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Oxygen partial pressure modulates 67-kDa laminin receptor expression, leading to altered activity of the green tea polyphenol, EGCG

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ABSTRACT

(−)-Epigallocatechin-3-*O*-gallate (EGCG) exhibits anti-tumor activity mediated via the 67-kDa laminin receptor (67LR). In this study, we found that 67LR protein levels are reduced by exposure to low O_2 levels (5%), without affecting the expression of HIF-1α. We also found that EGCG-induced anti-cancer activity is abrogated under low O_2 levels (5%) in various cancer cells. Notably, treatment with the proteasome inhibitor, prevented down-regulation of 67LR and restored sensitivity to EGCG under 5% O_2. In summary, 67LR expression is highly sensitive to O_2 partial pressure, and the activity of EGCG can be regulated in cancer cells by O_2 partial pressure.
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1. Introduction

Tea (*Camellia sinensis* L.) is one of the most widely consumed beverages in the world. (−)-Epigallocatechin-3-*O*-gallate (EGCG), the major green tea catechin present in the leaves, is primarily responsible for the health benefits attributed to green tea. EGCG possesses remarkable cancer chemopreventive and therapeutic potential in various cancers. Moreover, EGCG is relatively safe [1], and green tea extract containing 60% EGCG was approved by the US Food and Drug Administration as the first botanical drug [2].

The microenvironment of malignant solid tumors is vastly different from normal tissues, and is characterized by extreme diversity in ionic strength, pH, the distribution of nutrients and O_2 concentration [3]. Increasing experimental evidence indicates that low O_2 conditions have a profound impact on malignant progression and response to therapy. O_2 conditions can be categorized as "low O_2 (not hypoxia)", "chronic hypoxia", "acute hypoxia" and "cycling hypoxia", according to causative factors and duration of cancer cell exposure to hypoxic conditions [4]. Activation of hypoxia-inducible factor 1 alpha (HIF-1α) in cancer cells induces the expression of various genes responsible for adaptation to hypoxic conditions and resistance to chemotherapy and radiation therapy. Pre-hypoxic regions (HIF-1-independent) are also known to exist within tumors. However, to date there are no studies regarding the effects of EGCG on cancer cells under hypoxic conditions.

The 67-kDa laminin receptor (67LR) was originally identified as a non-integrin cell surface receptor for the extracellular matrix molecule, laminin [5]. Previous studies showed that 67LR is overexpressed in various cancers, including multiple myeloma [6], acute myelogenous leukemia [7], colorectal carcinoma [8] and cervical cancer [9]. Pathological studies revealed that increased expression of 67LR is correlated with the histological severity of lesions and tumor progression [8,9]. Notably, 67LR was identified as a cell-surface EGCG receptor capable of mediating the anti-tumor effects of EGCG in vivo [10–12]. Recently, we identified the 67LR extracellular domain corresponding to the 161–170 region as the EGCG binding site [13]. Additionally, mass spectrometric analysis revealed that intact (chemically non-altered) EGCG forms the non-covalent complex with $67LR^{161-170}$ peptide, and there is no ion peak corresponding to the covalent complex of chemically altered EGCG with the 67LR peptide, indicating that EGCG activates 67LR by forming a non-covalent complex with the receptor [13].

Abbreviations: EGCG, (−)-epigallocatechin-3-*O*-gallate; 67LR, 67-kDa laminin receptor; HIF-1a, hypoxia-inducible factor 1 alpha; MRLC, myosin II regulatory light chain; ROS, reactive oxygen species

