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Original Article

Hypoxia accelerates the progression of angiosarcoma through the regulation of angiosarcoma cells and tumor microenvironment

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ABSTRACT

Background: Angiosarcoma is a rare malignant tumor with a poor prognosis. It is known that hypoxic condition activates tumor progression in several cancers. Additionally, hypoxic tumor microenvironment accelerates immune escape. However, the presence and significance of hypoxia in angiosarcoma has not been adequately investigated.
Objective: To study the role of hypoxia in the progression of angiosarcoma.
Methods: The protein level of hypoxia inducible factor-1α (HIF-1α) in angiosarcoma was examined using immunohistochemistry and immunoblotting. To study the effect of hypoxia on tumor progression, cell proliferation, migration, invasion, and tube formation assays were performed in angiosarcoma cells. The influence of tumor cell supernatant in hypoxia from angiosarcoma cells on immune escape and angiogenesis was analysed to investigate the modulatory effect of hypoxia on tumor microenvironment of angiosarcoma. The molecular mechanism related to these results was investigated using immunoblotting and real time RT-PCR.
Results: HIF-1α protein was over-expressed in angiosarcoma tissues and cell lines under hypoxic conditions, and there was heterogeneity of oxygen supply in angiosarcoma. Hypoxia enhanced the proliferation, migration, and invasion abilities and inhibited tube formation in angiosarcoma cells. Tumor cell supernatant in hypoxia from angiosarcoma cells activated the monocyte invasion ability, facilitated its differentiation into M2-like macrophages, and suppressed cell-adhesion. These *in vitro* results were compatible to the pathological findings of angiosarcoma patients.
Conclusion: Hypoxia plays a major role in progression of angiosarcoma cells by enhancing cell proliferation, migration, and invasion and by modulating the tumor microenvironment.

1. Introduction

Angiosarcoma is a malignant tumor derived from endothelial cells, and most angiosarcoma often occurs in the skin of the head and neck in elderly patients. The prognosis of angiosarcoma is poor because standard and effective therapeutic methods for metastatic angiosarcoma have not been established [1]. To resolve this problem, it is necessary to accumulate new observations of the pathogenesis in angiosarcoma.

There are several studies about the etiology of angiosarcoma. For example, overexpression of heat shock protein 90 [2] and survivin [3] accelerate tumor progression in angiosarcoma.

Abbreviations: HIF-1α, hypoxia inducible factor-1α; OH- HIF-1α, hydroxy-hypoxia inducible factor-1α; TME, tumor microenvironment; HDMEC, human dermal microvascular endothelial cells; PD-L1, programmed cell death-1 ligand-1; TCS, tumor cell supernatant; TAM, tumor-associated macrophage; ERK, extracellular signal-regulated kinase; FAK, focal adhesion kinase; MMP-2, matrix metalloproteinase-2.

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Further, the NUP160-SLC43A3 fusion gene has been shown to be responsible for angiosarcoma [4]. Moreover, PTPRB and PLCG1 mutations have been identified in angiosarcoma tissues [5]. Because tumor cells produce several cytokines, the tumor microenvironment (TME) has also attracted attention as an aggravating factor in many cancers [6]. TME consists of angiogenesis, immune suppression, and extracellular matrix remodeling [7,8]. In regards to angiosarcoma, the infiltration of immune escape-related cells, such as M2-like macrophages [9] and programmed cell death-1 ligand-1 (PD-L1) positive cells [10,11], have been detected in angiosarcoma tissues. These cells accelerate immune escape, and their expression is predictive of poor prognosis in several cancers [12,13].

The progression of tumor cells is maintained by tumor angiogenesis through the abundant supply of oxygen and nutrients [14]. Tumor angiogenesis is mainly based on hypoxia, and hypoxic conditions activate cancer progression, including cell proliferation, migration, and invasion in several malignant tumors [15]. Additionally, a hypoxic tumor microenvironment can help cancer cells to inhibit immune function [16,17]. The presence of hypoxia inducible factor (HIF)-1 is an indication of a hypoxic environment [7], and HIF-1 protein expression has been detected in angiosarcoma tissues [18,19].

However, the presence and significance of hypoxia in angiosarcoma has not been adequately investigated, and further studies are needed. Thus, the purpose of the current study was to evaluate the effect of hypoxia in the progression of angiosarcoma.

2. Materials and methods

2.1. Tissue samples

Skin samples were obtained from the involved skin of 22 patients with angiosarcoma and three with senile angioma. Control skin samples were obtained from three healthy donors. According to the Declaration of Helsinki, institutional review board approval and written informed consent were obtained before patients entered into this study.

2.2. Cell cultures

Primary human dermal microvascular endothelial cells (HDMEC) and human pericytes were obtained from Takara (Shiga, Japan). We used two human cutaneous angiosarcoma cell lines (HAMON [20] and ISO-HAS [21]). HDMEC, HAMON, and ISO-HAS were cultured in Endothelial Cell Growth Medium (Lonza, Basel, Switzerland), and pericytes were cultured in Pericyte Growth Medium (Takara) at 37 °C under 5% CO_2, 95% air condition as a well-oxygenated condition (20% O_2). Hypoxic conditions (10%, 5%, 3%, and 1% O_2) were maintained in a Multigas incubator (Panasonic, Osaka, Japan) or INVIVO$_2$ 400 hypoxia workstation (Ruskinn, Leeds, UK).

2.3. Immunoblotting

Immunoblotting was performed as described previously [22]. Each antibody is described in detail below: antibodies for hypoxia inducible factor-1α (HIF-1α), hydroxy-HIF-1α (OH-HIF-1α), phospho-extracellular signal-regulated kinase (p-ERK), ERK, and β-actin were purchased from Cell Signaling Technology (Beverly, MA), phospho-focal adhesion kinase (p-FAK), FAK, phospho-paxillin (p-paxillin), and paxillin were from Abcam (Cambridge, UK), and matrix metalloproteinase-2 (MMP-2) were from Kyowa Pharma Chemical (Toyama, Japan), respectively. To analyze the degradation rate of the HIF-1α protein, cycloheximide (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) was used to inhibit *de novo* protein synthesis.

