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Differential Responses of Myogenic C2C12 Cells to Hypoxia between Growth and Muscle-Induction Phases: Growth, Differentiation and Motility

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Abstract. [Introduction] Local hypoxia plays a favorable role in muscle regeneration. [Subjects and Methods] The effect of hypoxia on cell growth, differentiation, and motility was examined in differentiating and growing C2C12 mouse myoblasts cells, respectively. [Results] Hypoxia induced growth suppression in the growth phase, but the suppression diminished in the differentiation phase. Hypoxia inhibited the expression of differentiation marker proteins, accompanying MyoD mRNA suppression. Expression of HIF-1α protein was induced only in the induction phase, regardless of oxygen concentration. Hypoxia did not alter motile activity in the growing phase, but augmented the motile property in the differentiation phase. Expression of autocrine motility factor mRNA was augmented under hypoxic conditions. [Conclusion] In differentiating cells, HIF-1α induced by myogenic differentiation may compensate for the cell growth suppression due to hypoxia, and support the motility augmentation. Hypoxia may shift differentiating cells into the growth phase which provides the ability to translocate to an appropriate area.
Key words: C2C12, Myogenesis, Hypoxia

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INTRODUCTION

Skeletal muscle has the ability to complete rapid and extensive regeneration in response to severe damage including excessive mechanical stimuli, ischemia, or toxicological tissue destruction[1)]. Physical exercise is a mechanical stimulus that induces muscle damage and regeneration. Local hypoxia, a drop in intramyocellular oxygen pressure, occurs with exercise[2)]. Also, low-intensity resistance exercise is effective at inducing muscular hypertrophy and concomitant increase in strength when combined with vascular occlusion[3)]. Taken together, local hypoxia in the muscle may play a favorable role in muscle regeneration. On the other hand, although the concentration of oxygen in the atmosphere is 21%, the level of oxygen in the tissue *in vivo* is very low[4–6)]. In skeletal muscle the partial saturation of oxygen is reported to be between 1% and 10%[7,8)]. Thus, reduced oxygen tension may be a normal oxygen condition for myogenic cells resident in muscle tissue. To avoid confusion, in the present study, we use "normoxia" for the atmospheric oxygen concentration of 21%, traditionally used in tissue culture, and "hypoxia" for conditions of reduced oxygen tensions. Although these terms are used for athletes training on the mountain[2)], this nomenclature has also been used for culture conditions in the literature as well[9–12)]. Against this background, many investigations have been performed to elucidate the effects of hypoxia on proliferation and differentiation of myoblasts[13)]. A hypoxic cycling test resulted in augmentation of HIF-1α RNA message in high-level endurance athletes[14)]. However, how hypoxia induces molecular metabolisms for muscle regeneration is still unclear.

Skeletal muscle differentiation is characterized by myoblast withdrawal from the cell cycle, induction of muscle-specific gene expression, and cell fusion into multinucleated myotubes. The antagonism between proliferation and differentiation implies that signaling pathways driving proliferation must be suppressed to allow induction of differentiation[9)]. Several studies have unraveled important mechanisms by which the four myogenic regulatory factors (MRFs), including MyoD,

Myf-5, myogenin, and MRF4, control the specification and the differentiation of the muscle lineage[15]. Proliferating myoblasts still express MyoD and Myf-5 before the onset of muscle differentiation[9,16,17]. The link between myoblast cell cycle withdrawal and differentiation is established through regulation of cyclin-dependent kinases (CDKs) and cyclin-dependent kinase inhibitors (CDKIs). The CDKI p21 and retinoblastoma protein (pRb) are critical for establishing the post-mitotic state during myogenesis[9,18,19]. In myogenic cells, p21 expression increases during the G_1 phase of the cell cycle and up-regulation is associated with permanent cell cycle arrest of muscle cells[9,20]. In addition, MyoD is a key transcription factor regulating both p21 and pRb gene expression during muscle differentiation. In myogenic cells, growth suppression induced by hypoxia has been demonstrated clearly by Di Carlo et al., showing G_1 arrest of the cell cycle responding to hypoxia[9]. This growth arrest is associated with p27 accumulation in C2C12 myoblasts[9]. However, the hypoxic effects on growth are still unclear in the differentiating cells, i.e., cells cultivated in the differentiation medium.

Hypoxia has profound effects on cell metabolism and growth. Recently, reports have shown that hypoxia can regulate the proliferation and differentiation of stem cells, and that mild hypoxia has salutary effects on stem/progenitor cells[21,22]. Cells can adapt to low oxygen (O_2) in part through activation of hypoxia-inducible factors (HIFs). HIF-1α is one of the key transcription factors in the response to hypoxia; it mediates a variety of adaptive cellular and systemic responses to hypoxia by up-regulating the expression of more than 50 different genes to assist animals in their adaptation and survival[22–24]. Several studies have found that myogenesis can adapt to hypoxia. It is noteworthy that, unlike the differentiation of other cell types such as preadipocytes or chondroblasts, the effect of hypoxia on myogenesis is independent of HIF-1α, while myogenesis can also adapt to glucose deprivation[25]. Interestingly HIF-1α protein is required for C2C12 myogenesis[26].

