低酸素下で発現するリン酸化酵素を標的とする膵癌治療法

平成 17 年度基盤的研究開発育成事業（共同研究）研究成果報告書

平成 18 年 8 月

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【はしがき】

ヒト悪性腫瘍の発生には、複数の癌遺伝子あるいは癌抑制遺伝子に段階的に異常が生じ、悪性形質を獲得して過程が重要であることが示されている。こうした癌細胞側の異常がどのようにして起こるのかについての明確な機構は依然として不明のままである。一方癌細胞が臨床癌にまで進展していく過程には、宿主側の正常細胞もその構成員となる腫瘍をとりまく Tumor Microenvironment の関与が重要であることも知られている。典型的なものとしては、癌が 1 mm をこえて増殖すためには血管新生が必須であるという事実が知られている。

申請者らは、癌組織での酸素分圧が正常組織の約５分の１程度であるために、こうした低酸素環境に適応するための HIF-1 を中心とした応答機構が臨床癌の形成には必須であるとの仮説をたてた。この仮説に基づいて、低酸素環境下で新たに発現する遺伝子や発現亢進する遺伝子を DNA マイクロアレイという手法を用いて網羅的に検索した。その結果、アドレノメジュリンという血管新生因子や Pim-1 というセリン・スレオニンカイネースの発現が亢進することを見出した。本研究では、Pim-1 というセリン・スレオニンカイネースの役割について検討したので、以下に得られた結果を報告する。

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	直接経費	間接経費	合計
平成17年度	1,000千円	0	1,000千円

【研究発表】

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(特許出願)

1. 発明の名称：低酸素条件下において遊走能を有する樹状細胞、およびその利用
   出願番号：特願2005-272279
   出願日：平成17年9月20日
   発明者：小林 正伸

2．発明の名称：Pim-1活性阻害剤
出願番号：特願2005-276648
出願日：平成17年9月22日
発明者：小林 正伸・陳 健
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【研究成果】

1．　Pim-1阻害による造腫瘍性の喪失

Pim-1の機能を阻害するC末端フラグメント（dominant negative Pim-1）を導入した膵癌細胞株を作成すると、導入株のみにdominant negative Pim-1蛋白が発現している（図1）。

Fig. 5B

この細胞をＳＣＩＤマウスに移植すると、導入株のみが造腫瘍性を喪失していた（図2）。

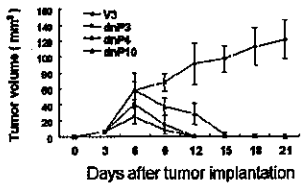

Fig. 5C

以上のデーターから、固形癌においてPim-1発現が造腫瘍性に必須であることが示唆された。

2．　Pim-1発現によるNIH3T3細胞のanchorage-independent growth能の獲得

ついで、NIH3T3細胞に全長のPim-1を発現させたときのtransformationをアガー中でのコロニー形成能で観察した。その結果、全長のPim-1を発現

させても正常酸素分圧下ではコロニー形成能は増加しないが、低酸素下では有意に増加した（図３）。

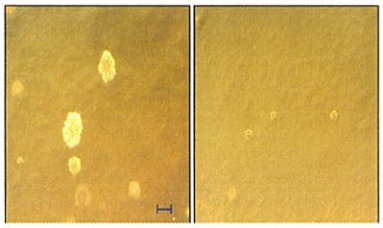

Fig. 4B

以上の結果は、Pim-1 蛋白の発現が低酸素下で亢進して造腫瘍性を増加させている可能性を示唆する。これらの結果から、Pim-1 蛋白は固形癌において造腫瘍性に重要な役割を果たしていることが示唆された。

３．　今後の検討課題

Pim-1 蛋白の低酸素下で発現亢進する機序として転写のみでは説明できず、転写以後の蛋白レベルにて制御されている可能性が高い。今後はこの機構を明らかにすることが必要である。発現様式が HIF-1 蛋白と同様に酸素分圧依存性であることから、分解機構が主な発現制御機構と思われる。ユビキチン化機構、プロリル水酸化酵素の関与などが候補として考えられ、今後一つ一つ検討していく。

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Acetylcholine from vagal stimulation protects cardiomyocytes against ischemia and hypoxia involving additive non-hypoxic induction of HIF-1α

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Abstract **Electrical stimulation of the vagal efferent nerve improves the survival of myocardial infarcted rats. However, the mechanism for this beneficial effect is unclear. We investigated the effect of acetylcholine (ACh) on hypoxia-inducible factor (HIF)-1α using rat cardiomyocytes under normoxia and hypoxia. ACh posttranslationally regulated HIF-1α and increased its protein level under normoxia. ACh increased Akt phosphorylation, and wortmannin or atropine blocked this effect. Hypoxia-induced caspase-3 activation and mitochondrial membrane potential collapse were prevented by ACh. Dominant-negative HIF-1α inhibited the cell protective effect of ACh. In acute myocardial ischemia, vagal nerve stimulation increased HIF-1α expression and reduced the infarct size. These results suggest that ACh and vagal stimulation protect cardiomyocytes through the PI3K/Akt/HIF-1α pathway.**

Keywords: Acetylcholine; Ischemia; Apoptosis; Protein kinases

1. Introduction

The prognosis of patients with chronic heart failure remains poor, due to progressive remodeling of the heart and lethal arrhythmia. Acute ischemia or hypoxia causes loss of cardiomyocytes, followed by remodeling in the chronic phase. Although various therapeutic approaches have been introduced, including implantable defibrillators [1], a more effective modality of therapy has been anticipated for several years. A recent animal study by Li et al. [2] demonstrated that vagal nerve stimulation prevented ventricular remodeling after myocardial infarction, suggesting a novel therapeutic strategy against heart failure. Furthermore, Krieg et al. [3] reported that acetylcholine (ACh) has a cardioprotective effect. Although nitric oxide (NO) is supposed to be a major signaling molecule induced by ACh, a mechanism for the beneficial effect of vagal nerve stimulation on cardiomyocytes remains to be clarified. To investigate this mechanism, we hypothesized that vagal stimulation or ACh directly triggers a cell survival signal that is subsequently amplified and leads to protection of the cardiomyocytes from acute ischemic conditions, and that this effect of ACh, if continued, could be responsible for chronic cardioprotection.

In the present study, we focused on demonstrating the cellular action of ACh through hypoxia-inducible factor (HIF)-1α. HIF-1α is a transcription factor that is important for cell survival under hypoxia. HIF-1α activates the expression of many genes indispensable for cell survival [4,5]. Under normoxia, the HIF-1α protein level is very low, due to proteasomal degradation through with von Hippel–Lindau tumor suppressor protein (VHL). However, HIF-1α escapes from this degradation under hypoxia, and this is recognized as the hypoxic pathway [6,7]. Recently, it was revealed that HIF-1α can be also induced via a non-hypoxic pathway by angiotensin II [8,9]. Taken together, it is conceivable that HIF-1α induction is one of the adaptation processes to hypoxia and ischemia, and that additional induction of HIF-1α during ischemia via a non-hypoxic pathway could provide further cardioprotection.

Therefore, we investigated the direct effects of ACh on survival signaling in cardiomyocytes and of vagal stimulation on hearts. The results suggest that ACh and vagal stimulation protect cardiomyocytes from acute hypoxia and ischemia via additional HIF-1α protein induction through a non-hypoxic pathway.

2. Materials and methods

2.1. Cell culture

To examine the effect of ACh on cardiomyocytes, H9c2 cells as well as primary cardiomyocytes isolated from neonatal rats were used. H9c2 cells, which are frequently used to investigate signal transduction and channels in rat cardiomyocytes, are derived from rat embryonic ventricular cardiomyocytes. H9c2 cells were incubated in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and antibiotics. Primary cardiomyocytes were isolated from 2–3-day-old neonatal WKY rats and incubated in DMEM/Ham F-12 containing 10% FBS. HEK293 cells and HeLa cells cultured in DMEM containing 10% FBS were also used.

2.2. Western Blot analysis

H9c2 cells and primary cardiomyocytes were treated with 1 mM ACh to evaluate expression of HIF-1α protein under normoxia or with 1 mM *S*-nitroso-*N*-acetylpenicillamine (SNAP) to study the signal transduction. To investigate the signal transduction, H9c2 cells were pretreated with a PI3K inhibitor, (wortmannin; 300 nM), a muscarinic receptor, (atropine; 1 mM), a transcriptional inhibitor, (actinomycin D; 0.5 μg/ml) or a protein synthesis inhibitor, (cycloheximide; 10 μg/ml), followed by ACh treatment. Cell lysates were mixed with a sample

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buffer, fractionated by 10% SDS–PAGE and transferred onto membranes. The membranes were incubated with primary antibodies against HIF-1α (Santa Cruz Biotechnology, Santa Cruz, CA, USA), Akt and phospho-Akt (Cell Signaling Technology, Beverly, MA, USA), and α-tubulin (Lab Vision, Fremont, CA, USA), and then reacted with an HRP-conjugated secondary antibody (BD Transduction Laboratories, San Diego, CA, USA). Positive signals were detected with an enhanced chemiluminescence system (Amersham, Piscataway, NJ, USA). In each study, the experiments were performed in duplicate and repeated 3–5 times ($n = 3$–5). Representative data are shown.