2.4. Immunohistochemical analysis

Immunohistochemistry was performed using primary antibodies against HIF-1α and programmed cell death-1 ligand-1 (PD-L1) (Abcam), ERG (Nichirei, Japan), CD163 (Leica Biosystems, Nussloch, Germany), CD204 (CosmoBio, Tokyo, Japan), and Iba1 (FUJIFILM Wako Pure Chemical Corporation), as described previously [23]. The number of stained cells was counted under a microscope at ×400 magnification.

2.5. RNA isolation, cDNA synthesis, and quantitative real-time polymerase chain reaction (real-time PCR)

Total RNA was extracted from culture cells using ISOGEN (Nippon Gene, Tokyo, Japan). cDNA synthesis and quantitative real-time PCR were performed as described previously [24]. Primer sets for HIF-1α, GM-CSF, IL-4, IL-10, IL-13, TGF-β1, CXCL12, VEGF-A, PD-L1, angiopoetin-2, CD31, and GAPDH were purchased from Qiagen (Hilden, Germany). Expression levels of every mRNA were corrected relative to GAPDH.

2.6. Cell proliferation, migration, invasion assay, and tube formation assay

HDMEC, ISO-HAS, and HAMON cells were seeded into 24-well plate, and cells were stained with trypan blue (Sigma Aldrich, St. Louis, MO) and counted using the TC20™ Automated Cell Counter (BIORAD, Berkeley, CA) from 24 to 72 h after culture. Migration and invasion assays were performed as described previously [2]. The ability of tube formation was determined using the Endothelial cell tube formation assay (Corning, NY, US), and microscope photographs were taken under a microscope at ×100 magnification.

2.7. Measurements of lactate concentration, MMP-2 activity, and caspase-3/7 activity

Lactate concentration, MMP-2 activity, and caspase-3/7 activity were evaluated using the Lactate assay kit (Cell Biolabs, San Diego, CA), MMP-2 fluorescent activity assay kit (AnaSpec, Fremont, CA), and Caspase-Glo 3/7 kit (Promega Corporation, Madison, WI) according to the manufacturer's protocols, respectively.

2.8. Assessment of monocyte invasion and differential macrophage polarization

Monocytes from healthy donors were isolated using RosetteSep Human Monocyte Enrichment Cocktail (STEMCELL technologies, Vancouver, Canada), and an invasion assay using the tumor cell supernatant (TCS) cultured in 20% or 1% O_2 was performed. Monocytes were differentiated into macrophages with AIM-V medium (Thermo Fisher Scientific, Waltham, MA) containing 2% human serum and 1 ng/mL GM-CSF as described previously [25]. Following, macrophages were stimulated with or without the aforementioned TCS, and cells were fixed with cold acetone and reacted with anti-CD163 antibody (clone AM-3 K, Transgenic, Kumamoto, Japan) and anti-CD204 antibody (clone SRA-E5, CosmoBio, Tokyo, Japan). HRP-labeled secondary antibody (HISTOFINE, Nichirei, Tokyo, Japan) was reacted and visualized using the DAB solution (Nichirei, Tokyo, Japan).

2.9. Statistical analysis

Data presented as bar graphs are the mean ± standard deviation (SD) of at least three independent experiments. The statistical analyses were performed using the Mann–Whitney *U* test and

Wilcoxon rank-sum test for comparisons of the medians. A p-value of less than 0.05 was considered statistically significant.

3. Results

3.1. HIF-1α protein overexpressed in angiosarcoma tissues and enhanced protein expression in hypo-vascular than in rich-vascular areas

We performed immunohistochemical staining to compare the expression levels of hypoxia inducible factor-1α (HIF-1α) in angiosarcoma tissues with those in senile angioma (benign vascular tumor) or normal vessels. Immunohistochemical staining showed that HIF-1α expression was not seen in senile angioma or normal vessels, but it was strongly detected in angiosarcoma tissues (Fig. 1a). Furthermore, there was heterogeneity in HIF1-α immunostaining intensity in angiosarcoma, and HIF-1α protein was enhanced in tumor cells distant from vessels than those around the vessels (Fig. 1b).

3.2. Hypoxia increased HIF-1α protein through the clearance of hydroxy-HIF-1α (OH-HIF-1α) in both time-dependent and oxygen-concentration manners in angiosarcoma

To clarify the mechanism of intra-tumor heterogeneity of HIF-1α protein expression levels, we investigated the effects of hypoxia