Cell motility is a key phenotype in fundamental cell functions such as embryonal development, chemotaxis, wound healing, invasion, and metastasis[27]. Myogenic cells show high motile activity once cells are activated in differentiation induction[28], implying the important role of cell motility in muscle regeneration. In the hypoxic condition, cell motility is augmented, especially for cancer cells, resulting in the promotion of metastasis[29]. One of the key molecules responsible for hypoxia-induced motility stimulation is autocrine motility factor (AMF), a protein secreted by cancer cells, which stimulates the motility of not only cancer cells but also normal fibroblasts via an autocrine route[30–33]. AMF has been identified to be identical to an intracellular glycolytic enzyme, glucose-6-phosphate isomerase (GPI), catalyzing the interconversion of glucose-6-phosphate and fructose-6-phosphate, the second step of the Embden-Meyerhof glycolytic pathway[32,34,35]. Furthermore, AMF/GPI is also identified as a neurotrophic factor, neuroleukin (NLK)[35,36]. This multi-functional molecule, a member of the ecto/exo-enzyme family, plays an important role in the enhancement of the motility induced by hypoxia[29]. So far, few reports have studied the effect of hypoxia on the motile properties of myogenic cells.

In this study, we investigated the effects of hypoxic conditions on myogenesis, using the mouse myoblast cell line C2C12, a model widely used to study postnatal skeletal muscle formation[37]. Especially, we focused on the effect of hypoxia on cell growth and motility in differentiating cells, since most previous studies have examined the effects on cells in growing medium[13]. The results showed a clear differential response to hypoxia in the muscle-induction phases compared to the growth phase, i.e., diminishment of growth suppression, and enhancement of cell motility. The response to hypoxia is discussed from the point of view of its favorable impact on muscle regeneration.

SUBJECTS AND METHODS

C2C12 mouse myoblasts were provided by the American Type Culture Collection (ATCC: Manassas, VA, USA). Cells were grown in growth medium, designated as GM, consisting of Dulbecco's modified Eagle's medium (DMEM, Grand Island Biological Co., N.Y. USA) supplemented with 20% fetal bovine serum (FBS, K. C. Biological Inc., Lenex) and 100 μg/ml of kanamycin (Banyu Pharmaceutical Co., Tokyo, Japan). Myogenic differentiation was induced at the confluent phase by serum deprivation[37]. The culture medium was replaced with induction medium, designated as IM, consisting of DMEM supplemented with 2% horse serum (HS) and 100 μg/ml of kanamycin at 37°C in a humidified incubator under 5% CO_2, which is the conventional 20% O_2 condition (normoxia), and the media were replaced every day. In the experiments for myogenic induction, GM was used instead of IM as a control. To ensure reproducibility, we performed all experiments with cultures grown for no longer than 6 weeks after recovery from frozen stocks.

To evaluate the effects of hypoxia on myogenesis, C2C12 cells were plated in an adjustable hypoxia chamber with real-time pO_2 readout (BL-40M, JUJI FIELD INC., Tokyo).

Growth curves of C2C12 cells in various conditions were determined by plating the myogenic cells in GM at 1×10^4 cells/well in 24-well plates (Corning Incorporated, NY, USA) and incubating for various lengths of time. Quadruplicate cultures were harvested, and the number of cells per well was determined by the trypan blue dye exclusion method and viable single cells were counted in a Burker-Turk hemocytometer. Although the MTT assay was tried, a comparison of the cell numbers in between normoxia and hypoxia could not be made because the products of the assay depend on the respiratory function[38].

To evaluate the growth of the myogenic cells in the differentiation phase, culture medium was replaced from GM to IM 24 hours after plating of the cells at a density of 2×10^4. Then, the cell number was determined at various times after the medium change.

Uniform carpets of gold particles were prepared on coverslips coated with bovine serum albumin, as described previously. Colloidal gold-coated coverslips were placed in

35-mm tissue culture dishes, and 3,000 cells in suspension culture were added to plates containing 1 ml of GM or IM, corresponding to growing or differentiating conditions, respectively. After 24 hours, the phagokinetic tracks were visualized using dark-field illumination under a Nikon inverted microscope. The area cleared of gold particles by at least 50 cells was measured using NIH image 1.62, and the standard error reflecting the 95% confidence level was calculated[39].

Total RNA was extracted with TRIzol Reagent (Invitrogen, US). Two hundred nanograms of total RNA in a 20 μl volume of each sample was reverse transcribed to obtain the cDNA used in each RT-reaction of SuperScript™ III Platinum Two-Step qRT-PCR Kit with SYBR Green (Invitrogen, US). Two-microliter cDNA templates in a total volume of 50 μl containing 1 unit of Taq DNA polymerase (TOYOBO, Osaka, Japan) was amplified. PCR primer pairs used in this study included glyceraldehydes-3-phosphatase dehydrogenase (GAPDH):
F, 5' -GTGGCAAAGTGGAGATTGTTGCC-3',
R, 5'-GATGATGACCCGTTTGGCTCC-3';
MyoD: F, 5'-GCAGGCTCTGCTGCGCGACC-3',
R, 5'-TGCAGTCGATCTCTCAAAGCACC-3';
myogenin: F, 5'-GAGCGCGATCTCCGCTACAGAG-3',
R, 5'-TCTGGCTTGTGGCAGCCCAGG-3',
HIF-1α: F, 5'-TGCTCATCAGTTGCCACTT-3',
R, 5'-TGGGCCATTTCTGTGTGTA-3' and
AMF: F, 5'-GCTTTGCTGCCTATTTCCAG-3',
R, 5'-AATGAAGGGTGTCAGCTTGG-3'.

