner, E., Lim, M., Hilton, D. A., Zagzag, D., Buechler, P., Isaacs, W. B., Semenza, G. L. and Simons, J. W. Overexpression of hypoxia-inducible factor 1alpha in common human cancers and their metastases. *Cancer Res.*, **59**, 5830–5835 (1999).
8) Talks, K. L., Turley, H., Gatter, K. C., Maxwell, P. H., Pugh, C. W., Ratcliffe, P. J. and Harris, A. L. The expression and distribution of the hypoxia-inducible factors HIF-1alpha and HIF-2alpha in normal human tissues, cancers, and tumor-associated macrophages. *Am. J. Pathol.*, **157**, 411–421 (2000).
9) Zagzag, D., Zhong, H., Scalzitti, J. M., Laughner, E., Simons, J. W. and Semenza, G. L. Expression of hypoxia-inducible factor 1alpha in brain tumors: association with angiogenesis, invasion, and progression. *Cancer*, **88**, 2606–2618 (2000).
10) Ryan, H. E., Poloni, M., McNulty, W., Elson, D., Gassmann, M., Arbeit, J. M. and Johnson, R. S. Hypoxia-inducible factor-1alpha is a positive factor in solid tumor growth. *Cancer Res.*, **60**, 4010–4015 (2000).
11) Birner, P., Schindl, M., Obermair, A., Plank, C., Breitenecker, G. and Oberhuber, G. Overexpression of hypoxia-inducible factor 1alpha is a marker for an unfavorable prognosis in early-stage invasive cervical cancer. *Cancer Res.*, **60**, 4693–4696 (2000).
12) Brown, J. M. The hypoxic cell: a target for selective cancer therapy—eighteenth Bruce F. Cain Memorial Award lecture. *Cancer Res.*, **59**, 5863–5870 (1999).
13) Bos, R., Zhong, H., Hanrahan, C. F., Mommers, E. C. M., Semenza, G. L., Pinedo, H. M., Abeloff, M. D., Simons, J. W., van Diest, P. J. and van der Wall, E. Levels of

hypoxia-inducible factor-1alpha during breast carcinogenesis. *J. Natl. Cancer Inst.*, **93**, 309–314 (2001).
14) Kurebayashi, J., McLeskey, S. W., Johnson, M. D., Lippman, M. E., Dickson, R. B. and Kern, F. G. Quantitative demonstration of spontaneous metastasis by MCF-7 human breast cancer cells cotransfected with fibroblast growth factor 4 and LacZ. *Cancer Res.*, **53**, 2178–2187 (1993).
15) Kurebayashi, J., Kurosumi, M. and Sonoo, H. A new human breast cancer cell line, KPL-1 secretes tumour-associated antigens and grows rapidly in female athymic nude mice. *Br. J. Cancer*, **71**, 845–853 (1995).
16) McLeskey, S. W., Zhang, L., El-Ashry, D., Trock, B. J., Lopez, C. A., Kharbanda, S., Tobias, C. A., Lorant, L. A., Hannum, R. S., Dickson, R. B. and Kern, F. G. Tamoxifen-resistant fibroblast growth factor-transfected MCF-7 cells are cross-resistant *in vivo* to the antiestrogen ICI 182,780 and two aromatase inhibitors. *Clin. Cancer Res.*, **4**, 697–711 (1998).
17) Otsuki, T., Yamada, O., Kurebayashi, J., Moriya, T., Sakaguchi, H., Kunisue, H., Yata, K., Uno, M., Yawata, Y. and Ueki, A. Estrogen receptors in human myeloma cells. *Cancer Res.*, **60**, 1434–1441 (2000).
18) Harvey, J. M., Clark, G. M., Osborne, C. K. and Allred, D. C. Estrogen receptor status by immunohistochemistry is superior to the ligand-binding assay for predicting response to adjuvant endocrine therapy in breast cancer. *J. Clin. Oncol.*, **17**, 1474–1481 (1999).
19) Mazure, N. M., Chen, E. Y., Yeh, P., Laderoute, K. R. and Giaccia, A. J. Oncogenic transformation and hypoxia synergistically act to modulate vascular endothelial growth factor expression. *Cancer Res.*, **56**, 3436–3840 (1996).
20) Forsythe, J. A., Jiang, B. H., Iyer, N. V., Agani, F., Leung, S. W., Koos, R. D. and Semenza, G. L. Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1. *Mol. Cell. Biol.*, **16**, 4604–4613 (1996).
21) Poller, D. N., Snead, D. R., Roberts, E. C., Galea, M., Bell, J. A., Gilmour, A., Elston, C. W., Blamey, R. W. and Ellis, I. O. Oestrogen receptor expression in ductal carcinoma *in situ* of the breast: relationship to flow cytometric analysis of DNA and expression of the c-erbB-2 oncoprotein. *Br. J. Cancer*, **68**, 156–161 (1993).
22) Knight, W. A., Livingston, R. B., Gregory, E. J. and McGuire, W. L. Estrogen receptor as an independent prognostic factor for early recurrence in breast cancer. *Cancer Res.*, **37**, 4669–4671 (1977).
23) Gasparini, G., Toi, M., Miceli, R., Vermeulen, P. B., Dittadi, R., Biganzoli, E., Morabito, A., Fanelli, M., Gatti, C., Suzuki, H., Tominaga, T., Dirix, L. Y. and Gion, M. Clinical relevance of vascular endothelial growth factor and thymidine phosphorylase in patients with node-positive breast cancer treated with either adjuvant chemotherapy or hormone therapy. *Cancer J. Sci. Am.*, **5**, 101–111 (1999).
24) Gagliardi, A. and Collins, D. C. Inhibition of angiogenesis by antiestrogens. *Cancer Res.*, **53**, 533–535 (1993).
25) Evans, S. M., Koch, C. J., Laughlin, K. M., Jenkins, W. T., Van Winkle, T. and Wilson, D. F. Tamoxifen induces hypoxia in MCF-7 xenografts. *Cancer Res.*, **57**, 5155–5161 (1997).
26) Oikawa, T., Hiragun, A., Yoshida, Y., Ashino-Fuse, H., Tominaga, T. and Iwaguchi, T. Angiogenic activity of rat mammary carcinomas induced by 7,12-dimethylbenz[a]anthracene and its inhibition by medroxyprogesterone acetate: possible involvement of antiangiogenic action of medroxyprogesterone acetate in its tumor growth inhibition. *Cancer Lett.*, **43**, 85–92 (1998).
27) Brown, J. M. Exploiting the hypoxic cancer cell: mechanisms and therapeutic strategies. *Mol. Med. Today*, **6**, 157–162 (2000).