2.3. MTT activity assay

To evaluate the effects of hypoxia and ACh on the mitochondrial function of cardiomyocytes, we measured 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reduction activity in H9c2 or HEK293 cells under hypoxia (1% oxygen concentration), in the presence or absence of ACh. The cells were pretreated with 1 mM ACh for 12 h, and then subjected to hypoxia for 12 h. At 4 h before sampling, the MTT reagents were added to the culture and incubated.

2.4. Caspase-3 activity assay

Caspase-3 activity was measured using a CPP32/Caspase-3 Fluorometric Protease Assay Kit, (Chemicon International, Temecula, CA, USA). Hypoxia-treated H9c2 cells with or without 1 mM ACh pretreatment were lysed and the cytosolic extract was added to the caspase-3 substrate. A fluorometer equipped with a 400-nm excitation filter and 505-nm emission filter was used to measure the samples.

2.5. DePsipher assay

To examine the effects of hypoxia and ACh on the mitochondrial electrochemical gradient, we analyzed cardiomyocytes using a DePsipher™ Mitochondrial Potential Assay Kit (Trevigen, Gaithersburg, Maryland, USA). Apoptotic cells, which undergo mitochondrial membrane potential collapse cannot accumulate the DePsipher reagent in their mitochondria. As a result, apoptotic cells show decreased red fluorescence in their mitochondria, and the reagent remains in the cytoplasm as a green fluorescent monomer. Therefore, apoptotic cells were easily differentiated from healthy cells, which showed more red fluorescence.

2.6. Evaluation of NO production

NO production was measured using the 4,5-diaminofluoresceindiacetate (DAF-2DA; Alexis, Lausen, Switzerland) fluorometric NO detection system as previously reported [10]. The intensity of the DAF-2DA green fluorescence in ACh-treated cells was measured and compared with that in non-treated cells (λ_{Ex} 492 nm; λ_{Em} 515 nm).

2.7. Transfection

To investigate the direct contribution of Akt phosphorylation to HIF-1α stabilization or that of HIF-1α to the ACh effect, HEK293 cells were transfected with an expression vector for wild-type Akt (wt Akt), dominant-negative Akt (dn Akt) [11], wild-type HIF-1α (wt HIF-1α) [12] or dominant-negative HIF-1α (dn HIF-1α), using Effectene (Qiagen, Valencia, CA, USA) according to the manufacture's protocol. After transfection, HEK293 cells were pretreated with 1 mM ACh for 12 h, followed by evaluating the HIF-1α protein level or by hypoxia for 12 h and MTT activity in each group was evaluated. As a control, cells were transfected with a vector for green fluorescent protein (GFP).

2.8. RT-PCR

Total RNA was isolated from H9c2 cells according to a modified acid guanidinium–phenol–chloroform method using an RNA isolation kit (ISOGEN; Nippon Gene, Tokyo, Japan), and reverse-transcribed to obtain a first-strand cDNA. This first-strand cDNA was amplified by specific primers for HIF-1α, and the PCR products were fractionated by electrophoresis.

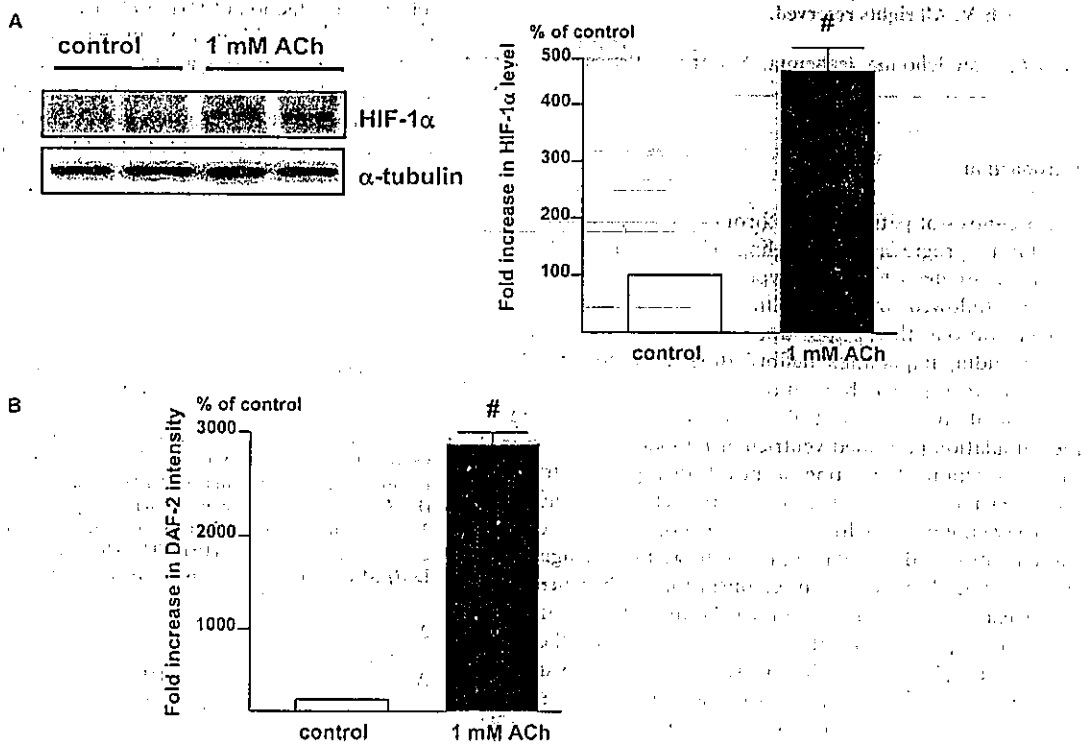

Fig. 1. HIF-1α is induced by ACh in rat cardiomyocytes even under normoxia. (A) After treatment of H9c2 cells with 1 mM ACh for 8 h, the HIF-1α protein level is increased ($^{\#}P < 0.05$ vs. control, $n = 4$). (B) ACh (1 mM) increases the intensity of DAF-2DA fluorescence ($^{\#}P < 0.01$ vs. control, $n = 3$).

2.9. Vagal nerve stimulation in myocardial ischemia

Left ventricular myocardial ischemia (MI) was performed by 3 h of left coronary artery (LCA) ligation in anesthetized 9-week-old male Wistar rats under artificial ventilation previously described [2]. Sham-operated (control) rats did not undergo LCA ligation. For vagal nerve stimulation (VS), the right vagal nerve in the neck was isolated and cut. Only the distal end of the vagal nerve was stimulated in order to exclude the effects of the vagal afferent. The electrode was connected to an isolated constant voltage stimulator. VS was performed from 1 min before the LCA ligation until 3 h afterwards, using 0.1 ms pulses at 10 Hz (MI-VS). The electrical voltage of the pulses was adjusted to obtain a 10% reduction in the heart rate before LCA ligation, but VS (MI-VS) was not associated with any blood pressure reduction during the experiments, compared with MI. At the end of the experiments, the rats were either injected with 2 ml of 2% Evans blue dye via the femoral vein to measure the risk area followed by determination of the infarct size with 2% triphenyl tetrazolium chloride (TTC) staining or the heart was excised for protein isolation and subsequent Western Blotting to detect HIF-1α protein. The percentage of the infarcted area of the left ventricle was calculated as the ratio of the infarcted area to the risk area.

2.10. Densitometry

The Western Blotting data were analyzed using Kodak 1D Image Analysis Software (Eastman Kodak Co., Rochester, NY, USA).

2.11. Statistics

The data were presented as means ± S.E. The mean values between two groups were compared by the unpaired Student's *t* test. Differences among data were assessed by ANOVA for multiple comparisons of results. Differences were considered significant at $P < 0.05$.