PCR amplification conditions for GAPDH, MyoD and myogenin were a 2-min initial denaturation step at 94°C followed by 25 cycles at 94°C for 1 min, 56°C for 1 min, and 72°C for 1 min[40]. PCR amplification conditions for HIF-1α were 94°C for 30 s, 55°C for 1 min, 72°C for 1 min for 22 cycles, with the last cycle including an extension for 10 min at 72°C[26]. PCR amplification conditions for AMF were a 2-min initial denaturation step at 94°C followed by 21 cycles at 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min. The PCR products were separated by electrophoresis on 1% agarose gel, stained with ethidium bromide, and visualized with a UV transilluminator. To analyze the data statistically, densitometric analysis was performed using ImageJ. Statistical analyses were performed using data from three independent experiments.

Immunoblot analyses were performed, as described previously[34]. Briefly, C2C12 cells grown in GM or IM were harvested by trypsin-EDTA, and washed twice in PBS. The cells were resuspended at 1×10^7 cells/ml in lysis buffers: PBS containing 1 mM EDTA, 0.5%NP-40 and protease inhibitor cocktail (Sigma, St. Louis) for anti-mouse myosin heavy chain antibody (MF-20) (Developmental Studies Hybridoma Bank, University of Iowa), and 40 mM Tris (pH7.5) containing 300 mM KCl, 1%TritonX-100, 2 mM EDTA, and protease inhibitor cocktail for anti-mouse HIF-1α antibody (H-206) (Santa Cruz Biotechnology, Inc. California). They were then lysed for 40 min on ice. The extracts were cleared by centrifugation at 15,000 g for 30 min[26]. Equal amounts of protein in the supernatant (20 μl protein/lane), estimated by cell count (2×10^5 cells), were boiled at 90°C for 5 min, electrophoresed on a 10% SDS gel and sequentially electrophoretically transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA). After blocking with quench solution (15% non-fat milk in PBS containing 0.2% sodium azide) for 24 hours at room temperature, membranes were incubated with either MF-20 mouse antibody (1:500), or anti-HIF-1α rabbit polyclonal antibody (1:100) in quench solution for 1 hour at room temperature and washed three times for 10 min with PBS. Then, the membranes were incubated with HRP-conjugated secondary anti-rabbit IgG (1:200) in quench solution for 1 hour at room temperature, washed three times for 10 min with quench solution and washed once for 10 min with PBS, then visualized by chemiluminescence using an ECL plus immunoblotting kit (Cell Signaling Technology) with a digital luminescent image analyzer LAS-3000 (Fujifilm, Tokyo, Japan). To analyze the data statistically, densitometric analysis was performed using ImageJ, as described for the PCR analysis. Statistical analyses were performed using data from three independent experiments.

C2C12 cells were harvested by cell scraper and the cell pellet was made by centrifugation at 15,000 g for 5 min. After fixation in 20% neutral buffered formalin, the cell blocks were embedded in paraffin, then sectioned to approximately 2 μm and stained with hematoxylin & eosin (H.E.) and subjected to immunostaining. Antigen retrieval was performed by microwave (citric acid buffer, 90°C, 15 min). Blocking of endogenous peroxidase was performed, and these slides were washed twice for 5 min with PBS. After blocking in protein block serum-free (DAKO) for 15 min at room temperature, these slides were incubated with anti-mouse myosin heavy chain (MF-20, 1:200) at 4°C overnight and washed twice for 5 min with PBS. Then, the slides were incubated with secondary antibody, simple stain MAX-PO MULTI (Nichirei, Tokyo, Japan), for 30 min at room temperature and washed twice for 5 min with PBS, then visualized by 3,3'-diaminobenzidine-4HCl (DAB: Wako, Osaka, Japan). Lastly, counterstaining was performed with hematoxylin.

RESULTS

Table 2 and 3 show representative growth curves of C2C12 cells under normal and hypoxic conditions in experiments performed in triplicate. C2C12 cells grew exponentially with a doubling time of approximately 13.5 hours and kept this growth rate until 3 days later, as shown in Table 1. It has been reported that hypoxia of approximately 1% O_2 arrested cell growth of C2C12 cells, resulting in G_1 arrest in the cell cycle[9]. In the present study, however, the hypoxia condition used did not induce cell growth arrest, and instead, suppressed the growth rate (Table 2). In GM, cell growth rate was the same as that of cells under the normal condition for 2 days after plating, even under the hypoxic condition, then the cells showed a reduced growth rate for approximately 18.5 hours, resulting in approximately 60% inhibition four days after plating (Table 1). This growth suppression of hypoxia was dependent on O_2 concentration, as shown in Table 1. The

Table 1. Inhibition of cell growth of hypoxia on O_2 concentration[a]

O_2 concentration	cell number (×10,000)		inhibition (%)
	normoxia (20% O_2)	hypoxia	
2%	50.0 ± 6.9	30.2 ± 4.8	39.6%*
5%	48.4 ± 2.8	36.1 ± 4.1	25.5%**
10%	47.8 ± 12.1	41.3 ± 2.7	13.6%

[a] Ten thousands cells were plated in 24-well plates in GM medium under normoxic or hypoxic (2%, 5%, 10%) condition. After cultured for 72 hours, the number of cells per well was determined by trypan blue dye exclusion method and viable single cells were counted in a Burker-Turk hemocytometer. * The number of cells were compared under normoxic or hypoxic condition ($p<0.01$). ** The number of cells were compared under normoxic or hypoxic condition ($p<0.05$).

