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## Original Paper

# Hepatic Artery Embolization Induces the Local Overexpression of Transforming Growth Factor $\beta$ 1 in a Rat Hepatoma Model

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## Keywords

Hepatic arterial embolization · Hepatoma · Hypoxia-inducible factor 1 $\alpha$  · Hypoxia · Transforming growth factor  $\beta$ 1

## Abstract

**Introduction:** The underlying mechanism involved in the recurrence of hepatoma after hepatic arterial embolization (HAE) is not adequately examined. An immunosuppressive cytokine, transforming growth factor  $\beta$ 1 (TGF- $\beta$ 1), can lead to tumor progression and is affected by hypoxia in various cancers. The study aimed to assess the effect of HAE on the expression of TGF- $\beta$ 1 in a rat hepatoma model. **Methods:** Sprague-Dawley rats bearing N1S1 hepatoma cells underwent HAE (HAE group,  $n = 5$ ) or sham treatment (sham group,  $n = 4$ ). The animals were euthanized at 48 h, and liver tissues were harvested. Immunohistochemistry (IHC) and quantitative polymerase chain reaction (qPCR) were performed to compare the expression of TGF- $\beta$ 1 and hypoxia-inducible factor 1 $\alpha$  (HIF-1 $\alpha$ ) between the HAE and sham groups. In vitro experiments with the N1S1 cell line were also performed under normoxic (21% O<sub>2</sub>) or hypoxic (1% O<sub>2</sub>) conditions for 48 h, and the expression of TGF- $\beta$ 1 and HIF-1 $\alpha$  was assessed with western blotting and enzyme-linked immunosorbent assay. Statistical data comparisons were performed by Student *t* test. **Results:** IHC showed that both the TGF- $\beta$ 1-positive and HIF-1 $\alpha$ -positive tumor peripheral areas were larger in the HAE group ( $6.59 \pm 2.49$  and  $10.26 \pm 4.14\%$ ;  $p < 0.001$ , respectively) than in the sham group ( $0.34 \pm 0.41$  and  $0.40 \pm 0.84\%$  respectively). Similarly, qPCR showed that the mRNA expression levels of TGF- $\beta$ 1 and HIF-1 $\alpha$  were higher ( $1.95 \pm 0.38$ -fold and  $1.62 \pm 0.37$ -fold;  $p < 0.001$  and  $p = 0.002$ , respectively) in the HAE group

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than those in the sham group. TGF- $\beta$ 1 expression was suppressed when HIF-1 $\alpha$  inhibitors were added ( $p = 0.001$ ), and HIF-1 $\alpha$  expression was upregulated when exogenous TGF- $\beta$ 1 was added ( $p = 0.033$ ) in N1S1 cells. **Conclusion:** HAE enhanced local TGF- $\beta$ 1 expression in a rat hepatoma model. In vitro experiments suggest that HAE-induced hypoxic stress may trigger the interdependent expression of TGF- $\beta$ 1 and HIF-1 $\alpha$ .

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## Introduction

Hepatoma is the second leading cause of cancer-related death globally [1]. Hepatic arterial embolization (HAE) plays a central role in the management of intermediate stage hepatoma [2, 3]. As hepatomas are predominantly supplied by the hepatic artery, the blockage of their arterial supply by HAE can induce tumor hypoxia and necrosis. However, even if a complete response is achieved, >40% of patients develop recurrence at 1 year after HAE, and the prognosis of these patients is unfavorable [4]. Therefore, identifying the underlying mechanism involved in recurrence after HAE is important to establish a more effective therapeutic strategy and improve patient survival.

Recently, several studies reported that the overexpression of transforming growth factor  $\beta$ 1 (TGF- $\beta$ 1) is an independent risk factor of poor prognosis in patients with hepatoma [5–7]. TGF- $\beta$ 1 is a cytokine that is strongly associated with cell growth and proliferation [8]. Moreover, TGF- $\beta$ 1 also functions as an immunosuppressive cytokine in the cancer microenvironment and drives immune evasion, leading to resistance to anti-cancer treatment [9–11]. The expression of TGF- $\beta$ 1 is affected by various stimulations. Recent studies have shown that hypoxic stress may induce the overexpression of TGF- $\beta$ 1 [12]. Based on previous findings, we hypothesized that hypoxic stress induced by HAE may trigger the overexpression of TGF- $\beta$ 1 in the tumor microenvironment. The study aimed to assess the effect of HAE on the expression of TGF- $\beta$ 1 in a rat hepatoma model.

## Materials and Methods

### *Tumor Cell Line*

The N1S1 rat hepatoma cell lines were obtained from the manufacture (ATCC, Manassas, VA, USA) and used to in vivo and in vitro in this study. These N1S1 cells were cultured in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10% fetal bovine serum (FBS) at 37 °C, 5% CO<sub>2</sub> in a humidified incubator.