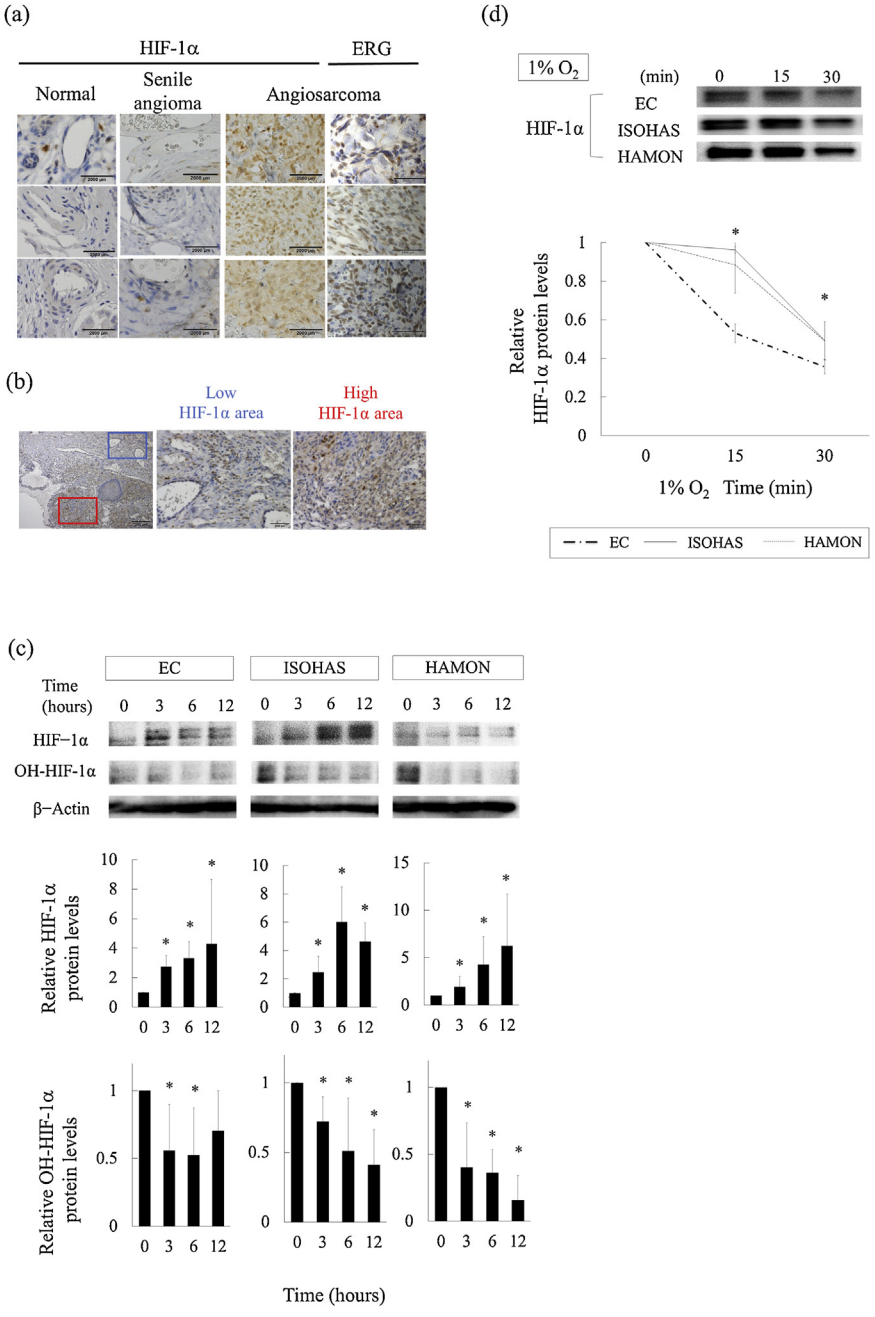

Fig. 1. Overexpression of HIF-1α in angiosarcoma induced by hypoxia.
(a) Anti-HIF-1α and anti-ERG (the positive marker of endothelial cells) immunohistochemistry in normal vessels, senile angioma, and angiosarcoma. (b) The heterogeneity of HIF-1α immunostaining in angiosarcoma. (c) The effect of hypoxia was analyzed on HIF-1α protein using immunoblotting under 1% O_2 for 0–12 h. (d) Degradation rate of the HIF-1α protein determined by immunoblotting. Data represent the mean and SD from three independent experiments. * $p < 0.05$ versus control subjects.

on the HIF-1 protein in cultured angiosarcoma cells (ISO-HAS and HAMON). Overexpression of the HIF-1α protein is generally maintained by HIF-1α hydroxylation in hypoxic conditions [26,27]. In normoxic conditions (20% O_2), there was no significant difference of HIF-1α and OH-HIF-1α protein expression levels between angiosarcoma cells and human dermal microvascular endothelial cells (HDMEC) (Supplementary Fig. 1a). In several O_2 conditions (20%, 10%, 5%, 3%, and 1% O_2), HIF-1α protein expression levels were significantly increased whereas OH-HIF-1α protein expression levels were significantly decreased, correlating to the decreased oxygen concentration in ISO-HAS and HAMON. In HDMEC, hypoxic condition induced the overexpression of HIF-1α and OH-HIF-1α protein (Supplementary Fig. 1b).

Additionally, we examined time dependency of HIF-1α and OH-HIF-1α protein expression levels under hypoxic conditions (1% O_2). HIF-1α protein expression levels were significantly increased, and OH-HIF-1α protein expression levels were significantly decreased in a time-dependent manner in HDMEC, ISO-HAS, and HAMON (Fig. 1c). Following, we investigated relative HIF-1α mRNA levels in hypoxic conditions. HIF-1α mRNA expression levels in the hypoxic condition were unexpectedly decreased in HDMEC, ISO-HAS, and HAMON (Supplementary Fig. 1c). Additionally, *de novo* protein synthesis was blocked with cycloheximide under 1% oxygen tension, and we analyzed the degradation ability of the HIF-1α protein. The rate of degradation of the HIF-1α protein was significantly decreased in angiosarcoma cells compared to HDMEC (Fig. 1d). Based on these results, overexpression of HIF-1α protein may rely on protein degradation more than mRNA production.

3.3. Hypoxia enhanced proliferation, migration, and invasion ability and inhibited tube formation in angiosarcoma cells

To assess the effect of hypoxia on the function of angiosarcoma cells, we studied proliferation, migration, invasion, and tube formation under normoxic or hypoxic conditions. The proliferation of HDMEC and angiosarcoma cells was significantly increased in hypoxic conditions than normoxic conditions (Fig. 2a). Further, hypoxia significantly enhanced both migration (Fig. 2b) and invasion (Supplementary Fig. 2a) in HDMEC, ISO-HAS, and HAMON. Moreover, the tube formation assay showed that the tube formation