Table 2. Inhibition of cell growth of hypoxia in GM

time (hour)	cell number (×10,000)	
	normoxia (20% O_2)	hypoxia (2% O_2)
24.0	3.1 ± 1.2	21.1 ± 0.6
48.5	9.4 ± 1.9	8.7 ± 0.7
74.0	38.6 ± 9.3	23.4 ± 3.3
98.5	50.0 ± 6.9	30.2 ± 4.8
122.0	32.4 ± 4.8	2.1 ± 1.9

Table 3. Inhibition of cell growth of hypoxia in IM

time (hour)	cell number (×10,000)	
	normoxia (20% O_2)	hypoxia (2% O_2)
24	9.0 ± 1.0	9.0 ± 1.0
40.5	17.6 ±3.2	16.9 ±1.8
47.5	20.4 ± 2.5	21.7 ± 2.7
63.5	23.8 ± 1.8	22.6 ± 3.8
72.5	28.1 ± 1.1	28.7 ± 1.9
89.0	29.4 ± 6.9	27.1 ± 2.9

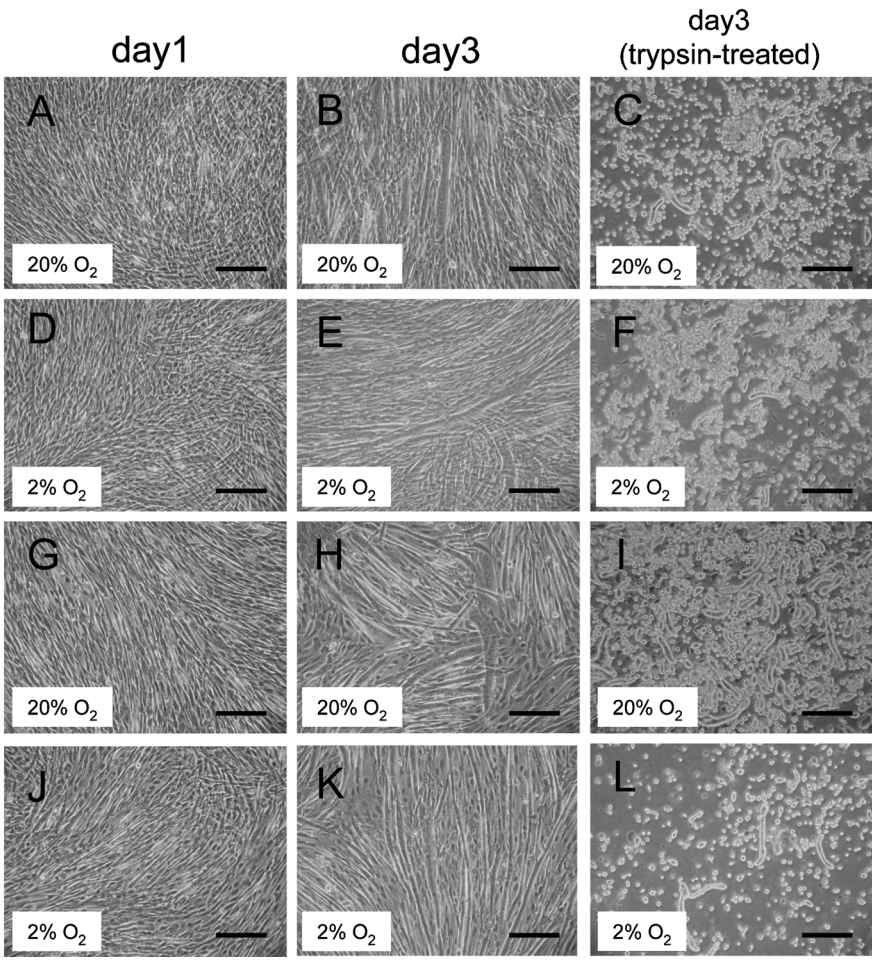

Fig. 1. The morphology of C2C12 cells *in vitro*. C2C12 cells were cultured in GM for three days to reach confluence, and the medium was replaced with GM (A-F) or IM (G-L). The cells were incubated under normoxic (20% O_2, A-C, G-I) or hypoxic (2% O_2, D-F, J-L) conditions. The cultivated cells were photographed one day (A, D, G, J) or three days (B, E, H, K) after medium replacement. The cells cultivated for three days after medium replacement were trypsinized (C, E, I, L). Bar indicates 200 μm.