### *Rat Hepatoma Model*

Sprague-Dawley rats (SLC, Hamamatsu, Japan) were used in this study. Animals were housed in cages with food and water available ad libitum. The room was maintained with 12-h light/dark cycle and kept at 25 °C. All animal experiments were approved by the Institutional Animal Care and Use Committee, and were conducted according to the Fundamental Guidelines for Proper Conduct of Animal Experiments and Related Activities in Academic Research Institutions in Japan. Surgical procedures in this study were performed under the anesthesia with an intraperitoneal injection of 0.3 mg/kg of medetomidine (Domitol; Nippon Zenyaku Kogyo Co., Ltd., Fukushima, Japan), 4.0 mg/kg of midazolam (Dormicum injection; Astellas Pharma Inc., Tokyo, Japan), and 5.0 mg/kg of butorphanol (Butorphanol Tartrate; FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan).

Rat orthotopic hepatoma model was established under the minilaparotomy. After anesthesia, the Sprague-Dawley rats were placed in a supine position. The abdominal skin incision was made and the left lobe of the liver was exposed. Then, a total of  $2.0\text{--}2.5 \times 10^6$  N1S1 cells in 50  $\mu$ L of phosphate-buffered saline were injected under the liver capsule by using a 26-gauge needle. After tumor cell injection, the injection site

was manually compressed for 5–10 min to avoid the reflux of injected cells. Then, the skin was closed by sutures and the rats were allowed to recover. The growth of the implanted tumors was monitored by ultrasonography.

#### *Hepatic Artery Embolization*

When the implanted liver tumor became >1 cm, rats were assigned to receive HAE (group,  $n = 5$ ) or sham treatment (sham group,  $n = 4$ ). After anesthesia, the left neck was dissected through a 2-cm-long skin incision and the left common carotid artery was isolated carefully from the vagus nerve. Then, the left common carotid artery was cannulated by a 20-gauge intravenous catheter (Angiocath; BD Biosciences, San Jose, CA, USA), and a 1.6-Fr custom made 40-cm long microcatheter (Carnelian Marvel; Tokai Medical Products, Aichi, Japan) with a 0.014-inch guidewire (Transcend; Boston Scientific, Marlborough, MA, USA) was inserted through the intravenous catheter. The microcatheter was advanced in to the proper hepatic artery under the fluoroscopic guidance. Then, HAE was performed with 75  $\mu$ m microsphere (Embozene TANDEM; Merit Medical Systems, South Jordan, UT, USA) diluted by contrast agent (Iopamiron 370, Bayer Yakuhin, Osaka, Japan). Embolization was ceased when the blood flow of proper hepatic artery became to-and-fro. Sham procedure was performed in the same way of inserting catheter without embolization. After embolization, the intravenous catheter was removed, the left common carotid artery was ligated, and the neck incision was closed by an uninterrupted suture.

Animals were euthanized at 2 days after HAE or sham treatment. Implanted tumors with surrounding liver tissues were harvested for the histopathological and the quantitative polymerase chain reaction (qPCR) analyses. For the enzyme-linked immunosorbent assay (ELISA), blood samples were collected from inferior vena cava and the blood serums were cryopreserved until the time of assay.

#### *Histopathological Analysis*

The harvested liver tissues were fixed in 4% paraformaldehyde for TGF- $\beta$ 1 staining and in Bouin solution for hypoxia-inducible factor 1 $\alpha$  (HIF-1 $\alpha$ ) staining. After dehydration by ethanol, tissues were embedded in paraffin, sectioned at 5  $\mu$ m thickness, and mounted on the microscope slides. One section was stained with hematoxylin and eosin and the contiguous sections were immunofluorescently stained as following; the sections were heated at 120 °C for 10 min to facilitate antigen retrieval. After deparaffinization and rehydration, slides were incubated with 1% hydrogen peroxide solution. After washing and blocking by skim milk, the slides were incubated with the primary antibodies overnight at 4 °C. The rabbit polyclonal antibodies to TGF- $\beta$ 1 (250576; ABBIOTEC, San Diego, CA, USA) diluted at 1:200 and HIF-1 $\alpha$  (GTX127309; GeneTex, Irvine, CA, USA) diluted at 1:100 were used as primary antibodies. After rinses in buffer, the slides were incubated with the biotinylated secondary antibody (Ultra-Sensitive ABC Peroxidase Rabbit IgG Staining Kit; Thermo Fisher Scientific K.K., Tokyo, Japan). Tissue staining was visualized with a DAB substrate chromogen solution (Thermo Fisher Scientific K.K., Tokyo, Japan). Then, slides were counterstained with hematoxylin, dehydrated, and sealed under a coverslip. Mouse liver samples that are known to express HIF-1 $\alpha$  were used as a positive control.

The immunohistochemistry-stained slides were scanned with a microscope (BX53M; Olympus, Tokyo, Japan) at high resolution, and accumulative 10 regions (5 regions from peripheral and intratumoral areas respectively) on each slide were acquired at high magnification ( $\times 40$ ). The TGF- $\beta$ 1 and HIF-1 $\alpha$  positive area were detected by Image J software (National Institutes of Health, Bethesda, MD, USA, <http://rsb.info.nih.gov/ij/>). The software was used to perform the quantification using the built-in “Color Deconvolution” tool to extract brown color channels [13]. Quantifications of TGF- $\beta$ 1 and HIF-1 $\alpha$  expressions were performed by calculating the positive proportion (%) divided tissue staining positive area by the total area in each sample.