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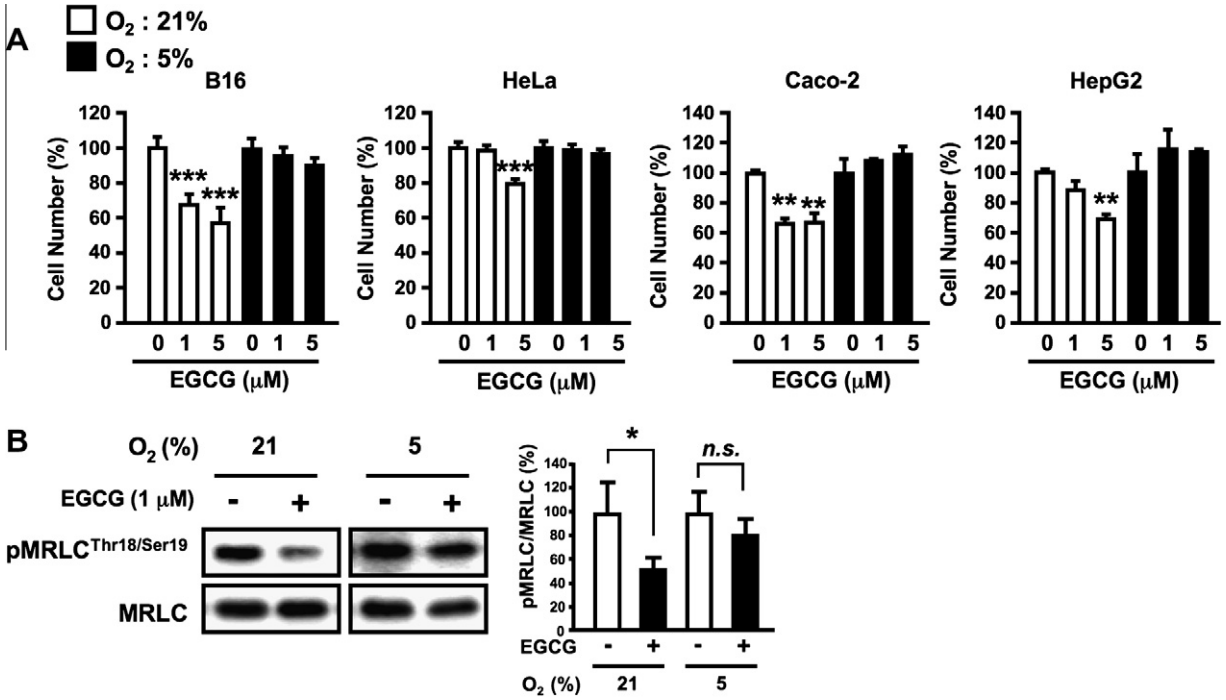

Fig. 1. (−)-Epigallocatechin-3-*O*-gallate (EGCG)-induced anti-proliferative activity is abrogated by exposure to 5% O_2 in various cancer types. (A) Solid tumor cell lines were treated with the indicated concentrations of EGCG under 21% or 5% O_2 levels for 96 h, and cell proliferation was measured by cell counter. (B) B16 cells were pre-cultured under 21% or 5% O_2 for 24 h, and treated with 1 μM EGCG at the indicated levels of O_2 for 24 h. Myosin II regulatory light chain (MRLC) phosphorylation levels were evaluated by Western blot. Band intensities were quantified using Photoshop and KyPlot 4.0. Data represent the mean ± SD of biological triplicate experiments. Asterisks indicate significance compared with control values at $^{**}p < 0.01$, $^{***}p < 0.001$ (Tukey's test).

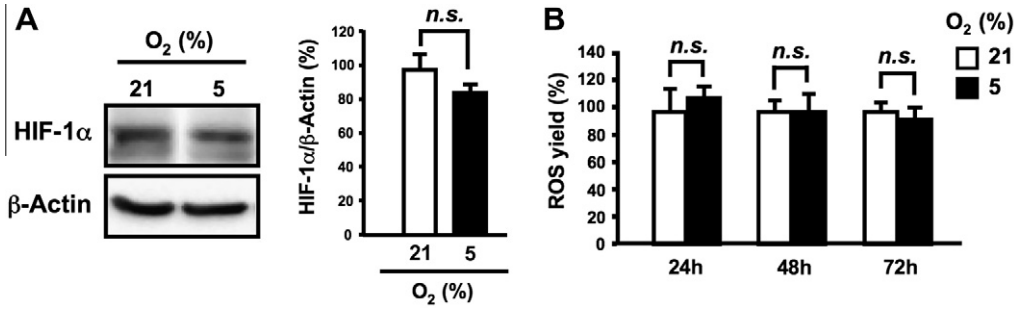

Fig. 2. Effect of exposure of B16 cells to 5% O_2 on Hypoxia inducible factor (HIF)-1α accumulation and intracellular reactive oxygen species (ROS) yield. (A) B16 cells were cultured under the indicated levels of O_2 for 24 h, and HIF-1α protein levels were evaluated by Western blot. (B) B16 cells were cultured for the indicated time, and intracellular ROS levels were evaluated by staining cells with H_2DCFDA and FACS analysis. Data shown represent the mean ± SD of biological triplicate experiments (Tukey's test).