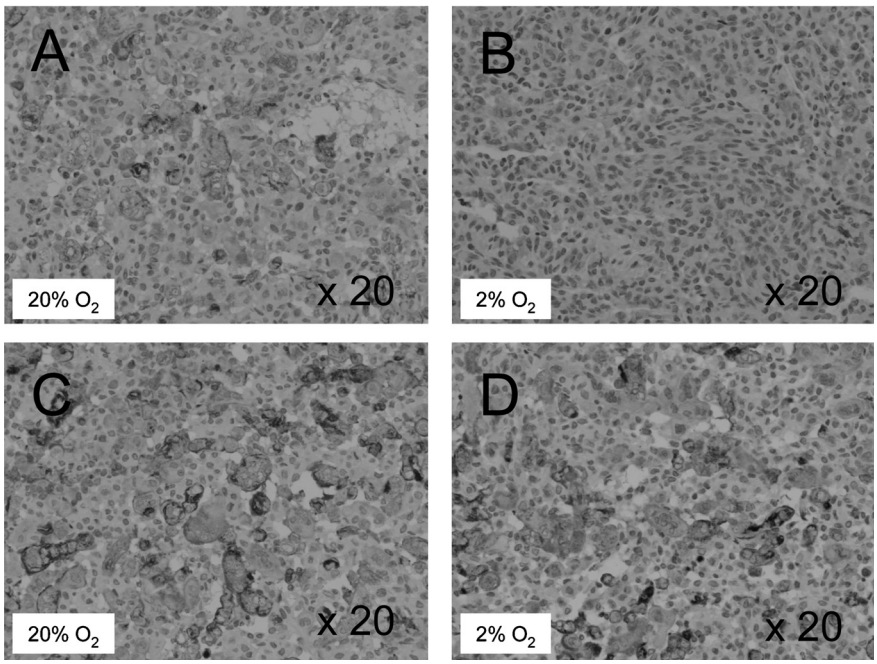

Fig. 2. Immunohistochemical analyses of mouse myosin heavy chain (mMHC) in C2C12 cells cultivated three days after medium replacement with GM (A, B) or IM (C,D) under normoxia (20% O_2, A, C) or hypoxia (2% O_2, B, D). Cells were harvested by cell scraper and a cell pellet was made, and immunostained, as described in Subjects and Methods (magnification, 20X). To analyze the data statistically, the ratio of nuclei in the cells with positive staining to those without staining (% positive nuclei) were evaluated in 4 areas selected at random.

significant suppression of cell growth disappeared when the O_2 concentration reached 10% (Table 1).

Under the normal O_2 concentration, cell growth ceased one day later when the medium was replaced with IM containing low serum concentration, as shown in Table 3. Interestingly, growth suppression induced by hypoxia, as exhibited in GM (Table 2), was not observed in the differentiating phase in C2C12 myogenic cells (Table 3).

On the basis of these growth analyses, we used 2% O_2 for inducing significant growth-suppression in GM but not in IM, as a hypoxic condition in the following experiments.

Muscle differentiation is evidenced by the presence of multinucleated large cells which are created by fusion of myogenic cells[41]. We therefore examined the morphology *in vitro* in the differentiating condition. There were no differences in cell morphology *in vitro* among cells one day after differentiation induction, as shown in Fig. 1 (A, D, G, J). Three days after medium replacement to IM, abundant large multi-nucleated cells were observed in IM under the normal oxygen condition (H), and multi-nucleated cells were also detected under other conditions (B, E, K). This was confirmed by visualization of trypsin-treated cells (C, F, I, L), indicating that hypoxia may inhibit muscle differentiation of C2C12 cells.

To confirm the differentiating status, we collected cells by cell scraper, and examined the expression of muscle myosin heavy chain by immunohistochemistry using anti-myosin heavy chain antibody (MF-20), which is a marker of muscle differentiation. The results corresponded well to those obtained for *in vitro* cell morphology. Myosin heavy chain recognized by MF-20 was positive in the cytoplasm of multi-nucleated myofibers. The cells expressing the differentiation marker were sparsely observed only in cells cultivated in IM for one day, while few such cells were seen in GM-cultured cells (data not shown). Three days after differentiation induction, many cells strongly expressed the differentiation marker in IM the under normal oxygen condition (Fig. 2C). Interestingly, cells grown in GM also expressed the marker, although there were fewer of these cells (Fig. 2A). It should be noted that the degree of expression of the marker was lower under hypoxic conditions in both GM and IM, confirming that hypoxia may inhibit muscle differentiation of C2C12 cells. These results were confirmed by statistical analysis of the distribution of % positive nuclei (data not shown).

The differentiation status was confirmed by immunoblot analyses. One day after differentiation induction, the myosin heavy chain molecules were detected only in IM in reduced numbers in response to hypoxia (Fig. 3). Three days after the induction, although the molecules were detected in cells under all culture conditions, cells cultivated in IM expressed the myosin heavy chain molecule more strongly than those in GM, and hypoxia reduced the expression (Fig. 3). Reduced expression of the myosin heavy chain molecules in response to hypoxia was confirmed by the densitometric analysis (data not shown)

We investigated the effect of hypoxia on the cell motility of C2C12 cells cultured in GM and IM, since hypoxia

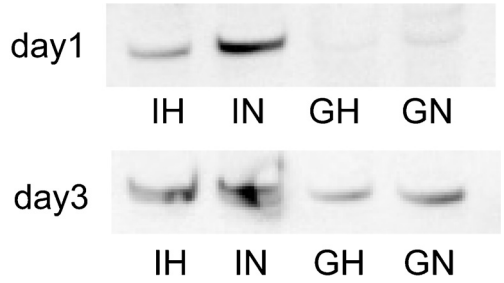

Fig. 3. Immunoblot analysis of muscle myosin heavy chain (mMHC) protein. C2C12 cells were cultured in GM for three days to reach confluence, then the medium was replaced with GM (GH, GN) or IM (IH, IN). The cells were incubated under normoxic (20% O_2, GN, IN) or hypoxic (2% O_2, GH, IH) conditions for one (day 1) or three days (day 3). To analyze the data statistically, densitometric analysis was performed at day 1 or day 3.