#### *mRNA Extraction and Quantitative Real-Time PCR*

The tumors of the harvested liver tissues were stored in the RNA stabilization solution (RNA save; Biological Industries, Cromwell, CT, USA). qPCR was performed to determine the changes in mRNA expressions of TGF- $\beta$ 1 and HIF-1 $\alpha$  in the rat livers after embolization. In addition, the mRNA expressions of  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) and collagen type-1 $\alpha$ -1 chain were also evaluated because  $\alpha$ -SMA positive cells are activated by TGF- $\beta$ 1 and promote the collagen deposition [14]. Extraction of total RNA was carried out by using a NucleoSpin RNA kit including DNase (TAKARA BIO, Shiga, Japan) in accordance with the manufacturer's instructions. cDNA was synthesized using the PrimeScript First Strand cDNA Synthesis Kit (TAKARA BIO, Shiga, Japan). Quantitative real-time PCR was performed using 2.0 ng/ $\mu$ L cDNA, SYBR Premix Ex Taq II (Takara Bio, Japan) and 0.4  $\mu$ M primers with the QuantStudio™ 12K Flex Real-Time PCR System (Thermo

Fisher Scientific). The reactions were incubated at 95 °C for 30 s followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s, with a final dissociation curve at 95 °C for 15 s, 60 °C for 60 s and 95 °C for 15 s. Dissociation curve analysis of amplified products was performed at the end of each PCR reaction to confirm that only one PCR product was amplified and detected.

The specific forward and reverse primer pairs for TGF- $\beta$ 1 (5'-caacaattcctggcgcttacct-3' and 5'-aaagccctgattccgtctcc-3'), HIF-1 $\alpha$  (5'-ctccatacaaggcagcagaaa-3' and 5'-caaaacaaccaacagaaacgaaac-3'),  $\alpha$ -SMA (5'-ggatcagcgccttcagtct-3' and 5'-gggctagaagggttagcacat-3'), collagen type-1 $\alpha$ -1 chain (5'-cagtcgattcacctacagcagc-3' and 5'-gggatggagggtttacacg-3'), and  $\beta$ -actin (5'-cactttctacaatgagctgcg-3' and 5'-ctggatggctacgtacatgg-3') were designed for rat sample respectively. Gene expression was normalized by  $\beta$ -actin as an internal control.

#### Cell Culture

$1 \times 10^6$  N1S1 cells were incubated at 10 cm dish with 5 mL IMDM medium including 10% FBS. After overnight incubation in the normoxic condition (21% O<sub>2</sub>), IMDM medium including 10% FBS was replaced. Then, cells were incubated under the normoxic or hypoxic (1% O<sub>2</sub>) condition for up to 48 h.

As a positive control, the N1S1 cells added with CoCl<sub>2</sub> (Nacalai Tesque, Kyoto, Japan), a chemical inducer of HIF-1 $\alpha$ , with concentration of 100  $\mu$ mol/L were used [15]. In order to evaluate the effect of HIF-1 $\alpha$  on TGF- $\beta$ 1 expression, LW6 (20  $\mu$ mol/L; Merck Millipore, Massachusetts, MA, USA) and FM19G11 (300 nmol/L; Sigma-Aldrich, St. Louis, MO, USA), HIF-1 $\alpha$  inhibitors that block different parts of HIF-1 $\alpha$  pathway, were used [16, 17]. Similarly, in order to evaluate the effect of TGF- $\beta$ 1 on HIF-1 $\alpha$  expression, recombinant TGF- $\beta$ 1 (Peprotech, Texas, TX, USA) with concentration of 5 mg/mL in IMDM medium without FBS was used [18].

#### Western Blotting

Immunoblot analysis was performed to quantify the protein levels of TGF- $\beta$ 1 and HIF-1 $\alpha$  in rat hepatoma cells (N1S1). Cell lysates were prepared using a radioimmunoprecipitation assay buffer (RIPA Buffer [10 $\times$ ]; Nacalai Tesque, Kyoto, Japan) supplemented with a phosphatase inhibitor. Protein concentration was quantified by using a protein quantification kit (Dojindo, Kumamoto, Japan), and samples were processed by using general protocol for western blotting. Membranes were incubated overnight at 4 °C with the following antibodies in Can Get Signal Solution 1 (TOYOBO, Osaka, Japan): anti-TGF- $\beta$ 1 (1:1,000, ABB250576; ABBIOTEC), HIF-1 $\alpha$  (1:1,000; GTX127309; GeneTex) and anti- $\beta$ -actin antibody (1:1,000; Sigma-Aldrich). After washing, membranes were incubated with the appropriate secondary antibody conjugated to horseradish peroxidase, and exposed to the ECL Plus Western blotting detection system (GE Healthcare Bio-Sciences, Piscataway, NJ, USA). The signals were detected by using a Chemilumino analyzer LAS-4000 (Fujifilm, Tokyo, Japan). The relative densities of gel bands were semiquantified by using Image J software. The adjusted relative density of TGF- $\beta$ 1 and HIF-1 $\alpha$  protein was then obtained after dividing its values with the relative density of  $\beta$ -actin expression as a normalized loading control.

#### Enzyme-Linked Immunosorbent Assay

ELISA was used to quantify the TGF- $\beta$ 1 secretion of N1S1 cells. The concentration of TGF- $\beta$ 1 in the supernatant of N1S1 cell culture was measured using the TGF- $\beta$ 1 ELISA kit (Quantikine ELISA kit; R&D Systems, Minneapolis, MN, USA) according to the manufacturer's protocol. The optical density measurements were performed at 490 nm in a microplate reader (SPECTRA max; Molecular Devices, San Jose, CA, USA).