3. Results

3.1. Posttranslational regulation of HIF-1α by ACh through a non-hypoxic pathway

ACh (1 mM) increased HIF-1α protein expression in H9c2 cells under normoxia (Fig. 1A). ACh increased NO production, as evaluated by DAF-2DA (Fig. 1B), suggesting that

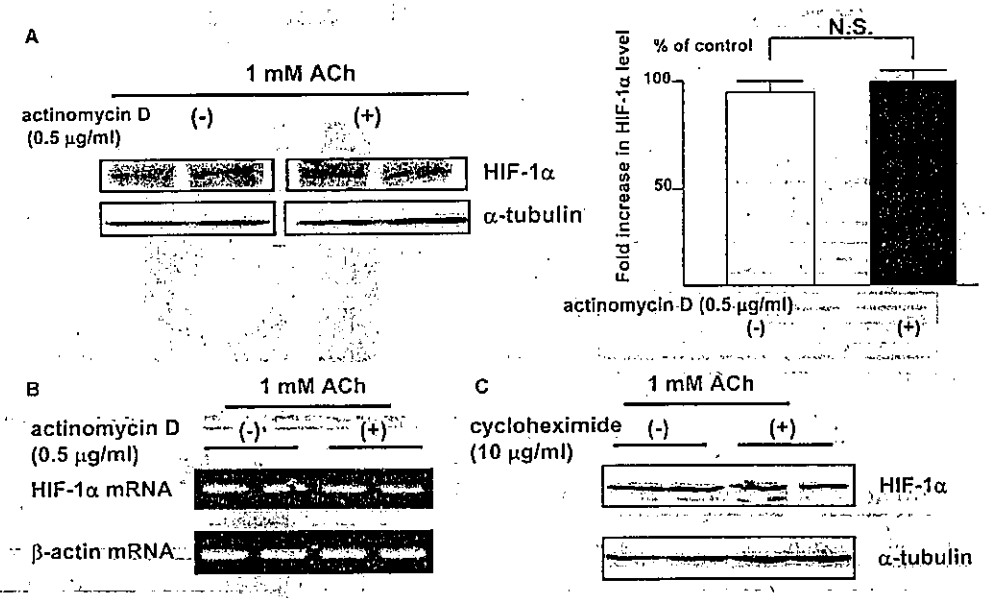

Fig. 2. HIF-1α induction by ACh is posttranslationally regulated in rat cardiomyocytes under normoxia. (A) The HIF-1α protein level in H9c2 cells in the presence of 0.5 μg/ml of actinomycin D is increased by 1 mM ACh to a comparable level to that in the absence of actinomycin D (N.S., not significant, $n = 3$). (B) Actinomycin D does not decrease the HIF-1α mRNA level, as evaluated by RT-PCR. (C) Cycloheximide (10 μg/ml) does not affect the HIF-1α protein level ($n = 3$).

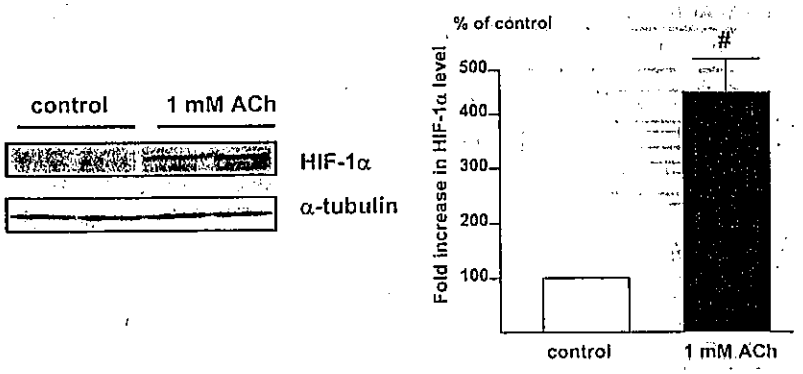

Fig. 3. Rat primary cultured cardiomyocytes show comparable HIF-1α induction by 1 mM ACh to that in H9c2 cells ($^{\#}P < 0.05$ vs. control, $n = 3$).

NO is involved in the signal transduction of HIF-1α induction. Actinomycin D (0.5 μg/ml; Figs. 2A and B) and cycloheximide (10 μg/ml; Fig. 2C) did not decrease the HIF-1α level under normoxia, suggesting that HIF-1α degradation is regulated by ACh. Furthermore, ACh increased HIF-1 α level in primary cardiomyocytes without reducing their beating rate (Fig. 3). Since H9c2 cells did not beat, these results suggest that HIF-1 induction is independent of the heart rate-decreasing effect of ACh.

3.2. Akt phosphorylation by ACh

ACh had no effect on the total Akt protein level, but increased Akt phosphorylation (Fig. 4A) as effectively as SNAP (data not shown). The ACh-induced Akt phosphorylation was inhibited by atropine in a dose-dependent manner (Fig. 4B). ACh-induced Akt phosphorylation and its inhibition by atropine were also observed in rat primary cardiomyocytes (Fig. 4C).

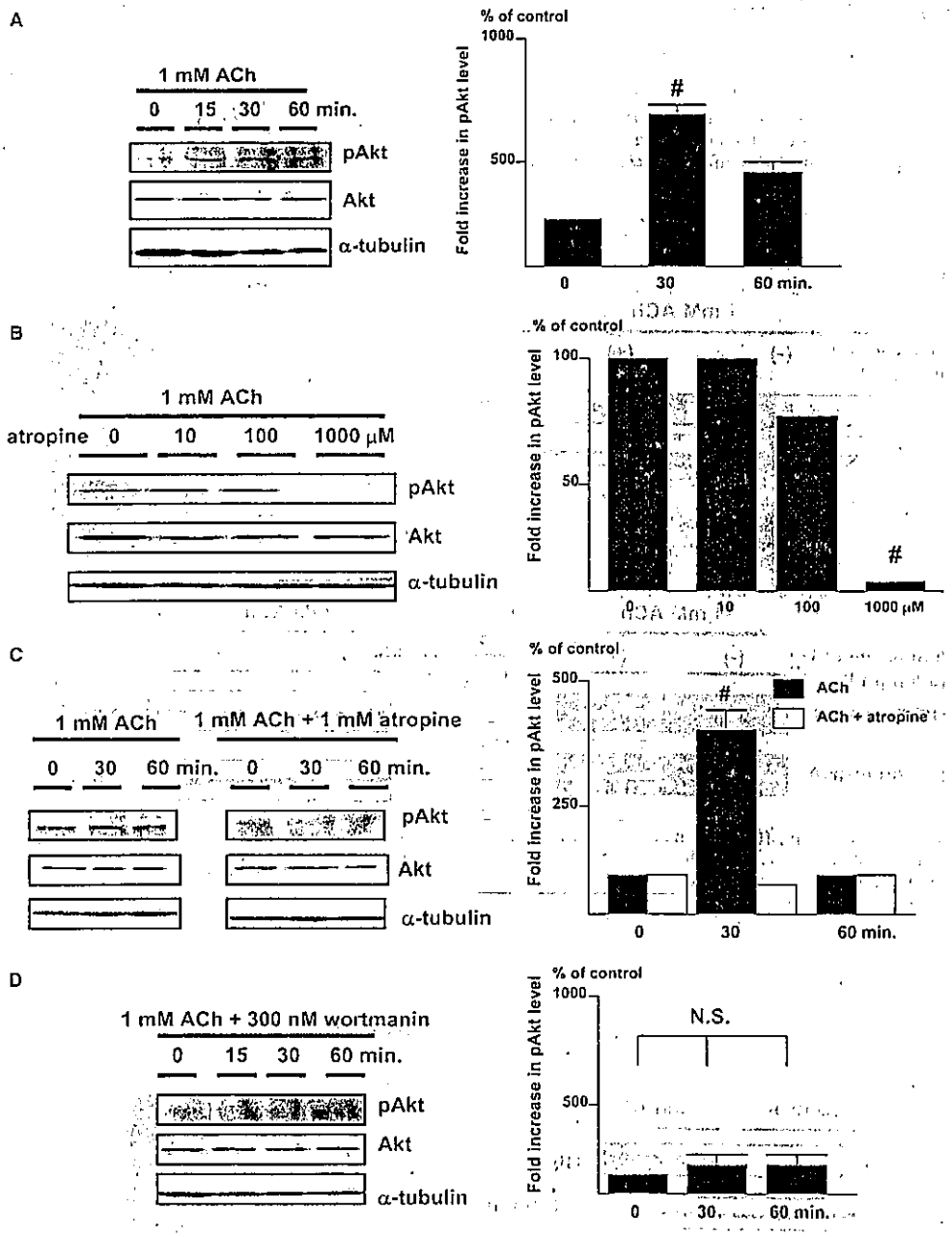

Fig. 4. Akt is activated by ACh in rat cardiomyocytes, leading to HIF-1α induction. (A) Akt phosphorylation in H9c2 cells is rapidly increased by 1 mM ACh ($^{\#}P < 0.05$ vs. baseline, $n = 4$), whereas the total protein level of Akt remains unaffected. (B) The ACh-induced increase in Akt phosphorylation is blocked by 1 mM atropine ($^{\#}P < 0.05$ vs. 0 μM atropine, $n = 3$). (C) ACh (1 mM) also increases Akt phosphorylation in rat primary cardiomyocytes ($^{\#}P < 0.05$ vs. baseline, $n = 3$), and atropine blocks this effect. (D) Pretreatment with 300 nM wortmannin completely inhibits ACh-induced Akt phosphorylation in H9c2 cells (N.S., not significant, $n = 3$). (E) Wortmannin (300 nM) also inhibits HIF-1α induction by ACh ($^{\#}P < 0.05$ vs. wortmannin (+), $n = 3$). Each figure shows a representative result from three independently performed experiments ($n = 3$). (F) In contrast to wt Akt, HIF-1α induction by ACh is blocked by dn Akt in HEK293 cells ($n = 4$).