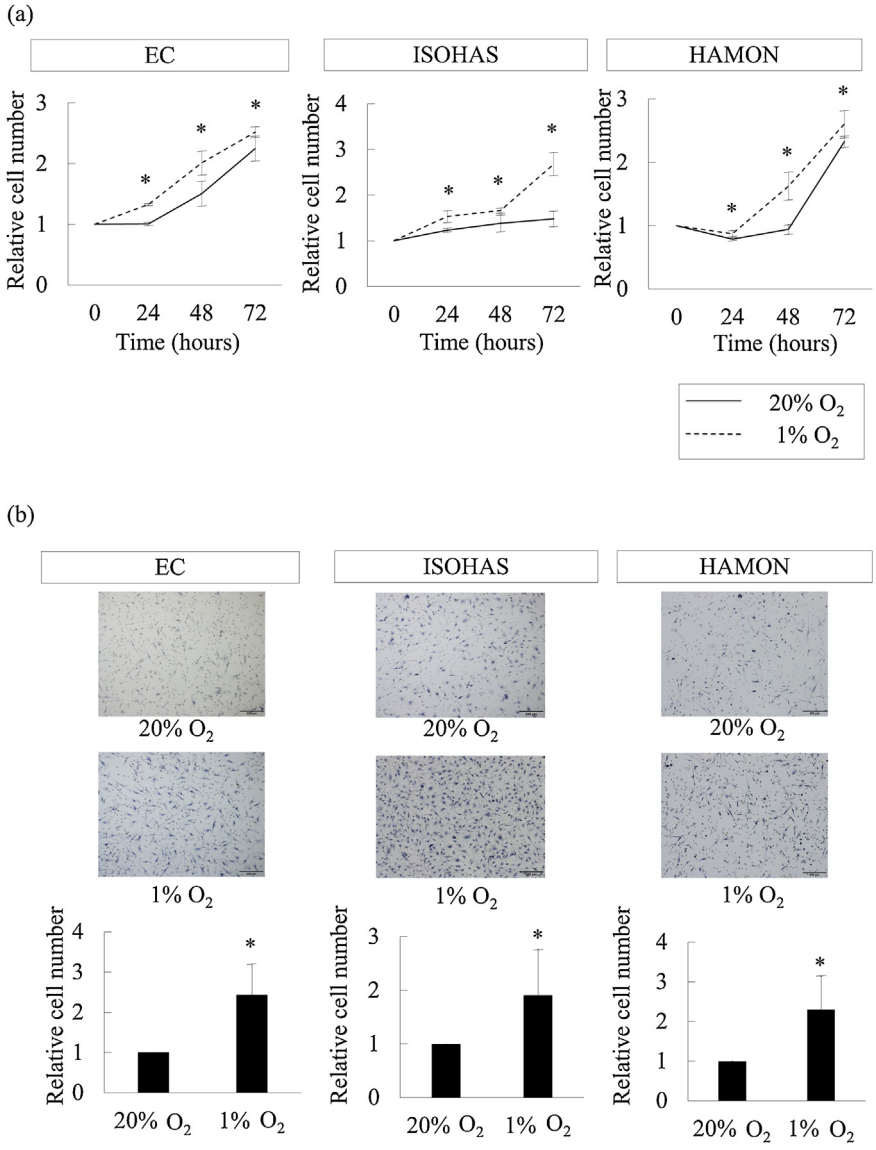

Fig. 2. Hypoxia enhances proliferation/migration in angiosarcoma cells.
(a) The relative cell numbers of HDMEC, ISO-HAS, and HAMON from 24 to 72 h under 20% or 1% O_2 conditions. (b) Migration assay were performed under 20% and 1% O_2 conditions. Data represent the mean and SD from three independent experiments. * $p < 0.05$ versus control subjects.

ability in HDMEC, ISO-HAS, and HAMON was inhibited under hypoxic conditions (Supplementary Fig. 2b). These results suggest that hypoxia accelerate the proliferation, migration, invasion and tube-formation of angiosarcoma as well as normal ECs.

3.4. Intra-tumor heterogeneity of tumor microenvironment in angiosarcoma patients

To evaluate the tumor microenvironment (TME) in angiosarcoma, we performed immunohistochemical staining in angiosarcoma tissues of five cases. CD163 and CD204 (both markers of M2-like macrophages), Iba-1 (total M1 and M2-like macrophages), and PD-L1 were detected in all angiosarcoma tissues (Fig. 3a: a representative case). The number of CD163 (Fig. 3b) and CD204 (Fig. 3c) positive cells and the rate of CD163/Iba-1 (Supplementary Fig. 3a) and CD204/Iba-1 (Supplementary Fig. 3b) in hypoxic areas were significantly increased compared to normoxic areas. In addition, PD-L1 was strongly detected in hypoxic areas. These results indicated that the immune escape might be formed by accumulation of tumor-associated macrophages (TAM) and PD-L1 positive cells affected by the hypoxic environment. Additionally, the vascular architecture was broken down (Fig. 3a) in the HIF-1α high area compared with the HIF-1α low area. Taken together, there may be an intra-tumor heterogeneity of TME regulated by hypoxia in angiosarcoma. Furthermore, we identified the presence of HIF-1α strongly positive cells in high HIF-1α areas of angiosarcoma tissues (Supplementary Fig. 3c) and assessed the relationship between the presence of HIF-1α strongly positive cells and clinical manifestations (sex, age, overall survival period, metastasis of lymph node/organ) (Supplementary Table 1). There was no correlation in sex or overall survival period. Angiosarcoma patients with HIF-1α strongly positive cells were significantly younger and had more lymph node/organ metastasis compared to those without.

3.5. Tumor cell supernatant (TCS) in hypoxic conditions promoted monocyte invasion and monocyte differentiation into M2-like macrophages and inhibited pericyte and HDMEC proliferation and tube formation ability in HDMEC

To investigate whether hypoxic conditions affected TME in angiosarcoma cells, we examined the effect of TCS obtained in normoxic and hypoxic conditions on macrophages, pericytes and HDMEC. TCS in hypoxia significantly enhanced the invasion ability of monocytes compared to normoxia (Fig. 4a). Further, we evaluated the differentiation potency of monocytes into M2-like macrophages under hypoxic conditions, and TCS in hypoxia significantly facilitated the differentiation into CD163-positive macrophages. TCS in hypoxia did not affect CD204 expression on cultured macrophages (Fig. 4b). In addition, TCS in hypoxia significantly inhibited the proliferation of pericytes (Fig. 4c), HDMEC (Fig. 4d), and tube formation ability in HDMEC (Supplementary Fig. 4) compared to TCS in control medium and normoxia. Additionally, we studied the effect of cell supernatant (CS) from HDMEC in hypoxic conditions. CS in hypoxia induced similar results to TCS from angiosarcoma cells although it did not affect the differentiation into CD163-positive macrophages.