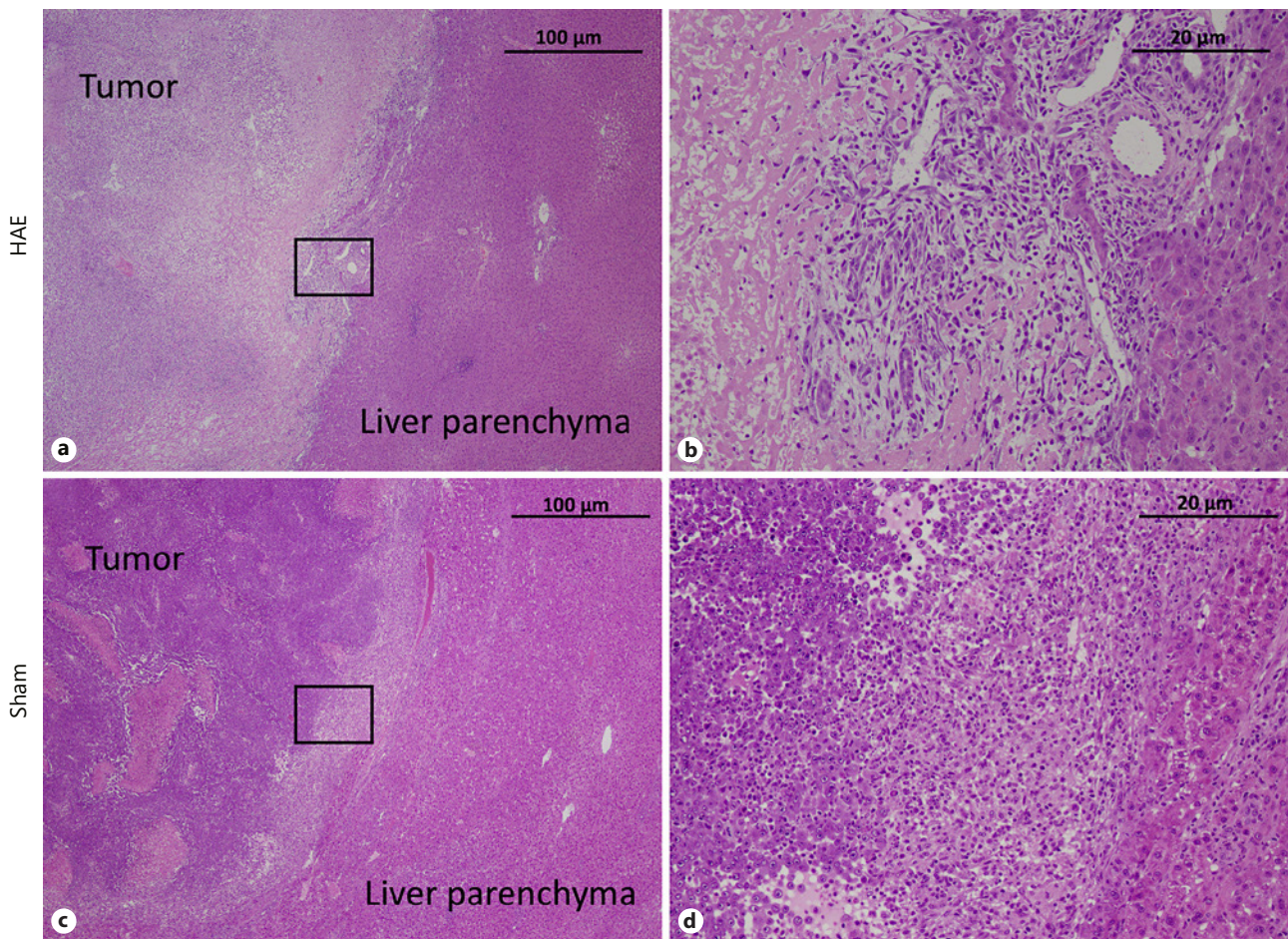
#### Statistical Analysis

All in vitro experiments were repeated in at least triplicate. Data were aggregated and expressed as means  $\pm$  SD unless otherwise indicated. Statistical analyses were performed using PRISM 8 software (2018, GraphPad; San Diego, CA, USA). Statistical data comparisons were performed by Student *t* test. All test results were considered significant at *p* values lower than 0.05.