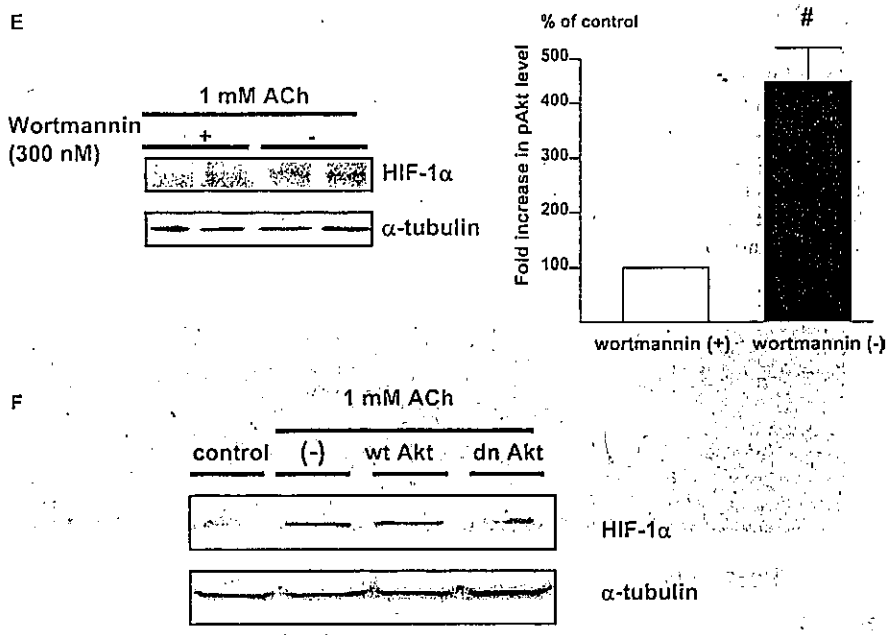

Fig. 4 (*continued*)

3.3. PI3K/Akt Pathway for HIF-1α induction by ACh

Wortmannin completely inhibited the ACh-induced Akt phosphorylation (Fig. 4D), in clear contrast to the data in Fig. 4A. Furthermore, it also attenuated the HIF-1α induction by ACh (Fig. 4E). To elucidate the contribution of Akt phosphorylation to HIF-1α protein level in normoxia, dn Akt was introduced into HEK293 cells, and found to partially inhibit the HIF-1α induction by ACh (Fig. 4F).

3.4. Effect of ACh on apoptosis during hypoxia

The DePsipher assay clearly showed that hypoxia (1% oxygen concentration) for 12 h caused mitochondrial membrane potential collapse leading to cell death, and that 1 mM ACh inhibited this collapse in H9c2 cells (Fig. 5A). ACh attenuated the decrease in MTT activity caused by 12 h of hypoxia in H9c2 cells (Fig. 5B; 103.4 ± 0.8% in ACh + hypoxia vs. 56.6 ± 0.7% in hypoxia, $P < 0.01$, $n = 8$) and HEK293 cells ($P < 0.01$ vs. hypoxia). The caspase-3 activity was increased by hypoxia in H9c2 cells, and pretreatment with 1 mM ACh inhibited this increase (Fig. 5C; 128 ± 2% in hypoxia vs. 90 ± 2% in ACh + hypoxia, $P < 0.01$, $n = 4$). To elucidate the dependency of the ACh-induced protective effect on HIF-1α, dn HIF-1α was transfected into HEK293 cells, followed by ACh pretreatment and then hypoxia. It was found that dn HIF-1α inhibited the protective effect of ACh from hypoxia (Fig. 5D; 115.1 ± 1.2% in wt HIF-1α and 111.8 ± 1.8% in GFP vs. 59.0 ± 3.4% in dn HIF-1α, $P < 0.05$, $n = 10$), suggesting that HIF-1α induction by ACh is partially responsible for the protective effect.

3.5. Effect of vagal stimulation on HIF-1α in myocardial ischemia

To evaluate the significance of ACh for cardioprotection in vivo, the vagal nerve was stimulated prior to the MI. Histological analysis demonstrated a tendency for the infarcted area from the vagal nerve-stimulated (MI-VS) hearts to be smaller than that from non-stimulated (MI) hearts (31.5 ± 4.6% in MI-VS vs. 40.9 ± 2.5% in MI, $n = 3$), even though the risk areas (non-perfused areas) were comparable (Fig. 6A; 59.2 ± 1.0% in MI-VS vs. 53.7 ± 1.0% in MI, $n = 3$). In the MI-VS hearts, the HIF-1α protein level was further elevated compared to that in the MI hearts (Fig. 6B; 244 ± 24% in MI-VS vs. 112 ± 1% in MI, $n = 3$). These results suggest that vagal nerve stimulation in the ischemic heart activates both the hypoxic and non-hypoxic pathways of HIF-1α induction, resulting in increased induction of HIF-1α.

3.6. Non-hypoxic induction of HIF-1α in other cells

The observed ACh-mediated HIF-1 induction was not limited to H9c2 or primary cultured cardiomyocytes, but also found in several other types of cell lines, including HEK293, and HeLa cells (Fig. 7). Since these cells did not beat spontaneously, the results suggest that the system of ACh-mediated HIF-1α induction is not only independent of the beating rate of cardiomyocytes, but also a generally conserved system in cells.

4. Discussion

4.1. Cardioprotective action by ACh and vagal stimulation via the muscarinic receptor

Using animal models, several studies have shown that accentuated antagonism against the sympathetic nervous system is a major mechanism for the beneficial effect of vagal tone on the ischemic heart [13]. Although ACh was involved in triggering preconditioning mechanisms in an ischemia-reperfusion model [3], it remained unclear whether vagal nerve stimulation in acute ischemia or hypoxia followed these mechanisms. In the present study, we have disclosed that ACh possesses a

protective effect on cardiomyocytes. In rat cardiomyocytes, ACh triggered a sequence of survival signals through Akt that eventually induced HIF-1α, inhibited the collapse of the mitochondrial membrane potential and decreased caspase-3 activity, thereby leading to the survival of cardiomyocytes under hypoxia. Furthermore, our results suggest ACh exerts this action through Akt in other cells. The current study therefore provides another insight into the cellular mechanism for the cardioprotective effects of ACh and vagal stimulation.

4.2. Signaling pathway of ACh via PI3K/Akt and antiapoptotic effects of ACh

Since previous studies demonstrated that a PI3K inhibitor greatly reduced HIF-1α induction in heart and renal cells [14,15] and a few studies have reported that MAP kinase is activated through ACh, we focused on the PI3K/Akt pathway, one of the important cell survival signaling pathways [16], and found that ACh directly activated Akt phosphorylation via PI3K. PI3K/Akt signaling has been reported to have an