3.6. Regulatory mechanism of angiosarcoma cells induced by hypoxia

We sought to clarify the mechanism of angiosarcoma progression induced by hypoxia. Under hypoxic conditions, phosphorylation of extracellular signal-regulated kinase (ERK) proteins was upregulated (Fig. 5a), and caspase-3/7 activity was suppressed (Fig. 5b), which indicated that hypoxia might enhance the proliferation of tumor cells through these pathways. Furthermore, the relative focal adhesion kinase (FAK) (Fig. 5c) and paxillin (Fig. 5d) protein expression levels were significantly increased

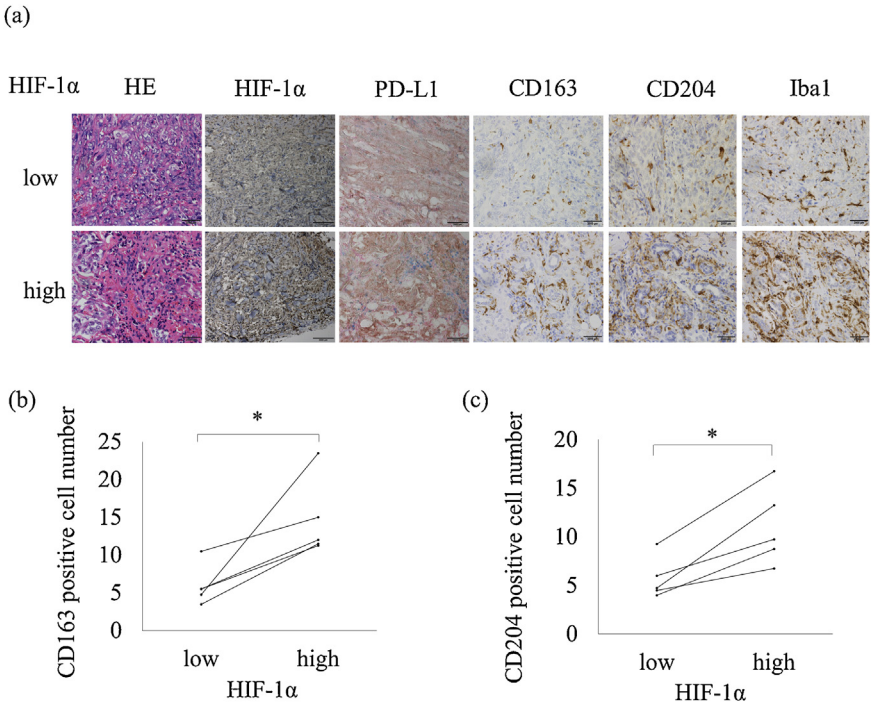

Fig. 3. Intratumor heterogeneity of tumor microenvironment in angiosarcoma patients.
(a) Immunohistochemistry with hematoxylin and eosin (HE), anti-HIF-1α, anti-CD163, anti-CD204, anti-Iba1, and anti-programmed cell death-1 ligand-1 (PD-L1) antibodies in angiosarcoma tissues, which were divided into HIF-1α high (hypoxic) and low (normoxic) areas (a representative case). (b–c) The comparison of CD163 (b) and CD204 (c) positive cell number in five angiosarcoma patients under low or high HIF-1α areas. Statistical analysis comparing five patients using the Wilcoxon rank-sum test. *$p < 0.05$ as compared with values in the low HIF-1α area.