## Results

### *HAE Enhanced both TGF- $\beta$ 1 and HIF-1 $\alpha$ Expression in the Peripheral Area of the Tumor*

Hematoxylin and eosin staining showed that necrotic areas were mainly located in the center of the tumor in the HAE group, while viable hepatoma cells were sparse in the peripheral

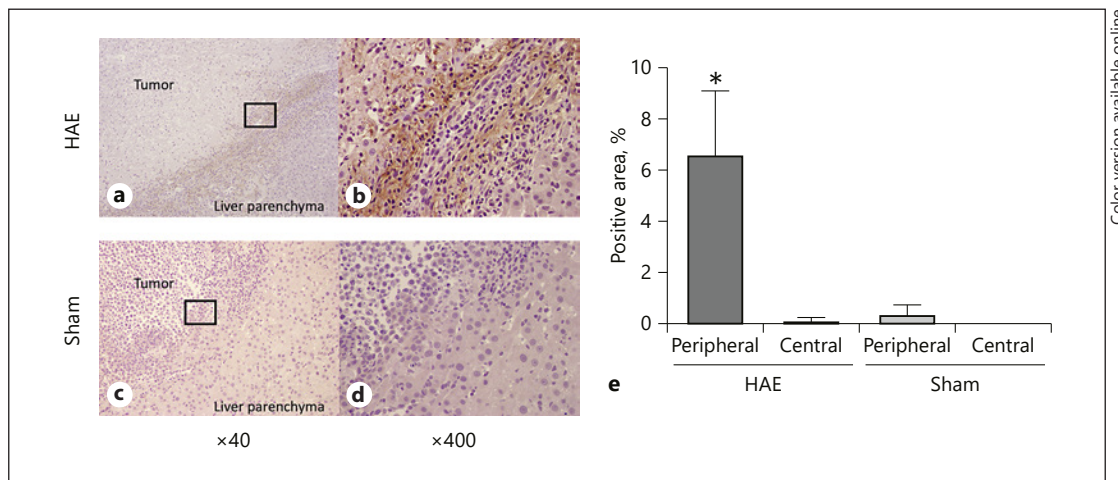


**Fig. 1.** Histological images of liver sections on day 2 after HAE and sham treatments. **a** The hematoxylin and eosin-stained liver sections revealed that necrosis was widespread in the tumoral area (tumor) and reduced in the liver parenchyma in the HAE group. **c** In the sham group, necrotic lesions were sparse in the tumoral area (tumor) and not observed in the liver parenchyma. **b, d** Magnified images ( $\times 200$ ) of the area in the black frame in **a** and **c**, respectively. HAE, hepatic arterial embolization.

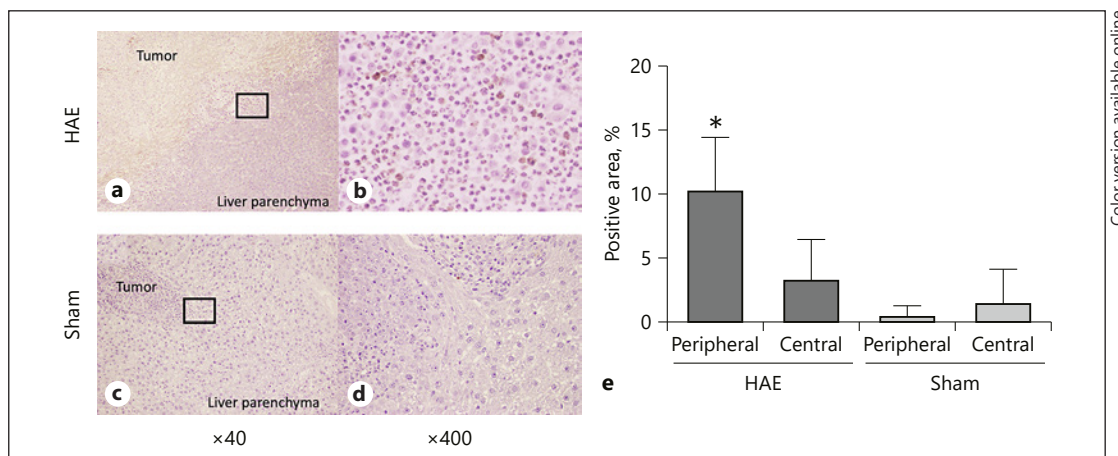
area (Fig. 1a, b). However, the necrotic area in the sham group was limited to the tumor, and the infiltration of inflammatory cells such as neutrophils was observed at the boundary of the tumor and liver parenchyma (Fig. 1c, d).

Immunohistochemistry showed that the TGF- $\beta$ 1-positive area in the peripheral area of the tumor was significantly larger in the HAE group than in the sham group ( $6.59 \pm 2.49$  and  $0.34 \pm 0.41\%$ , respectively;  $p < 0.001$ ; Fig. 2a–e). Similarly, the HIF-1 $\alpha$ -positive area in the peripheral area of the tumor was significantly larger in the HAE group than in the sham group ( $10.26 \pm 4.14$  and  $0.40 \pm 0.84\%$ ;  $p < 0.001$ ; Fig. 3a–e). On the other hand, TGF- $\beta$ 1 ( $p = 0.990$ ) and HIF-1 $\alpha$  ( $p = 0.134$ ) expression was not significantly different between the HAE and sham groups at the center of the tumor.