In this study, we investigated the effect of O_2 partial pressure on EGCG activity. We found that 67LR expression is highly sensitive to O_2 partial pressure, and that the activity of EGCG in cancer cells can be regulated by O_2 partial pressure under pre-hypoxic regions.

2. Materials and methods

2.1. Reagents

EGCG, catalase and superoxide dismutase (SOD) were purchased from Sigma (St. Louis, MO). Anti-HIF-1α antibody (NB100–105) was purchased from Novus Biologicals (Littleton, CO) and anti-67LR serum was obtained from rabbits immunized with synthesized peptide derived from human 67LR [14]. Anti-phospho myosin II regulatory light chain (MRLC) (Thr^{18}/Ser^{19}) antibody was purchased from Cell Signaling Technology (Beverly, MA). Anti-MLC2 (FL-172) antibody, anti-His tag antibody and anti-ubiquitin antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Alexa Fluor 488 and Alexa Fluor 555 anti-rabbit IgG antibodies and 2,7-dichlorodihydrofluorescein diacetate (H_2DCFDA) were obtained from Invitrogen (Carlsbad, CA). MG132, specific proteasome inhibitor, was purchased from Calbiochem (Calbiochem, CA). 67LR-His tag vector has been previously described [13].

2.2. Cell culture

HeLa (human cervical adenocarcinoma), HepG2 (human hepatocellular carcinoma) and B16 (mouse melanoma) cells were maintained in Dulbecco's modified Eagle medium (DMEM) (COSMO BIO Co., Ltd., Tokyo, Japan) supplemented with 5% (v/v) (B16 cells) or 10% (v/v) (HeLa and HepG2 cells) fetal bovine serum (FBS; Biological Industries, Kibbutz Beit Kaemek, Israel). Caco-2 cells (human colorectal adenocarcinoma) were maintained in DMEM supple-