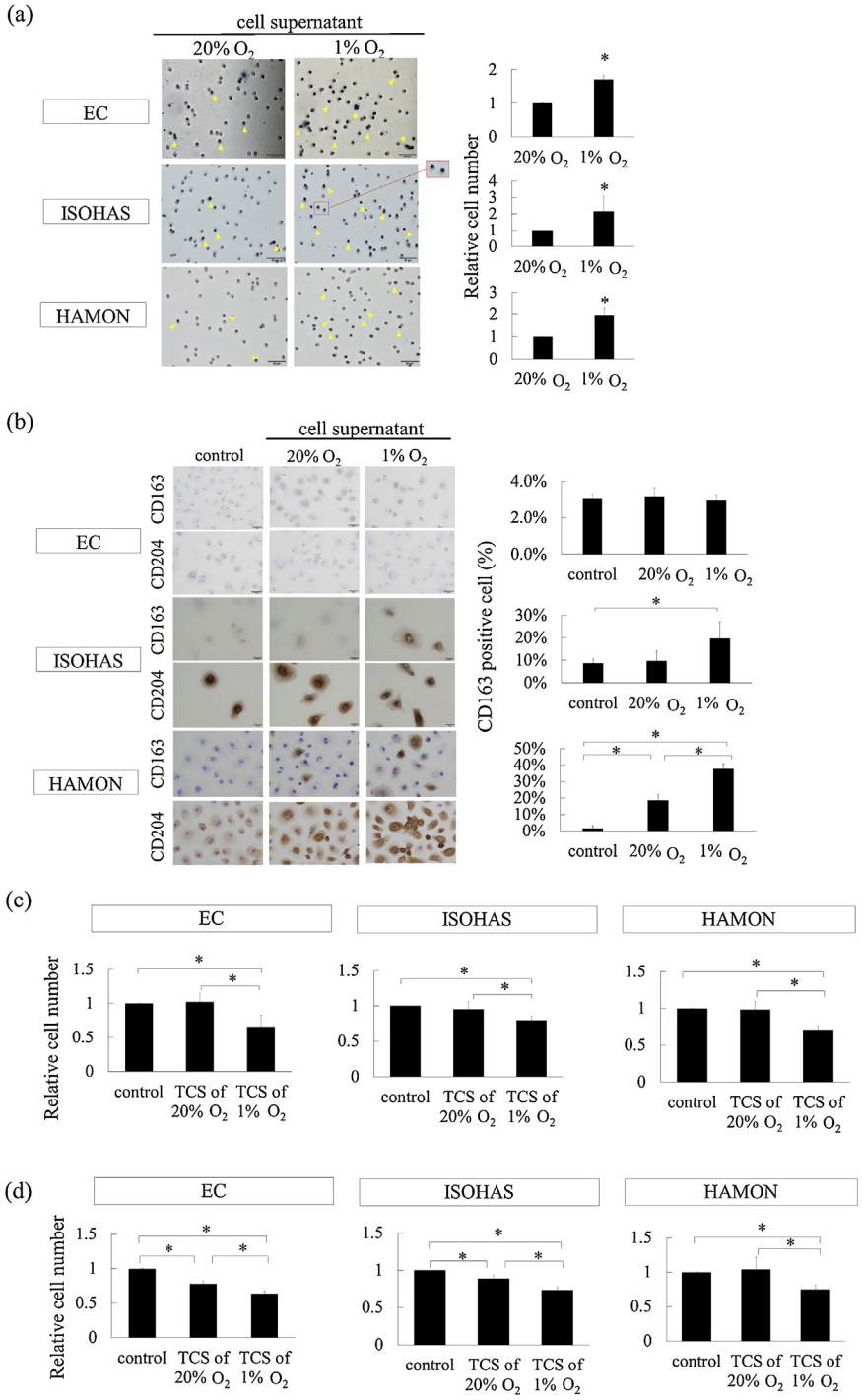

Fig. 4. The effect of tumor cell supernatant (CS) on monocyte invasion, macrophage functional polarization, pericyte/HDMEC proliferation.
(a) Monocyte invasion assay with CS from HDMEC, ISO-HAS and HAMON in normoxia or hypoxia. (b) Immature human macrophages were cultured, and cells were treated with 20% and 1% O_2 CS from HDMEC, ISO-HAS and HAMON for 2 days and stained with anti-CD163 or anti-CD204 antibodies. The relative cell number of pericytes (c) and HDMEC (d) after 48 h with CS from HDMEC, ISO-HAS and HAMON in normoxia or hypoxia. Data represent the mean and SD from three independent experiments. * $p < 0.05$ versus control subjects.

under hypoxic conditions. Thus, the activation of migration was due to hypoxia-induced increased phosphorylation of these factors. Furthermore, TCS from angiosarcoma in hypoxia significantly increased the activity of matrix metalloproteinase-2 (MMP-2) compared to normoxia, although relative MMP-2 protein levels were similar between normoxic and hypoxic conditions (Fig. 5e). These results indicate that hypoxic TCS contains soluble factors that promote invasive properties of cells of endothelial origin.

3.7. Regulatory mechanism of tumor microenvironment in angiosarcoma elicited by hypoxia

To reveal that hypoxic conditions affect TME in angiosarcoma, we investigated several mRNA expression levels associated with TME, such as immune escape and angiogenesis. Under hypoxic conditions, relative mRNA levels of GM-CSF, IL-4, IL-10, IL-13, TGF-β1, CXCL12, PD-L1 (immune escape-related cytokines), VEGF-A