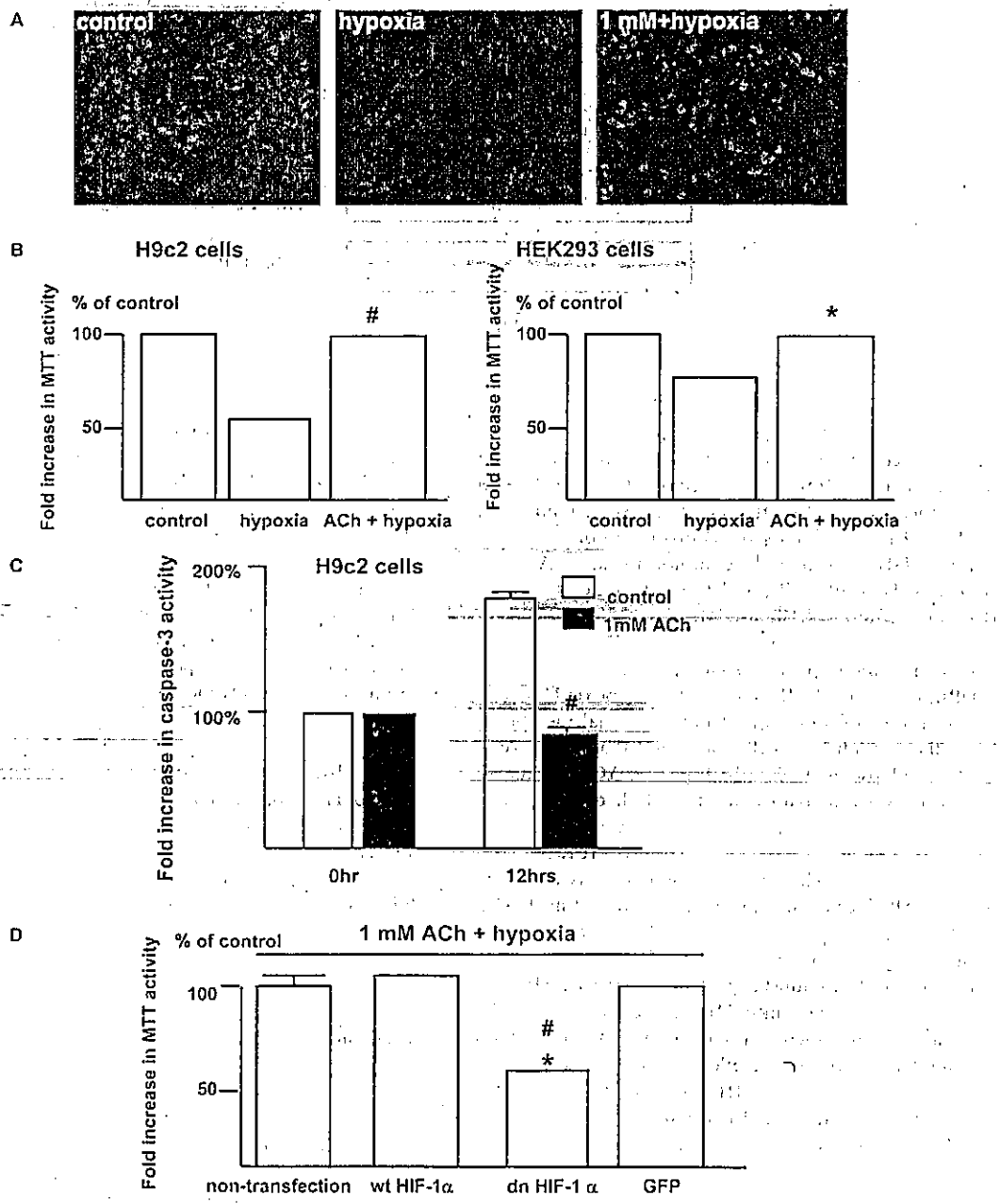

Fig. 5. Collapse of the mitochondrial membrane potential in rat cardiomyocytes under hypoxia is attenuated by ACh pretreatment. (A) Hypoxia decreases the mitochondrial membrane potential in H9c2 cells within 12 h. Red spots are decreased by hypoxia, whereas pretreatment with 1 mM ACh for 12 h inhibits this effect. (B) Pretreatment with 1 mM ACh inhibits the decrease in MTT reduction activity induced by 12 h of hypoxia not only in H9c2 cells ($^{\#}P < 0.01$ vs. hypoxia, $n = 8$) but also in HEK293 cells ($^{*}P < 0.01$ vs. hypoxia, $n = 8$). (C) Hypoxia increases caspase-3 activity, whereas pretreatment with 1 mM ACh inhibits this effect ($^{\#}P < 0.01$ vs. hypoxia, $n = 3$). (D) In contrast to wt HIF-1α or GFP, dn HIF-1α alone decreases the MTT activity under hypoxia after ACh treatment ($^{\#}P < 0.01$ vs. wt and GFP, $^{*}P < 0.05$ vs. non-transfection, $n = 10$).

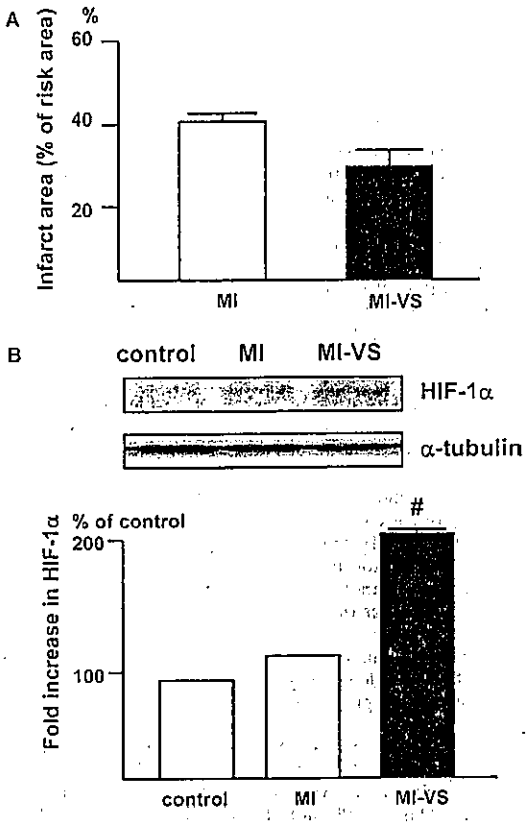

Fig. 6. Vagal nerve stimulation decreases infarcted area with increased HIF-1α expression. (A) A quantitative analysis reveals comparable non-perfused areas in both vagal-stimulated (MI-VS) and non-stimulated (MI) hearts, whereas the infarcted area identified by TTC staining is smaller in the MI-VS heart than in the MI heart. (B) HIF-1α induction in the ischemic heart is increased by vagal stimulation (MI-VS) compared with that in ischemia alone (MI) ($^{\#}P < 0.01$ vs. MI) ($n = 3$).

antiapoptotic activity through various features, such as inhibition of Bad-binding to Bcl-2, caspase 9, Fas and glycogen synthetase kinase-3 [17,18]. These facts imply a definite involvement of Akt activation in cell survival. As shown using dn HIF-1α, ACh inhibited hypoxia-induced cell death through HIF-1α induction via Akt phosphorylation. These results indicate that ACh actually protects cardiomyocytes from hypoxia at the cellular level.

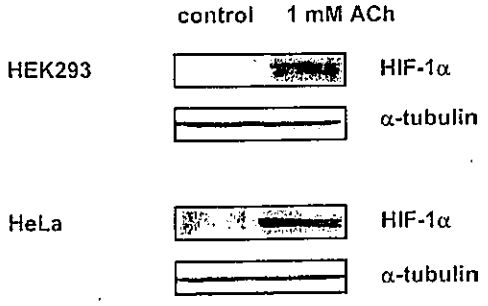

Fig. 7. HIF-1α is induced by ACh under normoxia in other cells. ACh (1 mM) increases HIF-1α protein level in HEK293 and HeLa cells ($n = 3$ each) under normoxia.

4.3. Additional induction of HIF-1α by ACh and vagal stimulation

HIF-1α regulates the transcriptional activities of very diverse genes involved in cell survival and is itself regulated at the posttranslational level by VHL [4,6,7]. Recent studies have shown that HIF-1α is also regulated through a non-hypoxic pathway involving angiotensin II, TNF-α and NO [8,9,19,20]. Therefore, it is speculated that cardiomyocytes possess a similar system for regulating HIF-1α through ACh, independent of the oxygen concentration. Induction of HIF-1α is a powerful cellular response against hypoxia, and further increases in its expression by other pathways may be beneficial. The present results indicate that the significance of ACh or vagal nerve stimulation in hypoxic stress can be attributed to additional HIF-1α induction through dual induction pathways, i.e., hypoxic and non-hypoxic pathways.

The present study has revealed that ACh-mediated HIF-1α induction is widely conserved in other cells. Consistent with a previous report [10], the current results suggest that NO is produced by ACh. According to a report that NO attenuates the interaction between pVHL and HIF-1α through inhibiting PHD activity [21], it is possible that ACh may increase the HIF-1α protein level through NO. Recent studies conducted by Krieg et al. [3] and Xi et al. [22], have provided supportive data compatible with our results, while another study by Hirota et al. [23] also revealed a non-hypoxic pathway for HIF-1α induction by ACh in a human kidney-derived cell line.

The signaling pathway of the muscarinic receptor has been studied extensively, and many pathways are involved in its specific biological effects. Therefore, possible involvement of other pathways in the non-hypoxic induction of HIF-1α cannot be excluded. However, it was demonstrated that dn Akt and dn HIF-1α decreased the effect of ACh. Consistent with a recent study [24], we have revealed that ACh or vagal stimulation protects cardiomyocytes in the acute phase. This observation suggests that the protective effect in the acute phase may result in inhibition of cardiac remodeling in the chronic phase, since vagal stimulation produces additional HIF-1α induction through a non-hypoxic pathway, which increases cell survival.

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Involvement of Transforming Growth Factor-β1 Signaling in Hypoxia-induced Tolerance to Glucose Starvation*

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Because survival and growth of human hepatoma cells are maintained by nutrient, especially glucose, glucose starvation induces acute cell death. The cell death is markedly suppressed by hypoxia, and we have reported involvement of AMP-activated protein kinase-α (AMPK-α), Akt, and ARK5 in hypoxia-induced tolerance. In the current study we investigated the mechanism of hypoxia-induced tolerance in human hepatoma cell line HepG2. ARK5 expression was induced in HepG2 cells when they were subjected to glucose starvation, and we found that glucose starvation transiently induced Akt and AMPK-α phosphorylation and that hypoxia prolonged phosphorylation of both protein kinases. We also found that hypoxia-induced tolerance was partially abrogated by blocking the Akt/ARK5 system or by suppressing AMPK-α expression and that suppression of both completely abolished the tolerance, suggesting that AMPK-α activation signaling and the Akt/ARK5 system play independent essential roles in hypoxia-induced tolerance. By using chemical compounds that specifically inhibit kinase activity of type I-transforming growth factor-β (TGF-β) receptor, we showed an involvement of TGF-β in hypoxia-induced tolerance. TGF-β1 mRNA expression was induced by hypoxia in an hypoxia-inducible factor-1α-independent manner, and addition of recombinant TGF-β suppressed cell death during glucose starvation even under normoxic condition. AMPK-α, Akt, and ARK5 were activated by TGF-β1, and Akt and AMPK-α phosphorylation, which was prolonged by hypoxia, was suppressed by an inhibitor of type I TGF-β receptor. Based on these findings, we propose that hypoxia-induced tumor cell tolerance to glucose starvation is caused by hypoxia-induced TGF-β1 through AMPK-α activation and the Akt/ARK5 system.