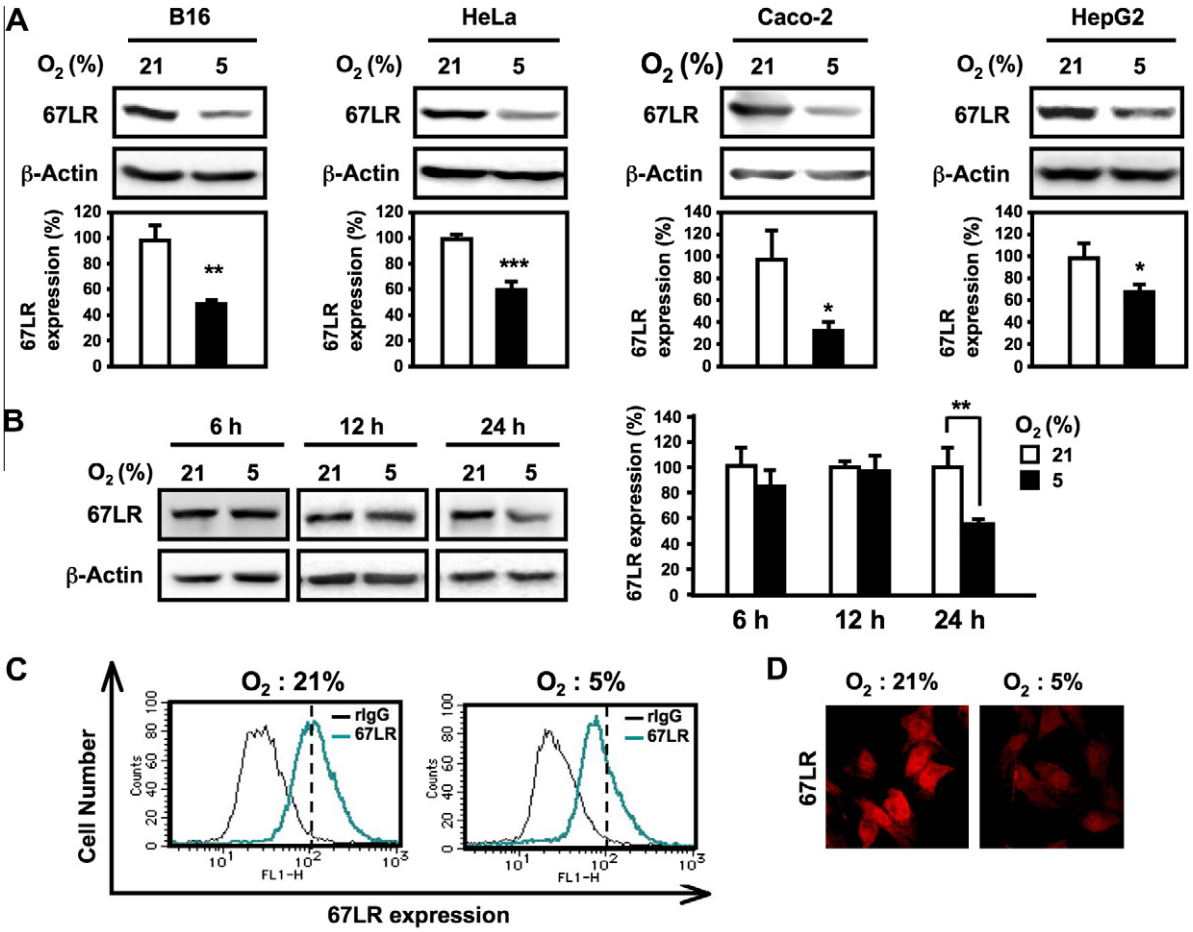

Fig. 3. 67LR expression is down-regulated by exposure to 5% O_2. (A) Solid tumor cell lines were cultured under the indicated levels of O_2 for 24 h, and 67LR protein levels were evaluated by Western blot. (B) B16 cells were cultured in 21% or 5% O_2 for the indicated time, and 67LR protein levels were evaluated by Western blot. (C and D) B16 cells were cultured under the indicated O_2 levels for 24 h and cell surface 67LR levels were detected by flow cytometry (C) or fluorescence microscopy (D). Data represent the mean ± SD of biological triplicate experiments. Asterisks indicate significance compared with control values at $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ (Tukey's test).

mented with 10% (v/v) FBS and 1% non-essential amino acids (NEAA; Hyclone Inc., Logan, UT). Cells were cultured under normoxia (21% O_2, 5% CO_2) or low O_2 (5% O_2, 5% CO_2) in a humidified incubator at 37 °C. To assess cell proliferation, cells were plated in 24-well plates (1×10^4 cells/well), and treated with the indicated concentrations of EGCG for indicated times in DMEM supplemented with 1% (v/v) FBS, 200 U/mL catalase, 5 U/mL SOD, 1% NEAA (for Caco-2 cells only) and 5 mg/mL bovine serum albumin (BSA). After treatment with EGCG, cells were counted by Sysmex F-520 (Sysmex, Kobe, Japan). The SOD and catalase have previously been shown to stabilize EGCG and to diminish H_2O_2 generation induced by catechins [15–17].

2.3. Western blotting

Immunoblot analysis was performed as previously described [10].

2.4. Measurement of intracellular reactive oxygen species (ROS)

B16 cells were incubated under normoxia or low oxygen conditions for the indicated times and stained with H_2DCFDA (10 μM) for 1 h. Cells were washed and resuspended in PBS. Intracellular ROS production was detected using a FACS Calibur (Becton Dickinson, Mountain View, CA).

2.5. Measurement of extracellular 67LR expression

B16 cells were fixed with 3.8% (v/v) paraformaldehyde, followed by incubation with 3% (w/v) BSA in PBS for 1 h on ice. Cells were incubated with anti-67LR antiserum (1:100) for 1 h on ice and washed with PBS. For flow cytometric analysis, cells were stained with anti-rabbit Alexa Fluor 488 antibody and extracellular 67LR expression was evaluated using a FACS Calibur. For fluorescence microscopy, cells were stained with anti-rabbit Alexa Fluor 555 antibody and 67LR expression was evaluated using a Keyence BZ-8100 fluorescence microscope (Keyence, Osaka, Japan).