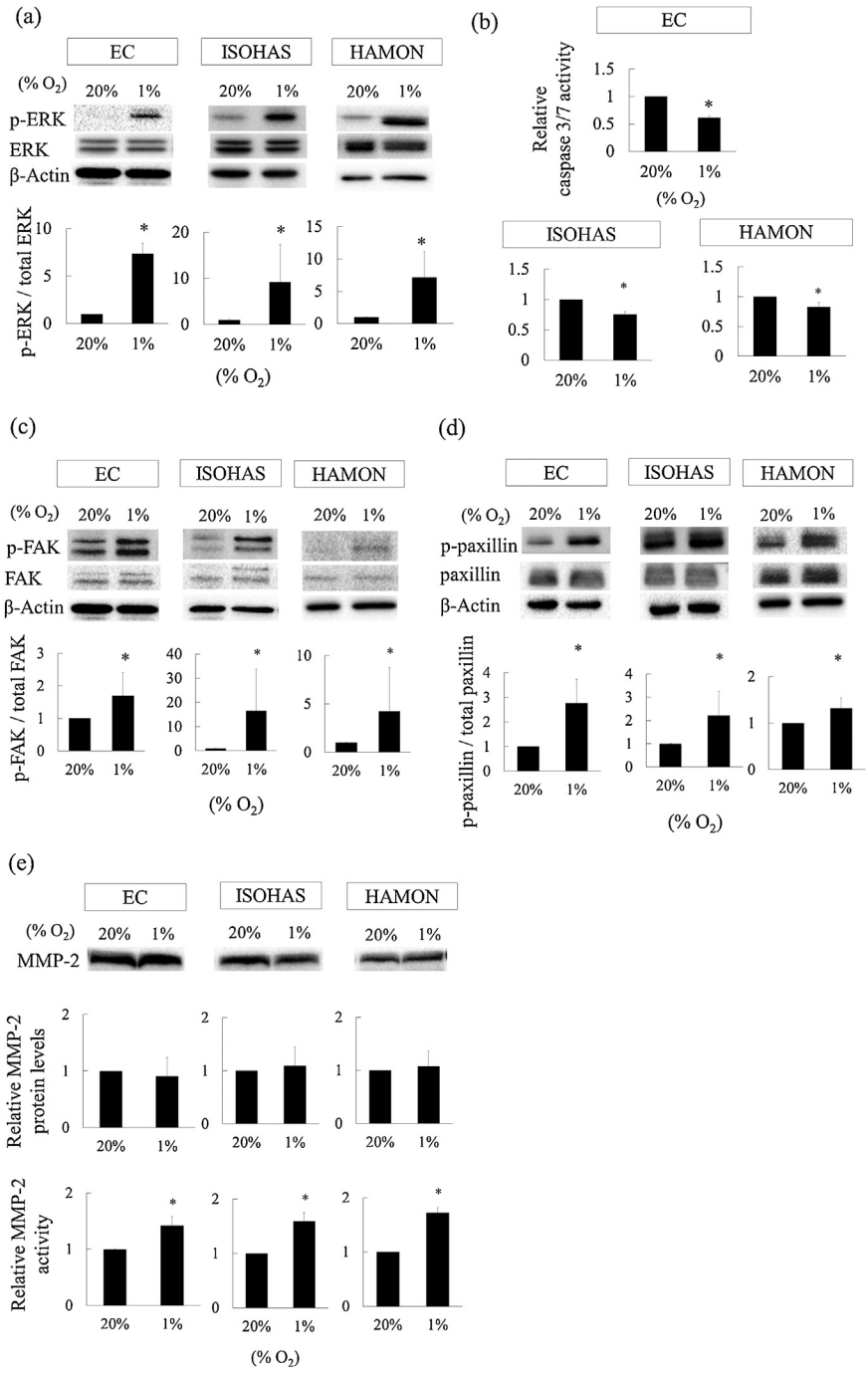

Fig. 5. Hypoxia regulation for several molecules associated with angiosarcoma cell progression.
(a, c–e) Immunoblotting of phosphorylated extracellular signal-regulated kinase (ERK), total ERK (a), phosphorylated focal adhesion kinase (FAK), total FAK (c), phosphorylated paxillin, total paxillin (d), extracellular matrix metalloproteinase-2 (MMP-2) (e) in HDMEC, ISO-HAS, and HAMON under 20% and 1% O_2. (b) Caspase-3/7 activity. (e) MMP-2 activity. Data are expressed as the mean ± SD of three independent experiments. $^*p < 0.05$ as compared with values in the 20% O_2 condition (1.0).

(angiogenesis factor), and angiopoietin-2 (function in pericyte detachment from the vascular wall) were significantly increased, although CD31 (endothelial cell adhesion molecule) was decreased in ISO-HAS and HAMON (Fig. 6a). In addition, TCS from angiosarcoma in hypoxia significantly enhanced lactate compared with TCS in normoxia (Fig. 6b).

4. Discussion

The behavior of malignant tumor cells under hypoxic conditions is generally exacerbated compared to normoxic conditions, and hypoxia affects not only cancer cells but also TME [28]. There have been no detailed investigations regarding the significance of hypoxic conditions in angiosarcoma cells. In this study, we identified three major findings of hypoxia in angiosarcoma: (1) the presence of hypoxic lesions in angiosarcoma tissues, (2) the effect of hypoxia on both angiosarcoma cells and the microenvironment, and (3) the molecular mechanism of angiosarcoma progression induced by hypoxia. Fig. 6c shows a hypothetic model about the effect of hypoxia in angiosarcoma.

The decreased rate of HIF-1α degradation has been shown to accumulate HIF-1α protein under hypoxic conditions [29,30],