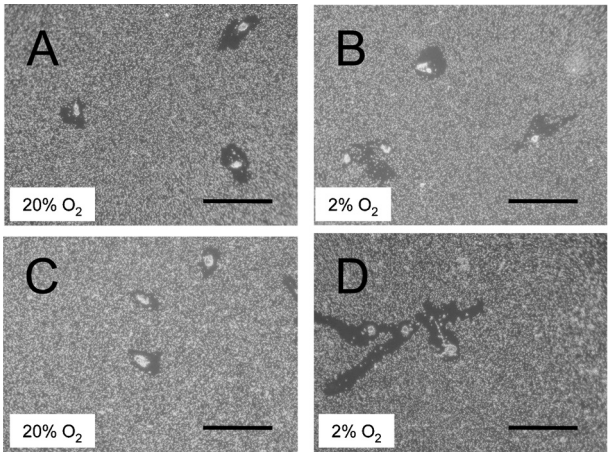

Fig. 4. Cell motility of C2C12 cells in response to hypoxia. Cells were plated on colloidal gold-coated coverslips in GM (A, B) or IM (C, D), and incubated under normoxia (20% O_2; A, C) or hypoxia (2% O_2; B, D) for 24 hours. Bar indicates 200 μm.

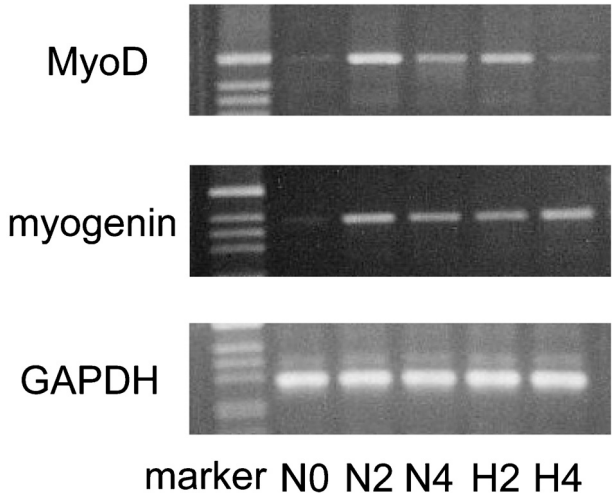

Fig. 5. RT-PCR of MyoD and myogenin mRNA during myogenesis. C2C12 cells were cultured in GM for three days to reach confluence, and then the medium was replaced with IM for differentiation. The cells were incubated under normoxic (20% O_2; N0, N2, N4) or hypoxic (2% O_2; H2, H4) conditions for zero (N0), 2 days (N2, H2), or 4 days (N4, H4). To analyze the data of MyoD and myogenin statistically, densitometric analysis was performed.

reportedly induces cell motility relating to metastatic capacity in neoplastic cells[29]. Cell motility was analyzed using Phagokinetic Tracks. C2C12 cells growing in GM were harvested by trypsin/EDTA and plated on colloidal gold-coated coverslips in GM or IM, and incubated under normoxia or hypoxia for 24 hours (Fig. 4). Under the growing condition, i.e., GM, there was no significant difference in the cell motility between the normal and hypoxic conditions. At a normal oxygen concentration, cell motility was inhibited under the differentiation condition, i.e., IM, probably due to reduction of serum. In contrast to the normal oxygen concentration, however, hypoxia augmented cell motility 2-fold, as compared with the normal oxygen concentration, indicating that the myogenic cells in the differentiation phase show high motility in response to hypoxia.

The myogenic differentiation of myoblasts is highly orchestrated by a family of MRFs. MyoD is one of the most important MRFs, and its mRNA expression was investigated. Although we tried to evaluate the protein expression level by both immunoblot and immunohistochemical analyses, specific expression of the protein was not detected, probably due to the lack of a high-quality antibody against mouse MyoD. As shown in Fig. 5, MyoD mRNA was not obvious on day 0. Two days later strong induction expression of MyoD mRNA was observed (Fig. 5, N2). This became weaker 4 days after induction, indicating that the regulator protein is expressed very early in the differentiation-induction[41]. In response to hypoxia, the mRNA expression was reduced both 2 and 4 days after induction. The reduced mRNA expression in response to hypoxia corresponded well to the protein expression of muscle heavy chain, as shown in Figs. 2 and 3.

Myogenin mRNA was also expressed at a relatively early phase. At either day 2 or 4 there was no significant difference in the RNA message expression by the cells under normoxia and hypoxia conditions.

The effect of hypoxia on myogenesis is independent of HIF-1α, while myogenesis can also adapt to glucose deprivation[25]. Interestingly, HIF-1α protein is required for C2C12 myogenesis[26]. Therefore we examined the response of protein expression of these cells to hypoxia using immunoblot analysis. As shown in Fig. 6, a small amount of HIF-1α protein was expressed 1 day later under either normoxia or hypoxia in GM. In contrast, HIF-1α protein was expressed slightly 1 day after induction under both normoxia and hypoxia in IM, but without significant difference. This expression pattern was more obvious 3 days after induction. In GM HIF-1α protein was expressed slightly. In IM, on the other hand, the expression of HIF-1α was augmented for 3 days. Furthermore, the protein

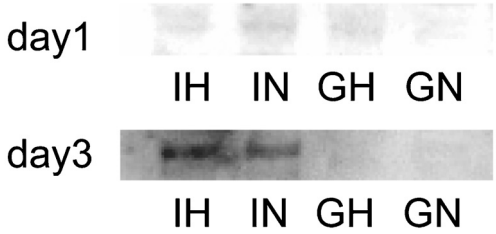

Fig. 6. Immunoblot analysis of HIF-1α protein (A). C2C12 cells were cultured in GM for three days to reach confluence, and then the medium was replaced with GM (GH, GN) or IM (IH, IN). The cells were incubated under normoxic (20% O_2; GN, IN) or hypoxic (2% O_2; GH, IH) conditions for one (day 1) or three days (day 3). To analyze the data at day 1 and day 3 statistically, densitometric analysis was performed.