2.6. Real-time quantitative reverse-transcription polymerase chain reaction (qRT-PCR)

Total RNA was extracted from cells using Trizol reagent (Invitrogen). RNA was treated with RNase-free DNase I (Takara Bio, Shiga, Japan) and quantified by spectrophotometry at A260 and A280 (Amersham Biosciences, Piscataway, NJ). Target gene expression was quantified by real-time qPCR using the relative standard curve method. In brief, 1 μL of cDNA was used as a template for qRT-PCR, which was performed on the Thermal Cycler Dice Real Time System (Takara Bio) using SYBR Premix Ex Taq (Takara Bio) according to the manufacturer's instructions. Sequences for the PCR primers are: 67LR, sense 5′-CTGTGCCCATCCAGCAGTTC-3′ and antisense 5′-

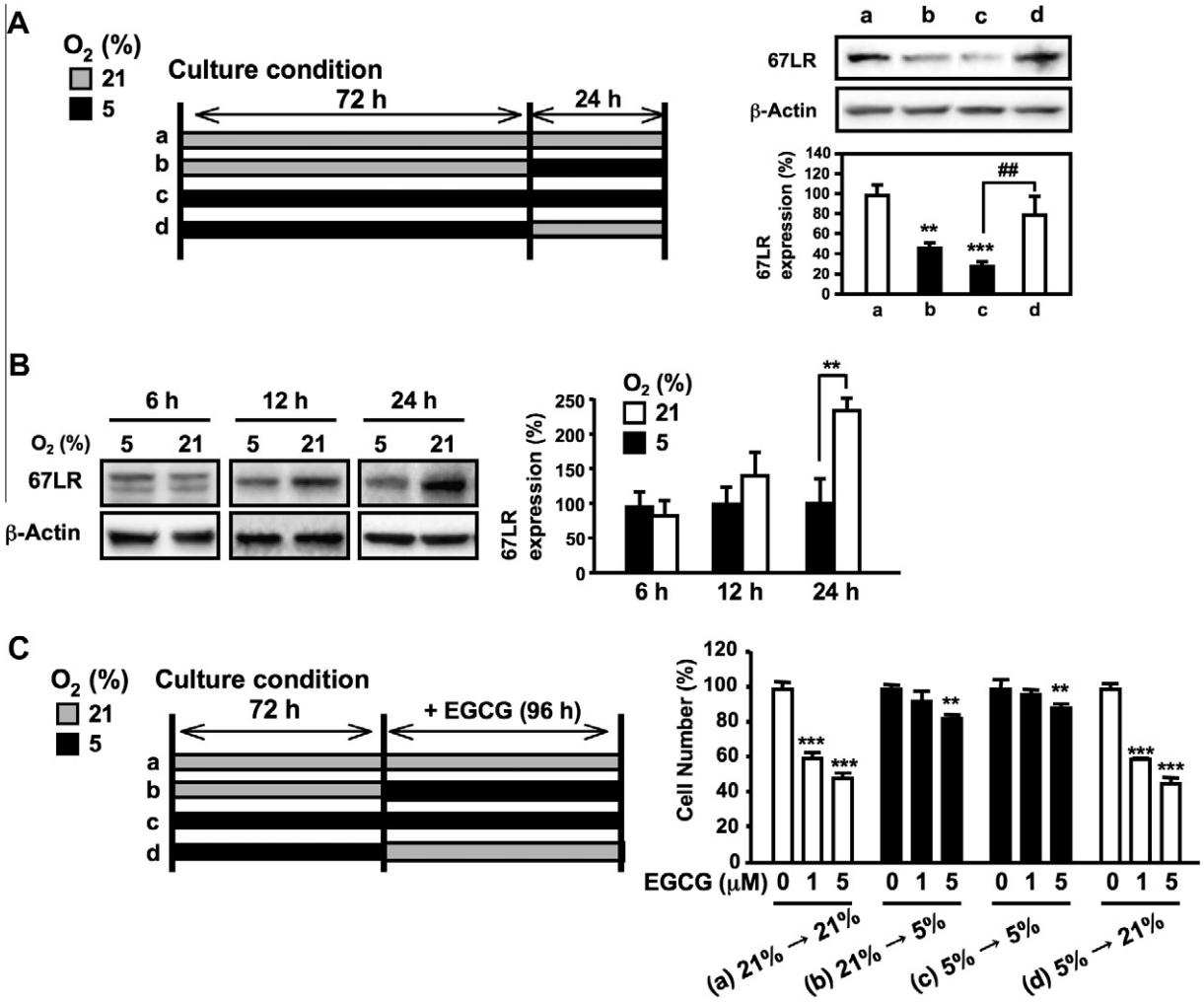

Fig. 4. 67LR expression is regulated by O_2 partial pressure. (A) B16 cells were pre-cultured under the indicated O_2 levels for 72 h, and exposed to 21% or 5% O_2 for 24 h (a–d). Cells were lysed and 67LR protein levels were evaluated by Western blot. Data represent the mean ± SD for biological triplicate experiments. Asterisks indicate significance compared with condition (a) at $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; Hash signs indicate significance compared with condition (c) at $^{\#\#}p < 0.01$ (Tukey's test). (B) B16 cells were pre-cultured under 5% O_2 for 24 h, and exposed to 21% or 5% O_2 levels for the indicated time. 67LR protein levels were evaluated by Western blot. (C) B16 cells were pre-cultured under the indicated O_2 levels for 72 h and treated with 1 μM EGCG under the indicated O_2 levels for 96 h. Data represent the mean ± SD of biological triplicate experiments. Asterisks indicate significance compared with control values at $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ (Tukey's test).