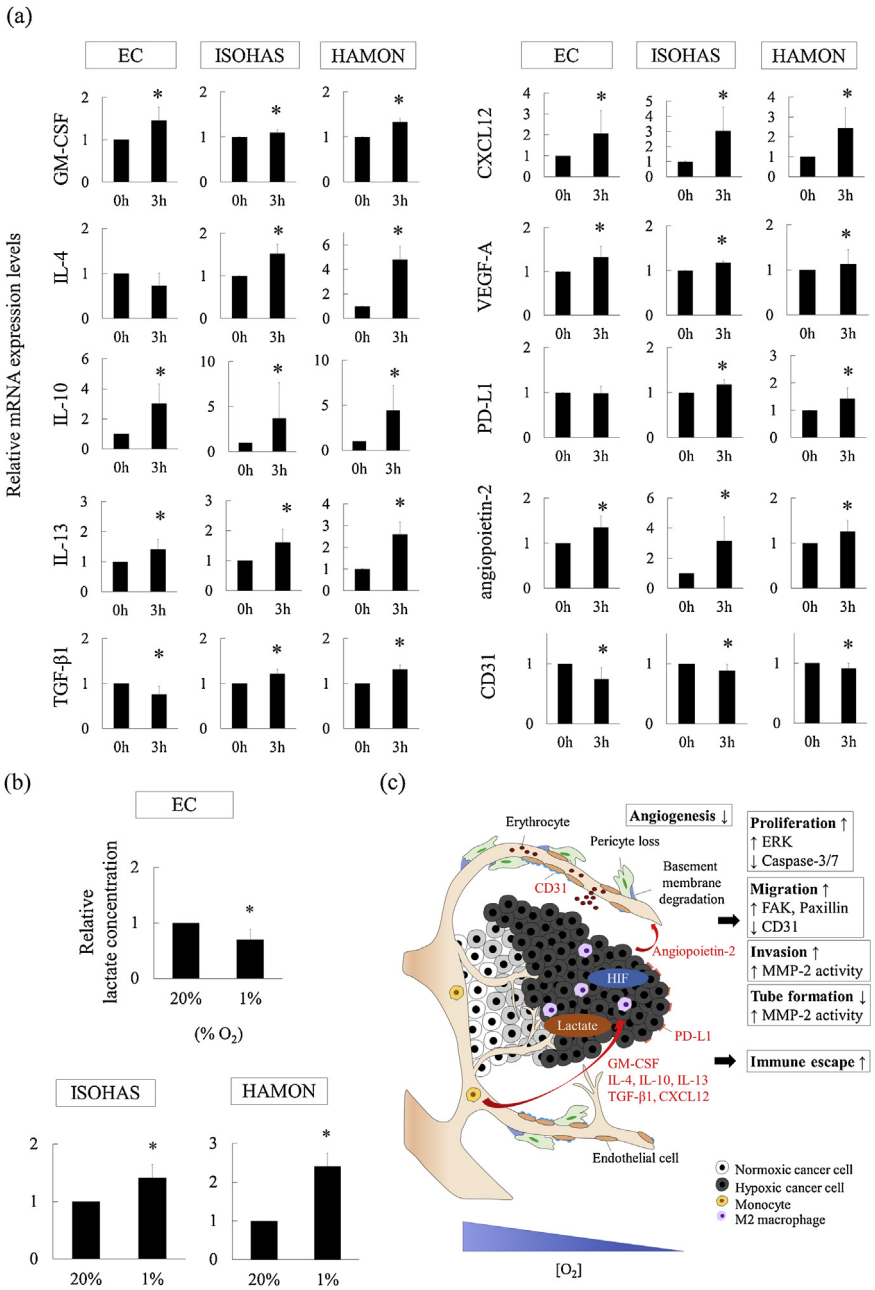

Fig. 6. Hypoxia regulation for several molecules associated with the tumor microenvironment.
(a) Relative mRNA levels of GM-CSF, IL-4, IL-10, IL-13, TGF-β1, CXCL12, VEGF-A, PD-L1, angiopoetin-2, and CD31 for 3 h under 1% O_2 in HDMEC, ISO-HAS, and HAMON. (b) The relative concentration of lactate in 20% and 1% O_2. Data are expressed as the mean ± SD of three independent experiments. *$p < 0.05$ as compared with values in the 20% O_2 condition (1.0). (c) A hypothetical model of hypoxia effects in angiosarcoma. Hypoxia accelerates the progression of angiosarcoma cells and its tumor microenvironment through the regulation of several molecules.

although HIF-1α protein is degraded through protein hydroxylation in normoxic conditions [31–33]. Enhanced HIF-1α protein is detected in many human cancers [34,35]. Because the origin of angiosarcoma is EC, we wondered whether the abundant supply of oxygen is maintained in angiosarcoma. Our results showed that increased HIF-1α protein was detected in angiosarcoma (Fig. 1a), and there was heterogeneity in the HIF-1α immunostaining intensity. Angiosarcoma cells distant from vessels had enhanced over-expression of the HIF-1α protein relative to those around vessels (Fig. 1b). Further, hypoxia increased HIF-1α protein in cultured angiosarcoma cells, although there was no significant difference in HIF-1α protein expression levels between HDMEC and angiosarcoma cells in normoxic conditions (Fig. 1c, Supplementary Fig. 1a–b). Based on these results, we concluded that HIF-1α protein is a cardinal protein essential for hypoxia in endothelial-lineage cells.

Next, our study demonstrated that hypoxia enhanced proliferation, migration, and invasion ability and inhibited the tube-forming ability of angiosarcoma cells (Fig. 2, Supplementary Fig. 2). Hypoxic conditions activated the ERK pathway (Fig. 5a), suppressed the caspase pathway (Fig. 5b), enhanced the phosphorylation of FAK and paxillin (Fig. 5c–d), decreased CD31 expression (Fig. 6a), and upregulated MMP-2 activity (Fig. 5e). Based on these findings, we speculated that hypoxia may activate angiosarcoma

cell progression by modulating several molecules, although further investigations (using each specific inhibitor) are needed.

Additionally, we revealed that the effect and regulatory mechanism of hypoxic TME in angiosarcoma cells. As shown in Fig. 4a–b, TCS in hypoxia activated the monocyte invasion ability and facilitated monocyte differentiation into M2-like (CD163-positive) macrophages compared to normoxia. Additionally, expression levels of GM-CSF, IL-4, IL-10, IL-13, TGF-β1, CXCL12, and lactate were significantly elevated in hypoxic conditions (Fig. 6a–b). These cytokines and lactate enhanced the accumulation of monocytes and the functional polarization of M2-like macrophages [36–39]. M2-like macrophages also expressed PD-L1 and PD-L2 via Stat3 activation [25] and lactate-induced M2 polarization is mediated by Stat3 signals [40], and these suggest that PD-L1 positive non-tumor cells in TME are M2-like macrophages. Furthermore, angiosarcoma cells in hypoxic conditions overexpressed PD-L1 compared to those in normoxic conditions (Fig. 6a). These findings suggested that the hypoxic tumor environment worsened immune escape of angiosarcoma.

Moreover, hypoxia augments angiopoietin-2 production, which results in abnormal angiogenesis in the presence of VEGF-A. Increased angiopoietin-2 production results in vascular destabilization [41–43]. In this study, hypoxic conditions accelerated production of VEGF-A and angiopoietin-2 compared to normoxic conditions (Fig. 6a). As shown in Fig. 4c–d and Supplementary Fig. 4, hypoxia suppressed the proliferation of pericytes/ HDMEC and the tube formation ability of HDMEC. The adhesion of each angiosarcoma cell was inhibited as stated previously (Supplementary Fig. 2b), and tumor angiogenesis was also unstable in the hypoxic environment of angiosarcoma. These results indicated that vascular structures in the hypoxic condition showed abnormal angiogenesis (the deterioration of luminal structure and the leakage of erythrocytes) through VEGF-A and angiopoietin-2 overexpression.

We identified the proliferation of angiosarcoma cells, the breakdown of vascular architecture, and the accumulation of M2-like macrophage and PD-L1-positive cells in hypoxic lesions of angiosarcoma tissues (Figs. 1b, 3 and Supplementary Fig. 3a–b). These clinical findings in angiosarcoma patients were compatible with our *in vitro* studies. Furthermore, the presence of HIF-1α strongly positive cells in hypoxic lesions correlated with metastasis of the lymph node and organ (Supplementary Fig. 3c, Supplementary Table 1). Based on these experimental data using both clinical samples and cultured angiosarcoma cells, we found the clinical importance of hypoxia in angiosarcoma.

There was a limitation in our study because we did not obtain data in mouse models, although we investigated the clinical significance of hypoxia in angiosarcoma patients. In conclusion, we revealed that the hypoxic condition plays a major role in the progression of angiosarcoma through the regulation of angiosarcoma cells and TME. Further studies should be necessary to clarify the pathogenesis of angiosarcoma.

Disclosure

The authors have no conflict of interest to declare.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jdermsci.2019.01.005.

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