Because tumor cell survival and growth are maintained by nutrients, especially glucose, and oxygen supplied by blood vessels, angiogenesis has been concluded to be essential for tumor malignancy (1, 2). Human hepatoma cell lines exhibited acute cell death when cells were subjected to glucose starvation (3, 4), but we have shown that hypoxic conditions allow tumor cells to survive under glucose starvation (5). Although an involvement of hypoxia-inducible factor-1 (HIF-1)[1] in hypoxia response has been known well (6, 7), we showed that hypoxia-induced tolerance to glucose starvation in tumor cells seemed to be caused by an HIF-1-independent mechanism because deferoxamine did not suppress cell death by glucose starvation (5). It has recently been found that most tumors have insufficient supplies of nutrients and oxygen because of an imbalance between demand, caused by both uncontrolled cell proliferation, and deformed vascularity (8), and it has been proposed that hypoxia is essential for tumor progression (7, 9–11). Our investigation of hypoxia-induced tumor cell tolerance to glucose starvation has demonstrated that both Akt and the AMPK family, especially AMPK-α1, are needed for signal transduction (4, 5); however, the precise molecular mechanism remains to be clarified.

AMPKs are a class of serine/threonine protein kinases, and their activation has been well documented in cells exposed to metabolic stress (12–14). Three subunits, α, β, and γ, have been identified in AMPKs, and the α-subunit has been demonstrated to bear their catalytic activity (12–14). At present two isoforms have been identified as the AMPK catalytic subunit family (AMPK-α1 and AMPK-α2 (15, 16)), and some related kinases, including MELK (17), SNARK (18, 19), and ARK5 (20), have also been identified as novel members of the AMPK catalytic subunit family. ARK5 is unique in that it is directly activated by Akt, and activated ARK5 suppresses cell death induced by glucose starvation and death receptor activation (20–22). We have also reported that ARK5 is closely involved in hypoxia-induced tolerance to glucose starvation in human hepatoma HepG2 cells (20).

Regulation of tissue development and homeostasis by TGF-β is well known (23). Recently, an induced expression of TGF-β under hypoxic condition in osteoblast was reported, and the induction was HIF-1-independent (24). The intracellular signaling induced by TGF-β is initiated by ligation to receptor (25) and is mediated by a unique pathway, Smad pathway (25). Smad mediated signaling from TGF-β receptor directly toward nuclei via phosphorylation (25). In addition to the Smad path-

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[1] The abbreviations used are: HIF-1: hypoxia-inducible factor-1; AMPK, AMP-activated protein kinase; PI, propidium iodide; TGF, transforming growth factor; RT, reverse transcription; PBS, phosphate-buffered saline; RNAi, RNA-mediated interference; DN, dominant negative; wt, wild type; ARK5, AMPK-related kinase 5; MELK, maternal leucine zipper kinase; SNARK, SNF1/AMPK-related kinase.

way, phosphatidylinositol-3 kinase stimulation via Ras/mitogen-activated kinase has been found to be an intracellular signaling pathway induced by TGF-β receptor (26). Recent investigations have revealed that TGF-β is closely involved in tumor malignancy via induction of cell survival, invasion, and metastasis (27–29).

In the present study we investigated the mechanism of hypoxia-induced tolerance to glucose starvation by human hepatoma HepG2 cells, and results showed that TGF-β expression is stimulated by hypoxia and that TGF-β is closely involved in hypoxia-induced tumor cell tolerance to glucose starvation through activation of Akt/ARK5 system.

EXPERIMENTAL PROCEDURES

Cell Lines and Transfection—Human hepatoma cell line HepG2 was maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (Sigma).

For transfection cells were seeded into a 6-well plates at 2.5×10^5/well, and transfection was performed with TransFast Transfection reagent (5 μg of DNA/well; Promega Corp.). Cells were exposed to transfection reagent for 4 h. Transfection yield was measured with green fluorescent protein-inserted expression plasmid and was 70–80%.

Recombinant Protein, Antibody, Chemical Inhibitor, and Plasmid—Recombinant human TGF-β1 was purchased from R&D Systems Ltd. Polyclonal antibodies against Akt (total and phosphorylated Ser-473), AMPK-α (total and phosphorylated Thr-172), and Smad2 (phosphorylated Ser-465/467) were purchased from Cell Signaling Technology Inc. The antibody for total Smad2 was purchased from Upstate Biotechnology, Inc. SB431542 was purchased from TOCRIS. Dominant-negative Akt1 was purchased from Upstate Biotechnology. The same antisense RNA expression vector of ARK5 and dominant negative ARK5 were used as in our previous studies (20). DE mutant of Smad3 was a generous gift from Drs. Miyazono and Imamura of The Cancer Institute of the Japanese Foundation for Cancer Research.

RNA Extraction and RT-PCR—Total RNA extraction was performed with Isogen purchased from Nippon Gene Co., Ltd. The concentration of extracted RNA was measured at A_{260}, and 0.5 μg of total RNA was reverse-transcribed with avian myeloblastosis virus transcriptase (TaKaRa Bio Co., Ltd.). After reverse transcription, PCR was performed with an LA PCR kit (Takara) using primer pairs for human TGF-β1 and type I/II/III TGF-β receptor. PCR was performed for 25 cycles.

Western Blot Procedure—Proteins were prepared for Western blot analysis by lysing cells for 30 min with PBS containing 1% Nonidet P-40 and centrifugation at 15,000 rpm for 15 min. All procedures were carried out at 4 °C. Concentrations were determined with a BCA protein assay kit (Pierce) with bovine serum albumin as a standard.

Sample proteins separated by SDS-PAGE were transferred onto nitrocellulose membranes by a semidry blotting system. Membranes were blocked at room temperature for 1 h with PBS containing 5% (w/v) skim milk (BD Biosciences), washed with a mixture of PBS and 0.05% Tween 20 (Sigma; Tween PBS), and then incubated overnight at room temperature with antibody diluted with PBS. After washing with Tween PBS, membranes were incubated at room temperature for 60 min with a 2000-fold diluted horseradish peroxidase-conjugated anti-rabbit IgG antibody (Santa Cruz Biotechnology Inc.). Membranes were then washed with Tween PBS and developed with ECL system (Amersham Biosciences.).

Cell Survival Assay—Cell viability was assessed by Hoechst 33342/PI staining procedure. Hoechst 33342 and PI were purchased from Molecular Probes, Inc. After incubation, cells were collected and stained with Hoechst 33342 and PI and then examined by fluorescence microscopy. Cell survival was measured as the ratio of cells carrying PI-unstained nuclei to all cells counted (~1000 cells).