AGCTGCTGACCAATCCTCAGTG-3′; β-actin, sense 5′-ATGGGTCAGAAGGACTCCTACG-3′ and antisense 5′-AGTGGTACGACCAGAGGCATAC-3′. mRNA levels were calculated using the cycle threshold values for specific genes and normalized to the cycle threshold of β-actin.

2.7. Statistical analysis

Values are expressed as the mean ± standard deviation compared with controls. Statistical analysis was performed using Tukey's *t* test. A value of $p < 0.05$ was considered significant.

3. Results

3.1. EGCG-induced anti-proliferative activity is blocked by exposure to 5% O_2 in various cancer cell types

We investigated the anti-proliferative effect of EGCG on several solid tumor cell lines including B16, HeLa, Caco-2 and HepG2 cells under low O_2 conditions (5% O_2). While EGCG inhibited cancer cell growth in a dose-dependent manner under normoxic conditions (21% O_2), exposure to 5% O_2 significantly abrogated EGCG-induced growth inhibition in all cell lines (Fig. 1A). Previously, we reported that dephosphorylation of MRLC mediates EGCG-induced anti-proliferative activity in B16 and HeLa cells [10,11]. EGCG induced dephosphorylation of MRLC at Thr^{18}/Ser^{19} residues in B16 cells cultured under 21% O_2. However, MRLC phosphorylation was not affected by EGCG treatment when cells were cultured under 5% O_2 (Fig. 1B). These results indicate that the anti-proliferative activity of EGCG is impaired under low O_2 growth conditions.

3.2. Effect of exposure to 5% O_2 on HIF-1α accumulation and intracellular ROS yield

HIF-1α and intracellular ROS levels play crucial roles in the resistance of cancer cells to chemopreventative agents [18,19]. Western blot and FACS analysis revealed that HIF-1α protein expression and intracellular ROS levels remained similar in B16