As shown in Figure 4a, the mRNA levels of  $\alpha$ -SMA and COL1A (fibrotic markers) were higher ( $6.95 \pm 2.00$ -fold and  $5.25 \pm 1.19$ -fold respectively;  $p < 0.001$ ) in the HAE group than in the sham group. Furthermore, in the HAE group, the TGF- $\beta$ 1 mRNA level was significantly increased by  $1.95 \pm 0.38$ -fold ( $p < 0.001$ ), and the HIF-1 $\alpha$  mRNA level was significantly increased by  $1.62 \pm 0.37$ -fold ( $p = 0.002$ ). Although HAE induced TGF- $\beta$ 1 mRNA expression in the tumor tissue, which was consistent with TGF- $\beta$ 1 protein expression



**Fig. 2.** TGF-β1 expression in liver sections. **a** HAE group and **(c)** sham group. **b, d** are magnified images (×400) of the area in the black frame in **(a, c)**, respectively. The TGF-β1-positive area (brown) was visible in the HAE group **(a, b)** but not in the sham group **(c, d)**. **e** Comparison of the TGF-β1-positive proportion (%) in the center and periphery of the tumor in the HAE group ( $n = 5$ ) and sham group ( $n = 4$ ). Data are presented as the mean ± SD. The significance level of the TGF-β1-positive proportion (%) in the periphery compared with the center of the tumor in the HAE group was set as \*  $p < 0.01$  using Student  $t$  test. HAE, hepatic arterial embolization.

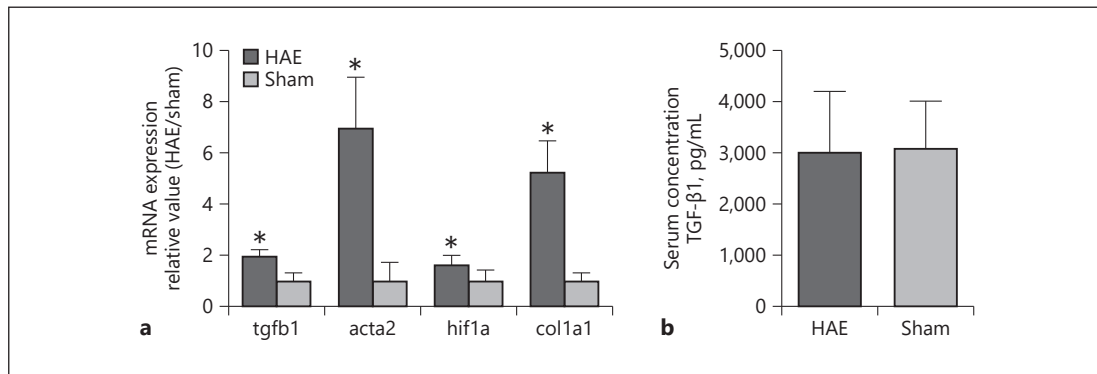


**Fig. 3.** HIF-1α expression in liver sections. **a** HAE group and **(c)** sham group. **b, d** are magnified images (×400) of the area in the black frame in **(a, c)**, respectively. The HIF-1α-positive area (brown) was visible in the HAE group but not in the sham group. **e** The histogram shows the stained positive area (%) of each slide calculated by ImageJ software. For HAE-treated tumors, the HIF-1α-positive area was the largest in the peripheral area followed by the intratumoral area (\*  $p < 0.01$ ). HAE, hepatic arterial embolization.

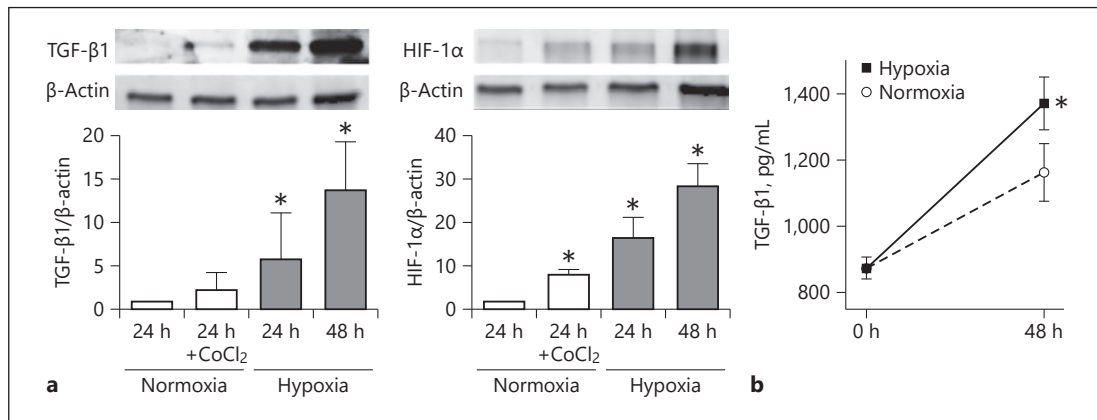
(Fig. 3), TGF-β1 protein secreted in the serum was not significantly different between the HAE and sham groups ( $3,029 \pm 1,173$  and  $3,099 \pm 903$  pg/mL respectively;  $p = 0.931$ ; Fig. 4b).