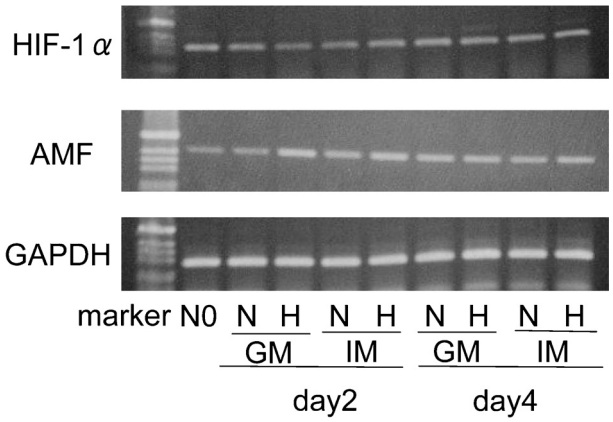

Fig. 7. RT-PCR of HIF-1α and AMF/PGI mRNA during myogenesis. C2C12 cells were cultured in GM for three days to reach confluence, and then the medium was replaced with GM or IM. The cells were incubated under normoxic (20% O_2; N0, N2, N4) or hypoxic (2% O_2; H2, H4) conditions for zero (N0), 2 days (day 2), or 4 days (day 4). To clarify the difference in the RNA expression of HIF-1α or AMF quantitatively, densitometric analysis was performed on the data obtained from PCR analyses of the AMF message obtained on day 2 and day 4.

expression was significantly higher under hypoxia than normoxia.

In contrast to protein expression of HIF-1α, mRNA of the protein was stable in C2C12 cells, suggesting that the level of HIF-1α protein expression is regulated by a proteasome- and chaperone-dependent pathways in C2C12 myoblasts[26]. In the present study, HIF-1α mRNA was expressed constantly, regardless of the culture conditions (Fig. 7).

AMF/PGI is a metastasis-related motility factor[34], which is induced by hypoxia, resulting in high motility[29]. Interestingly, motility related to metastatic capacity is associated with mRNA level, but not to protein expression level[36]. We therefore examined the expression of AMF/PGI mRNA for the possible linkage to the hypoxia-induced augmentation in cell motility found in IM. As shown in Fig. 7, mRNA was augmented significantly under hypoxia in both GM and IM 2 days after induction. However, the augmentation was diminished 4 days after induction.

DISCUSSION

In the present study, we found differential C2C12 cell responses of growth, differentiation, and motility to hypoxia in both the growth and induction phases. Hypoxia induced growth suppression in the growth phase, but the suppression was diminished in the differentiation phase. Hypoxia inhibited the expression of differentiation marker proteins, accompanied by MyoD mRNA suppression. Expression of mRNA of HIF-1α was not affected by the hypoxic condition, but the protein expression was induced only in the induction phase, regardless of oxygen concentration. Hypoxia did not alter motility in the growth phase, but augmented the motility in the differentiation phase. Interestingly, in the early induction phase, mRNA expression of AMF/PGI, a motility factor, was augmented under hypoxic condition.

In the present study, hypoxic conditions inhibited myogenic cell growth in an O_2 concentration-dependent manner in a growing medium. Proliferating responses to hypoxia varied among cell types. Hypoxia induces growth suppression by G_1/S cell cycle arrest in relatively differentiated cells: kidney epithelial cells[42], primary embryo fibroblasts[43], and splenic B lymphocytes[44]. HIF-1α has been shown to play an essential role in cell cycle arrest[44]. On the other hand, in mouse embryonic fibroblast hypoxia-induced G_1/S regulation, the resultant cessation of growth was due to enhanced expression of cyclin-dependent kinase inhibitor p27, and this growth inhibition was independent of HIF-1α[43]. In C2C12 myogenic cells, growth suppression induced by hypoxia has been demonstrated clearly by Di Carlo et al., who showed G_1 arrest in the cell cycle responded to hypoxia[9]. This growth arrest has been shown to be associated with p27 accumulation in C2C12 myoblasts[9]. In the present study, no accumulation of HIF-1α protein was observed, corresponding well to the report by Ono et al. that HIF-1α protein was hardly detectable in the growth phase while mRNA was constantly expressed[26]. These findings suggest that growth inhibition induced by hypoxia may be independent of HIF-1α protein expression, and is probably regulated by p27 expression.