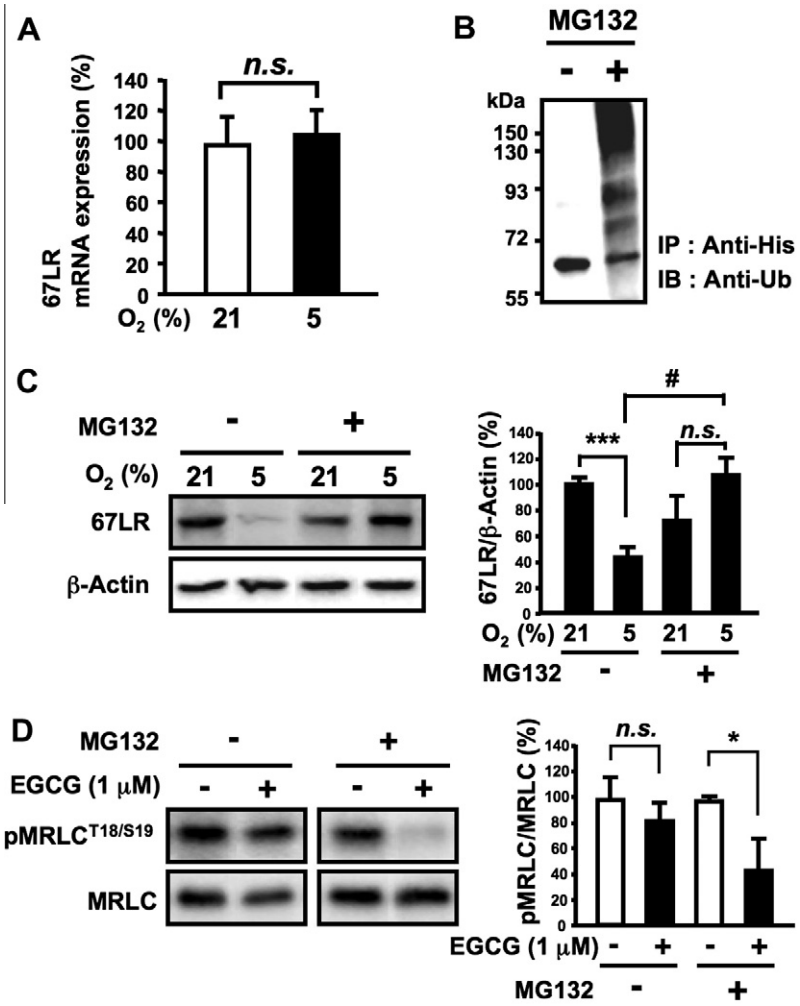

Fig. 5. Degradation of 67LR is mediated by modification of ubiquitination under 5% O_2.(A) B16 cells were cultured for 24 h under 21% or 5% O_2, and 67LR mRNA expression was determined by real-time polymerase chain reaction (PCR). 67LR mRNA levels were normalized to β-Actin. (B) HeLa cells were transfected with 67LR-His tagged vector for 72 h, and cells were treated with DMSO or 1 μM MG132 under 5% O_2 for 24 h. Ubiquitination of 67LR was detected by immunoprecipitation with anti-His antibody and samples were analyzed by Western blot using anti-ubiquitin and anti-67LR antibodies. (C) Cells were treated with DMSO or 1 μM MG132 at indicated O_2 levels for 24 h, and 67LR expression was evaluated by Western blot. (D) B16 cells were pre-cultured for 24 h under 5% O_2, and treated with DMSO or 1 μM MG132 for 6 h. Cells were treated with EGCG for 24 h under 5% O_2. MRLC phosphorylation was evaluated by Western blot. Data represent the mean ± SD of biological triplicate experiments. Asterisks or hash signs indicate significance compare.

cells cultured under normoxic and low O_2 conditions (Fig. 2A and B). These results indicate that HIF-1α and ROS are not involved in the observed resistance to EGCG treatment under 5% O_2.

3.3. 67LR expression levels are reduced by exposure to 5% O_2

EGCG is a ligand of 67LR and mediates tumor growth inhibition through this receptor in vivo [6,10–12]. Therefore, we investigated the involvement of 67LR in resistance to EGCG under low O_2 conditions. Western blot revealed that 67LR protein levels were decreased following exposure to 5% O_2 for 24 h in B16, HeLa, Caco-2 and HepG2 cells (Fig. 3A). Analysis of 67LR levels in B16 cells at earlier time-points revealed that this reducing effect was only observed after 24 h exposure to low O_2 conditions (Fig. 3B). Additionally, the cell surface expression of 67LR was also reduced in B16 cells following exposure to 5% O_2 for 24 h, as assessed by flow cytometry and immunofluorescence analysis (Fig. 3C and D).

3.4. 67LR expression is regulated by O_2 partial pressure

To further confirm the response of 67LR levels to changes in O_2 partial pressure, B16 cells were cultured under various O_2 conditions and the expression of 67LR was evaluated by Western blot. As shown in Fig. 4A (lanes a–c), the expression of 67LR decreased following culture in 5% O_2 in a time-dependant manner. Furthermore, this expression was dynamically regulated, since expression of 67LR was restored in cells pre-cultured in 5% O_2 for 72 h, upon increasing oxidative partial pressure to 21% (Fig. 4A, lane d). 67LR expression was up-regulated in response to increased O_2 partial pressure in a time-dependent manner, in cells pre-cultured in 5% O_2 for 72 h (Fig. 4B). The anti-proliferative activity induced by EGCG was observed in 21% O_2, independently of the pre-culture conditions (Fig. 4C). These results suggest that 67LR expression is highly sensitive to O_2 partial pressure, and is capable of altering the activity of EGCG in B16 cells.