*Hypoxic Condition Induced TGF-β1 and HIF-1α Expression in N1S1 Cells*

To elucidate the mechanisms of TGF-β1 expression, an in vitro cell culture experiment under hypoxic conditions, which mimic HAE, was performed. Western blot analysis of the in



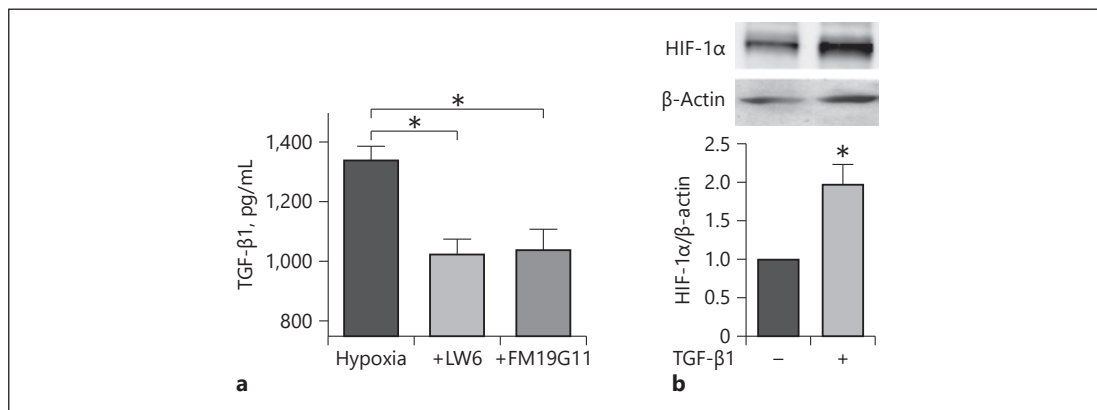
**Fig. 4.** Gene expression and serum concentration of TGF-β1 in tumors. **a** Quantification of TGF-β1, α-SMA, HIF-1α, and collagen type-1α-1 chain mRNAs normalized to β-actin in tumors of the HAE group (black bars,  $n = 5$ ) and the sham group (gray bars,  $n = 4$ ) by qPCR. Asterisks indicate a higher ( $* p < 0.01$ ) mRNA expression in tumors of the HAE group compared with the sham group according to Student  $t$  test. **b** Serum TGF-β1 protein of N1S1-bearing rats (HAE:  $n = 5$ ; sham:  $n = 4$ ). TGF-β1 secretion was not significantly different between the HAE and sham groups ( $p = 0.93$ ) according to Student  $t$  test. HAE, hepatic arterial embolization; TGF-β1, transforming growth factor β1.



**Fig. 5.** Hypoxia-induced expression of TGF-β1 and HIF-1α. **a** TGF-β1 and HIF-1α protein expression in rat hepatoma cells (N1S1) cultured under normoxic conditions (21% O<sub>2</sub>) with or without CoCl<sub>2</sub> (100 μmol/L; white bar) for 24 h and under hypoxic conditions (1% O<sub>2</sub>; black bar) for 24 and 48 h. Representative western blots for TGF-β1 and HIF-1α protein expression (upper panel) and densitometric analysis results normalized to β-actin (lower panel) are shown. The protein expression of TGF-β1 and HIF-1α was higher ( $* p < 0.01$ ) under hypoxic conditions than under normoxic conditions according to Student  $t$  test. **b** TGF-β1 secreted into the supernatant of N1S1 cells cultured under hypoxic conditions for 48 h was detected by ELISA. TGF-β1 secretion was higher ( $* p < 0.01$ ) under hypoxic conditions than under normoxic conditions according to Student  $t$  test. Each experiment was performed 4 times (**a**, **b**). TGF-β1, transforming growth factor β1; HIF-1α, hypoxia-inducible factor 1α.

vitro cell culture samples showed that the protein expression of both TGF-β1 and HIF-1α was higher ( $p = 0.031$  and  $p < 0.001$  respectively) when the cells were incubated under hypoxic conditions instead of normoxic conditions (Fig. 5a). Similarly, ELISA of the cell culture supernatant showed that TGF-β1 level was higher ( $p < 0.001$ ) in samples cultured under hypoxic conditions (1,397 ± 58 pg/mL) than in those cultured under normoxic conditions (1,119 ± 37 pg/mL; Fig. 5b).





**Fig. 6.** Relationship between TGF- $\beta$ 1 and HIF-1 $\alpha$  expression. Effect of HIF-1 $\alpha$  inhibitors on the hypoxia-induced expression of TGF- $\beta$ 1. **a** TGF- $\beta$ 1 secreted into the supernatant of N1S1 cells cultured under hypoxic conditions (1% O<sub>2</sub>) for 48 h without or with HIF-1 $\alpha$  inhibitors (20  $\mu$ mol/L LW6 or 300 nmol/L FM19G11) was detected by ELISA. The secretion of TGF- $\beta$ 1 under hypoxic conditions was significantly suppressed by HIF-1 $\alpha$  inhibitors (\* $p$  < 0.01). **b** A representative western blot of HIF-1 $\alpha$  expression in N1S1 cells stimulated with TGF- $\beta$ 1 (5 ng/mL) under normoxic conditions for 48 h (upper panel) and densitometric analysis results normalized to  $\beta$ -actin (lower panel). TGF- $\beta$ 1 significantly induced HIF-1 $\alpha$  protein expression under normoxic conditions (\* $p$  < 0.01). Each experiment was performed 4 times (**a**, **b**). TGF- $\beta$ 1, transforming growth factor; HIF-1 $\alpha$ , hypoxia-inducible factor 1 $\alpha$ .