RESULTS AND DISCUSSION

Involvement of Akt/ARK5 System in Hypoxia-induced Tolerance to Glucose Starvation—We have reported that glucose starvation induces cell death in HepG2 cells (3, 5, 21). In the current study we showed that cell survival of HepG2 cells was dependent on glucose concentration and that hypoxia delayed cell death induced by glucose starvation (Fig. 1*A*). In addition, hypoxia-induced tolerance to glucose starvation was also exhibited in non-tumor cells, fibroblast cell lines Rat1 and KMST6 (Fig. 1*B*). Because we had previously shown that AMPK-α, especially the AMPK-α1 subunit, Akt, and ARK5 are required for hypoxia-induced tolerance to glucose starvation (5, 20), we first investigated ARK5 protein expression by HepG2 cells by Western blotting. As shown in Fig. 1*C*, no protein expression of ARK5 was detected in HepG2 cells in the presence of glucose under normoxia, but ARK5 expression was induced when cells were exposed to 12 h of glucose starvation (Fig. 1*C*). Brief glucose starvation (<12 h) did not induce ARK5 expression (data not shown). Cells exposed to glucose starvation under hypoxic conditions, but not cells exposed to hypoxia alone, also expressed ARK5 (Fig. 1*C*), suggesting that ARK5 expression is regulated by glucose status, not by oxygen reduction. As stated above, we previously demonstrated that overexpression of ARK5 in HepG2 cells suppressed cell death during glucose starvation; however, results of the present study showed that HepG2 cells underwent cell death during glucose starvation even though ARK5 expression was markedly increased at 12 h. Because ARK5 activity is regulated by Akt (20, 21), we examined the effects of glucose starvation and/or hypoxia on Akt status in HepG2 cells to investigate why the newly expressed ARK5 did not suppress cell death during glucose starvation. We also investigated the phosphorylation status of AMPK-α, which is also required for hypoxia-induced tolerance to glucose starvation (5). As shown in Fig. 1*D*, Akt and AMPK-α phosphorylation increased when cells were exposed to glucose starvation, whereas there was no increase in phosphorylation of Akt and AMPK-α when cells were exposed to glucose-containing medium or hypoxia alone (Fig. 1*D*). Clear glucose starvation-induced phosphorylation of Akt and AMPK-α was observed after 3 h of glucose starvation, but their phosphorylation had decreased after 12 h and had disappeared by 24 h (Fig. 1*D*). Because HepG2 cells exposed to glucose starvation for 24 h undergo caspase-dependent cell death (21) and some protein kinases, including Akt, are caspase substrates (30), we suspect that decreased expressions of Akt and AMPK-α is caused by transduction of cell death signaling. No glucose starvation-induced phosphorylation of either molecule was observed after 3 h of glucose starvation during hypoxia, but phosphorylation of both was detected at 12 h, and it was observed even after 24 h of glucose starvation under hypoxic conditions (Fig. 1*D*). In the present study we observed transient phosphorylation of Akt in cells exposed to glucose starvation, but hypoxia prolonged phosphorylation of both Akt and AMPK-α during glucose starvation. ARK5 was newly expressed after 12 h of glucose starvation, but glucose starvation-induced phosphorylation of Akt was significantly decreased at 12 h. We, therefore, hypothesized that the newly expressed ARK5 in HepG2 cells exposed to glucose starvation under normoxic condition is inactive because of the absence of sufficient active Akt and that hypoxia is required for ARK5 activation through prolonged activation of Akt to allow cells to survive during glucose starvation. In addition, we found that even the first 3 h exposure to hypoxia suppressed cell death by glucose starvation, and some protein kinase phosphorylation and *de novo* protein synthesis occurred within these 3 h.[2] A more detailed mechanism that can explain an importance of this first 3-h reaction for hypoxia-induced tolerance to glucose starvation should be clarified. Our results showed that prolonged phosphorylation of Akt is required for ARK5 activation that is essential for hypoxia-induced tolerance to glucose starvation. In addition to these findings, the fate to survival or death after glucose starvation might be decided within a very early term.

To determine whether blocking of the Akt/ARK5 system

[2] A. Suzuki, G.-i. Kusakai, Y. Shimojo, J. Chen, T. Ogura, M. Kobayashi, and H. Esumi, unpublished data.

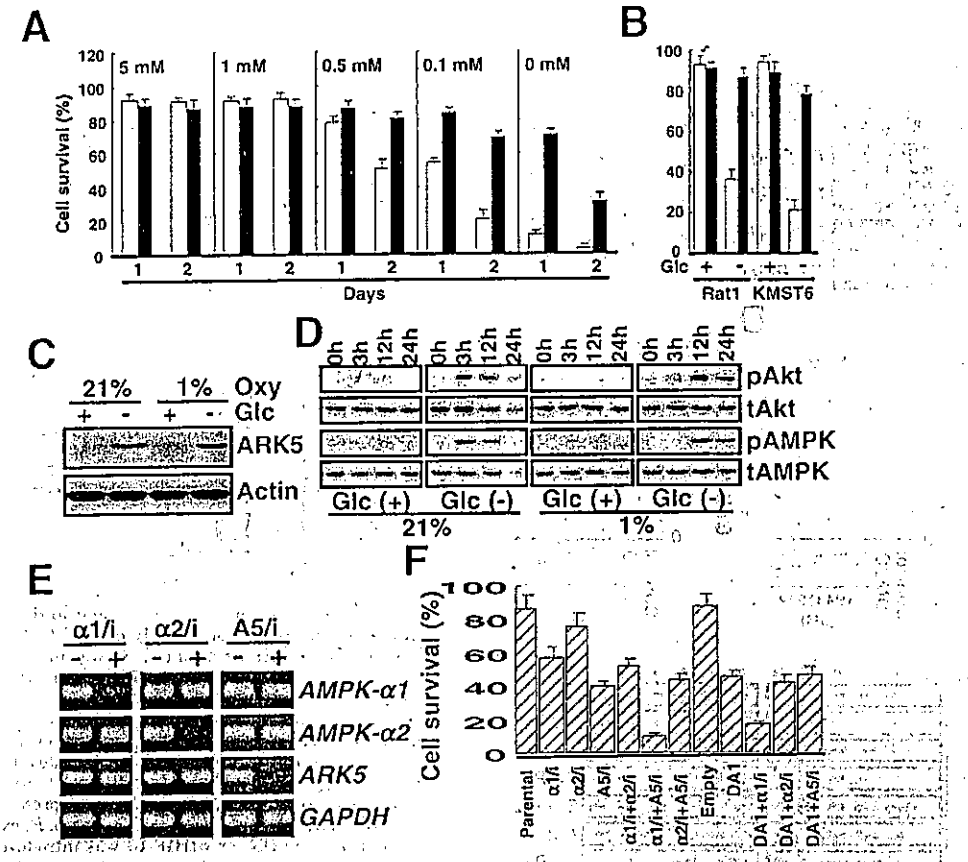

FIG. 1. **Involvement of AMPK-α, Akt, and ARK5 in hypoxia-induced tolerance to glucose starvation in HepG2 cells.** *A*, HepG2 cells were exposed for 1 or 2 days to medium containing 0–5 mM glucose under normoxic (*open*) or hypoxic (*closed*) conditions, and cell survival was measured by a Hoechst 33342/PI staining procedure. Cell survival is reported as the mean of data from three experiments, and *bars* represent S.E. values. *B*, rat fibroblast Rat1 or human fibroblast KMST6 cell lines were exposed for 24 h to medium containing (+) or not containing (−) glucose (*Glc*) under normoxic (*open*) or hypoxic (*closed*) conditions, and cell survival was measured by a Hoechst 33342/PI staining procedure. Cell survival is reported as the mean of data from three experiments, and *bars* represent S.E. values. *C*, HepG2 cells were exposed for 12 h to medium containing or not containing glucose (±*Glc*) under normoxic (*Oxy, 21%*) or hypoxic (*Oxy, 1%*) conditions, and cell extracts were collected for Western blotting with antibodies against ARK5 and β-actin (*Actin*). *D*, HepG2 cells were cultured in serum-free medium for 16 h and exposed to medium containing or not containing glucose (±*Glc*) under normoxic (*Oxy, 21%*) or hypoxic (*Oxy, 1%*) conditions for each period indicated, and cell extracts were collected for Western blotting with antibodies against phosphorylated (*pAkt* and *pAMPK*) and total (*tAkt* and *tAMPK*) Akt and AMPK-α. *E*, HepG2 cells were transfected (+) or not transfected (−) with RNAi for AMPK-α1 (*α1/i*), AMPK-α2 (*α2/i*), or ARK5 (*A5/i*), and RNA was extracted 48 h later for RT-PCR (AMPK-α1 and AMPK-α2, 30 cycles; ARK5, 35 cycles; glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), 28 cycles). *F*, HepG2 cells were transiently transfected or not transfected (*Parental*) with RNAi for AMPK-α1 (*α1/i*), AMPK-α2 (*α2/i*), or ARK5 (*A5/i*) or expression vector containing or not containing (*Empty*) the dominant-negative form of Akt1 (DN-Akt1). After 48 h cells were incubated for 24 h under hypoxic condition in medium without glucose, and cell survival was measured by a Hoechst 33342/PI staining procedure. Cell survival is reported as the mean from three experiments, and *bars* represent S.E. values.

and/or AMPK-α activation influences hypoxia-induced tolerance to glucose starvation in the present study, we prepared RNAi for AMPK-α1, AMPK-α2, and ARK5. When each RNAi was introduced into cells, it specifically suppressed mRNA expression of its target factor (Fig. 1*E*). As shown in Fig. 1*F*, introduction of RNAi for AMPK-α1 or ARK5 or DN-Akt1 partially suppressed hypoxia-induced cell survival, but RNAi for AMPK-α2 had no effect on hypoxia-induced cell survival. The combined introduction of RNAi for AMPK-α1 and ARK5 or of RNAi for AMPK-α1 and DN-Akt1 completely suppressed hypoxia-induced cell survival (Fig. 1*F*), suggesting that two distinct signaling pathways are required for hypoxia-induced cell survival against glucose starvation, one mediated by Akt/ARK5 and the other by AMPK-α1.