3.5. Degradation of 67LR is mediated by ubiquitination under 5% O_2

RT-PCR analysis revealed that 67LR mRNA levels were not affected by changes in O_2 culture conditions (Fig. 5A), suggesting that the decrease in 67LR expression occurs at the level of protein stability. To test this, we examined the involvement of the ubiquitin–proteasome proteolytic system in the decrease of 67LR under 5% O_2. HeLa cells were transfected with His-tagged 67LR, immuno-

precipitated with anti-His antibody and analyzed by Western blot using anti-ubiquitin antibody. Ubiquitinated 67LR was observed in cells cultured in 5% O_2 (Fig. 5B), and treatment with the proteasome inhibitor MG132, protected 67LR from degradation induced by exposure to 5% O_2 (Fig. 5C). Moreover, in cells treated with MG132, EGCG induced MRLC dephosphorylation at Thr^{18}/Ser^{19}, even at 5% O_2 (Fig. 5D). These observations indicate that exposure of cells to 5% O_2 accelerates 67LR degradation, thus abrogating the anti-cancer activity of EGCG.

4. Discussion

Tumor hypoxia, caused by an imbalance in O_2 supply and demand, occurs at a distance of 100–200 μm from blood vessels [20]. Previous studies showed that at least 25% of cancer cells are exposed to hypoxic conditions ($pO_2 < 2.5$ mmHg) in malignant solid tumor [3,21].

The molecular mechanisms underlying the anti-cancer properties of EGCG have been extensively studied; however, the majority of these studies were performed under normoxic conditions (21% O_2). Furthermore, the O_2 partial pressure in cell culture systems (160 mmHg) is much higher than that in the blood or tissues (< 40 mmHg). Therefore, the activity of EGCG in vivo remains to be elucidated. Our study is the first to investigate the activity of EGCG under low O_2 conditions and to elucidate the molecular mechanisms underlying EGCG resistance in solid tumor cells exposed to hypoxic conditions. Exposure of solid tumor cells to low O_2 levels led to the degradation of the EGCG-sensing molecule, 67LR, resulting in the abolishment of EGCG anti-proliferative activity.

EGCG is unstable, and generates oxidative products such as quinones, which form a covalent interaction with a nucleophilic cysteine residue in a peptide or protein [22]. It is known that SOD and catalase in the culture medium stabilized EGCG and increased its half-life to longer than 24 h [17]. Therefore, we examined the inhibitory effects of EGCG on cancer cell proliferation with addition of SOD and catalase to the cell culture medium. To examine whether or not O_2 condition is involved in the oxidation of EGCG, we evaluated the stability of EGCG under different O_2 conditions (21% or 5% O_2). LC/MS analysis revealed that O_2 condition did not affect on the amount of intact form of EGCG (data not shown). Additionally, in the cells treated by proteasome inhibitor, EGCG induced anti-melanoma activity even at 5% O_2 (Fig. 5D). These results suggested that the EGCG resistance under low O_2 condition is not due to the generation of oxidative products.

Our data indicate that 67LR expression is highly susceptible to O_2 partial pressure. 67LR plays a critical role in tumor cell attachment, metastasis and invasion [23]. EGCG is a ligand of 67LR, and exhibits tumor growth inhibition mediated through 67LR in vitro and in vivo [6,10–12]. Recently, upregulation of HIF-1α activity induced by intratumoral hypoxia, was shown to be involved in the upregulation 67LR mRNA, leading to chemotherapeutic resistance in gastric cancer [24]. However, in this study, we demonstrate that exposure to pre-hypoxic conditions induces 67LR degradation, abrogating EGCG-induced anti-proliferative activity in solid tumor cells. Our data indicate that under low O_2, 67LR is modified by ubiquitin, accelerating proteasomal degradation.

The proteasome is a large ~700-kDa complex responsible for the majority of protein degradation [25], and has been best characterized for its role in the ubiquitin-ATP dependent proteasome pathway [26,27]. Earlier reports indicated that the ubiquitin/proteasome pathway is responsible for generating oxidative conditions [28]. In our study, we found that the decrease in O_2 levels from 21% to 5% led to enhance degradation of 67LR via the ubiquitin/proteasome pathway. Additional studies are required to understand the mechanisms underlying 67LR ubiquitination following exposure of solid tumor cells to 5% O_2.

In conclusion, our findings demonstrate that 67LR protein levels are regulated by tumor oxidative conditions, thus affecting the activity of EGCG. Our results suggest that an increase in O_2 pressure leads to suppression of ubiquitin/proteasome-mediated 67LR degradation. Taken together, these data indicate that tumor oxidative stress may enhance the sensitivity of cancer cells to EGCG.

Acknowledgments

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