#### Association of TGF- $\beta$ 1 and HIF-1 $\alpha$ Expression in N1S1 Cells

When LW6 and FM19G11 (HIF-1 $\alpha$  inhibitors) were added to the N1S1 cell culture medium and incubated for 48 h under hypoxic conditions, the TGF- $\beta$ 1 level was lower in the groups with the inhibitors ( $p$  = 0.031 and  $p$  < 0.001 respectively) than in the control group without the inhibitors (1,340  $\pm$  46, 1,026  $\pm$  49, and 1,040  $\pm$  65 pg/mL in the control, LW6, and FM19G11 groups respectively; Fig. 6a). When recombinant TGF- $\beta$ 1 was added to the N1S1 cell culture medium and incubated for 48 h under normoxic conditions, the expression of HIF-1 $\alpha$  was significantly increased (1.97  $\pm$  0.26-fold;  $p$  = 0.033) compared with the expression in the control group (Fig. 6b).

#### Discussion

The results of our in vivo study showed that HAE could increase the local expression of TGF- $\beta$ 1. The expression of TGF- $\beta$ 1 has been reported to be affected by various stimulations such as inflammatory reactions [19]. Recently, Hung et al. [12] reported that hypoxic conditions induced the secretion of TGF- $\beta$ 1 by mesenchymal stem cells. In our study, TGF- $\beta$ 1-positive areas were predominantly located in the peripheral area of the tumor, where residual viable hepatoma cells were sparse, after HAE. Moreover, the overexpression of HIF-1 $\alpha$  was observed in the peripheral area of the tumor. HIF-1 $\alpha$  is known to function as a transcriptional regulator that upregulates several cancer-related genes [20]. Therefore, we hypothesized that hypoxic stress induced by HAE may trigger the overexpression of TGF- $\beta$ 1 in residual hepatoma cells in an HIF-1 $\alpha$ -dependent manner.

The results of our in vitro experiment support our hypothesis. The overexpression of TGF- $\beta$ 1 was observed when rat N1S1 hepatoma cells were cultured under hypoxic conditions, and it was downregulated when an HIF-1 $\alpha$  inhibitor was used. More interestingly, the expression of HIF-1 $\alpha$  was higher when exogenous TGF- $\beta$ 1 was added. These results suggest

that the expression of TGF- $\beta$ 1 and HIF-1 $\alpha$  may be interdependent. Similarly, Basu et al. [21] reported the interdependent signaling of HIF-1 $\alpha$  and TGF- $\beta$ /Smad3 in renal epithelial cells under hypoxic conditions, which could lead to renal fibrogenesis.

Notably, the mRNA expression of both  $\alpha$ -SMA and COL1A was also considerably upregulated after HAE in the rat hepatoma model. TGF- $\beta$ 1 is known to induce the transformation of hepatic stellate cells to myofibroblasts, which express  $\alpha$ -SMA [22, 23]. Moreover, these  $\alpha$ -SMA-positive myofibroblasts induce collagen deposition. Therefore, the high mRNA expression of both  $\alpha$ -SMA and COL1A may indicate the abundant secretion of TGF- $\beta$ 1 in the tumor microenvironment. However, serum TGF- $\beta$ 1 level was not higher in the HAE group in this study, suggesting that the overexpression of TGF- $\beta$ 1 after HAE was locally limited and more obvious compared with the serum level of TGF- $\beta$ 1.

TGF- $\beta$ 1 can promote cancer cell growth, proliferation, and immune evasion [9–11]. The overexpression of TGF- $\beta$ 1 has been reported to be associated with recurrence and poor prognosis in patients with hepatoma [5–7]. Similarly, the overexpression of HIF-1 $\alpha$  is known to be associated with poor prognosis in patients with hepatoma [24, 25]. Therefore, although HAE is a useful therapeutic option for the treatment of intermediate-stage hepatoma, the results of our study suggest that HAE may also adversely affect cancer treatment by causing the overexpression of TGF- $\beta$ 1 and HIF-1 $\alpha$ . Accordingly, the combined use of TGF- $\beta$ 1 and HIF-1 $\alpha$  inhibitors with HAE may be a reasonable approach for enhancing the effect of HAE; however, further studies are needed.

This study has several limitations including the small number of animals, the model of hepatoma (i.e., autograft model without liver cirrhosis), and short follow-up periods. For the *in vitro* experiment, the samples were assessed only after 48 h, and the kinetics of TGF- $\beta$ 1 and HIF-1 $\alpha$  was not examined. Moreover, our *in vitro* model did not accurately represent *in vivo* conditions.

In conclusion, HAE enhanced the local TGF- $\beta$ 1 expression in a rat hepatoma model. *In vitro* experiments suggest that HAE-induced hypoxic stress may trigger the interdependent expression of TGF- $\beta$ 1 and HIF-1 $\alpha$ .

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### Statement of Ethics

All animal experiments were approved by the Institutional Animal Care and Use Committee and were conducted according to the Fundamental Guidelines for Proper Conduct of Animal Experiments and Related Activities in Academic Research Institutions in Japan.

### Disclosure Statement

The authors have no conflicts of interest to declare.

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## Author Contributions

H.N., T.T., and K.K.: animal experiments. E.U., H.T., and H.K.: histology, image analysis, and quantification. E.U., H.T., and Y.H.: in vitro experiments. E.U., H.T., Y.H., T.O., and K.S.: statistical analysis and results. E.U., H.N., H.T., K.Y., M.Y., K.S., and T.M.: manuscript preparation.

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