In contrast to cells growing in GM, the suppressive effect of hypoxia on cell growth was diminished in differentiating cells. Since p27 expression has been shown to be augmented in response to hypoxia even in the differentiating condition[9], it is likely that growth suppressive signals may exist in C2C12 cells differentiating in IM. How the cells maintain a proliferating capacity under hypoxic conditions is unclear at present. Interestingly, hypoxia enhances proliferation in human mesenchymal stem cells[10]. Cell expansion of neutrophil precursors was enhanced at low O_2 concentration[45]. Zhao, T. et al.[22] reported that the numbers of neurospheres in 10% O_2 and 3% O_2 were increased 2.5-fold and 1.5-fold, respectively, as compared with that in 20% O_2 (normoxia). The level of HIF-1α mRNA and protein expression in embryonic neural stem/progenitor

cells (NPCs) increased significantly during proliferation of NPCs under hypoxia. It is also well known that hypoxia enhances the proliferation of cancer cells[45,46]. HIF-1α is one of the key molecules contributing to cancer progression[46–48], making it a target molecule for cancer therapies[47,48]. Induction of HIF-1α protein is required for the growth enhancement hypoxic cells[22,46–48]. In C2C12 cells, HIF-1α protein was induced by myogenic induction in the present study, in agreement with previous reports[26,49]. These findings suggest that HIF-1α may be stabilized independently of oxygen tension during C2C12 myogenesis, and the resultant HIF-1α up-regulated expression in IM may maintain the growth of differentiating C2C12 cells under the hypoxic condition.

We investigated the differentiation responses of C2C12 cells to hypoxia using *in vitro* morphology, immunohistochemstry, and immunoblotting. Cell shape changes to large multinucleated cells and the expression of a differentiation marker, muscle myosin heavy chain, in both immunohistochemical and immunoblot analyses clearly indicated the suppression of myogenic induction by hypoxia. The hypoxia-induced suppression of myogenesis was also demonstrated by Zhang, Y. et al.who showed that C2C12 cells after 3 days in DM at 21% O_2 differentiated into myocytes, while the myogenesis was inhibited at <2% O_2, with the strongest inhibition found at 0.01% O_2[25]. Furthermore, in the present study, MyoD mRNA was reduced by hypoxia, suggesting the possible association of MyoD withdrawal with hypoxia-induced differentiation suppression. This corresponds well with the finding that the MyoD expression is reduced under hypoxia[9]. Di Carlo et al. also demonstrated that ectopic expression of MyoD resulted in the activation of the myogenic differentiation program, identifying MyoD as a relevant target of hypoxia[9]. These results confirm that hypoxia-elicited MyoD proteasome-degradation may account for a mechanism in which low oxygen levels control myogenic differentiation.

It is interesting that the protein expression of HIF-1α was not correlated to hypoxia. Rather, the results of the immunoblot analysis indicate that the protein expression is associated with myogenic differentiation in C2C12 cells. Ono et al. demonstrated HIF-1α accumulated in the nucleus of myogenin-positive myoblasts on day 1 of differentiation, even at normal oxygen concentrations, while the amount of mRNA of the protein was constant[26], corresponding to our present results. HIF-1α has an essential role in myogenic differentiation, and the regulation of protein expression is independent of oxygen concentration[25,26]. HIF-1α protein expression was tightly regulated by HSP90[26]. It should be noted that this chaperone protein is tightly up-regulated by mechanical stress[50]. Taken together, HIF-1α protein may be related to muscle differentiation in specific response to mechanical stimulation; the elucidation of the detailed mechanisms will be necessary.

Phagokinetic track motility assay results indicate that hypoxia significantly stimulated cell motility in IM, while few effects of hypoxia were observed in GM. Hypoxia-related cell motility is associated with a cancer-related motility factor, AMF[29]. AMF has been identified as a motility-stimulating cytokine produced by cancer cells in an autocrine fashion[32], and is identical to a glycolytic enzyme, GPI, implying a relationship with glycolytic metabolism and then hypoxia[34]. In fact, in pancreatic cancer, hypoxia enhances the expression of AMF in various cancer cells and also enhances the random motility of pancreatic cancer cells[29,51]. Interestingly, motility related to cancer metastasis has been shown to be associated with mRNA expression but not protein expression[36]. In the present study, expression of mRNA of AMF/GPI was augmented in response to hypoxia. Thus, AMF/PHI may play, at least in part, an important role in hypoxia-induced motility enhancement. On the other hand, the reason why motility of C2C12 cells growing in GM did not respond to hypoxia regardless of the augmented expression of hypoxia-induced AMF/PHI mRNA is uncertain. Hypoxia-induced motility is dependent on HIF-1α[29]. Probably, motility signals stimulated via AMF/GPI under hypoxia are not expressed in cells growing in GM without expression of HIF-1α.

We used 2% O_2 as a hypoxic condition in the present study. Although the concentration of oxygen in atmosphere is 21%, the level of oxygen in the tissue *in vivo* is very low[4–6]. In skeletal muscle the partial saturation of oxygen is reported to be between 1% and 10%[7,8]. Thus the hypoxic condition used here may be a possible physiological environment for myogenic cells *in vivo*. At present, it is unclear whether the hypoxic condition used in the present study reflects on the drop in intramyocellular oxygen pressure induced by exercise[2], while the phenotypic changes in growth, differentiation, and motility of C2C12 myogenic cells were obviously related to the reduction of oxygen concentration.

Limitations of the present study include the lack of investigation for cell cycle distribution, and apoptotic induction. We have shown growth suppression under hypoxia, but did not evaluate whether the growth suppression was due to suppression of cell proliferation or induction of apoptosis. The molecules contributing to these growth inhibiting mechanisms are different. A survey of the candidate molecules, such as CDKIs and Rb, will be necessary. A similar survey will be required for the expression of molecules responsible for muscle differentiation in C2C12 cells. Lastly the role of AMF in the enhancement of cell motility needs to be evaluated in detail, for example in a molecule-functional approach including inhibitors. The limitations described here are important and not easily overcome, so the evaluation approaches are works in progress, as are other projects.

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