Involvement of TGF-β1 in Hypoxia-induced Tolerance to Glucose Starvation—TGF-β1 has been reported to be closely involved in tumor progression, characterized by induction of tumor cell survival, invasion, and metastasis (27–29), and TGF-β1 gene has been reported to be a hypoxia-responsible gene (24). In the present study we observed increased expression of TGF-β1 mRNA in HepG2 cells exposed to 12 h of hypoxia (Fig. 2*A*), and HepG2 cells exposed to glucose starvation under hypoxic conditions expressed TGF-β1 mRNA slightly more weakly than cells exposed to hypoxia alone (Fig. 2*A*). However, under normoxic conditions, HepG2 cells exposed to medium containing or not containing glucose did not show any increase in expression of TGF-β1 mRNA (Fig. 2*A*). By contrast with TGF-β1, neither glucose nor oxygen status affected expressions of TGF-β receptor I, II, and III mRNAs (Fig. 2*A*), and tolerance to glucose starvation was induced by recombinant TGF-β1 protein even under normoxic conditions (Fig. 2*B*). These findings suggest that TGF-β1 mRNA expression is responsible for oxygen status in HepG2 cells and hypothesize an involvement of TGF-β1 in the tolerance to glucose starvation.

To confirm the above hypothesis, we investigated an effect of specific inhibitor for TGF-β receptor 1 kinase activity SB431542 (31–33) on hypoxia-induced tolerance to glucose starvation. As shown in Figs. 2, *C* and *D*, tolerance of HepG2 cells to glucose starvation that was induced by hypoxia (Fig. 2*C*) or recombinant TGF-β1 (Fig. 2*D*) was suppressed by

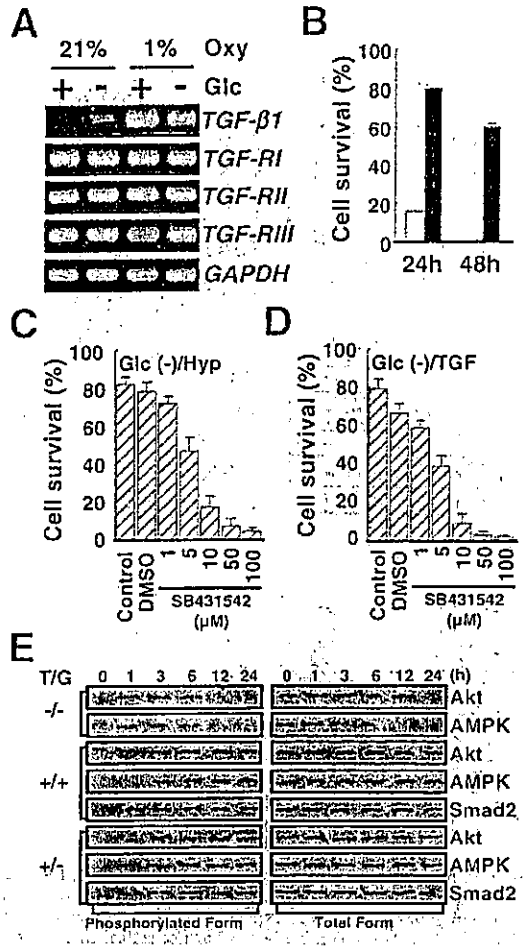

FIG. 2. **Involvement of TGF-β1 in hypoxia-induced tolerance to glucose starvation in HepG2 cells.** *A*, expression of TGF-β1, type I TGF-β receptor (*TGF-RI*), and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNAs was investigated by RT-PCR using total RNA extracted from HepG2 cells exposed for 12 h to medium containing (*Glc+*) or not containing (*Glc−*) glucose under normoxic (*Oxy*, 21%) or hypoxic (*Oxy*, 1%) conditions. *B*, HepG2 cells were exposed for 24 or 48 h to medium without glucose in the presence (*closed*) or absence (*open*) of human recombinant TGF-β1 (20 ng/ml) for 24 or 48 h. Cell survival is reported as the mean of data from three experiments, and *bars* represent S.E. values. *C*, HepG2 cells were incubated for 24 h in medium without glucose under hypoxic conditions in the presence or absence (*Control*) of SB431542 (0 mM Me_2SO (*DMSO*); 1–100 μM SB431542), and cell survival was measured by a Hoechst 33342/PI staining procedure. Cell survival is reported as the mean of data from three experiments, and *bars* represent S.E. values. *D*, HepG2 cells exposed to glucose starvation were incubated for 24 h in the presence or absence (*Control*) of SB431542 (0 mM Me_2SO; 1–100 μM SB431542), and cell survival was measured by a Hoechst 33342/PI staining procedure. Cell survival is reported as the mean of data from three experiments, and *bars* represent S.E. values. *E*, HepG2 cells subjected to serum-free medium for 16 h were exposed medium containing or not containing human recombinant TGF-β (*T*) and/or glucose (*G*), and cell extracts were collected at each period for Western blotting using antibodies for phosphorylated or total form of Akt, AMPK, and Smad2.

SB431542 in a dose-dependent manner, suggesting that hypoxia promotes TGF-β1 production, which in turn plays a central role in hypoxia-induced tolerance to glucose starvation in HepG2 cells.

In addition, we investigated whether TGF-β induces phosphorylation of Akt, AMPK-α, and Smad2 in HepG2 cells under the presence or absence of glucose using Western blotting. Although glucose starvation did not affect Smad2 status in HepG2 cells (data not shown), the phosphorylation status of

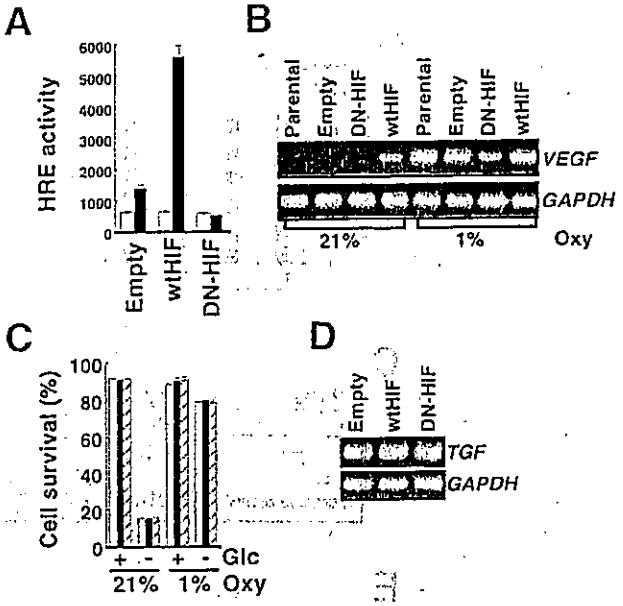

FIG. 3. **Hypoxia-induced tolerance and TGF-β1 mRNA expression are caused independently of HIF-1α.** *A*, transcriptional activity of wtHIF or DN-HIF forms of HIF-1α was investigated using the 5xHRE-containing luciferase vector (39). Luciferase activity was measured with cell extracts from HepG2 cells transiently transfected with expression vector containing or not containing (*Empty*) the dominant-negative form or wild-type HIF-1α under normoxic (*open*) or hypoxic (*closed*) conditions for 24 h. Transfection yield was normalized to 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal). Luciferase activity is shown as the mean of data from three experiments, and *bars* represent S.E. values. *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase. *B*, expression vector with or without (*Empty*) DN- or wtHIF-1α was introduced into HepG2 cells. At 48 h after transfection, cells were incubated for 24 h under normoxic (*Oxy, 21%*) or hypoxic (*Oxy, 1%*). Parental HepG2 cells (*Parental*) were also incubated under normoxic or hypoxic conditions, and expression of vascular endothelial growth factor (*VEGF*) or GAPDH mRNA was investigated by RT-PCR. *C*, HepG2 cells transiently transfected with vector containing or not containing (*open*) DN-HIF (*closed*) or wtHIF (*shaded*) were subjected for 24 h to medium containing (*Glc+*) or not containing (*Glc−*) glucose under normoxic (*Oxy, 21%*) or hypoxic (*Oxy, 1%*), and cell survival was measured by a Hoechst 33342/PI staining procedure. Cell survival is reported as mean of data from three experiments, and *bars* represent S.E. values. *D*, HepG2 cells transiently transfected with vector containing or not containing (*Empty*) the *DN-HIF*) or wtHIF. HIF-1α were incubated for 12 h under hypoxic condition, and then total RNAs were extracted, and RT-PCR for TGF-β1 or glyceraldehyde-3-phosphate dehydrogenase was performed.

Akt and AMPK-α exhibited drastic changes by glucose starvation and/or TGF-β treatment. As shown in Fig. 2*E*, TGF-β induced phosphorylation of Akt, AMPK-α, and Smad2 in the presence of glucose. Glucose starvation transiently induced phosphorylation of Akt and AMPK-α, and TGF-β sustained the phosphorylation (Fig. 2*E*). Thus, we showed that TGF-β induces sustained phosphorylation of protein kinases those are needed for hypoxia-induced tolerance to glucose starvation in HepG2 cells.

Hypoxia-induced Tolerance to Glucose Starvation Is Independent of HIF-1—Results of this study strongly suggested a critical role of TGF-β in hypoxia-induced tolerance to glucose starvation in HepG2 cells, and TGF-β mRNA expression has been reported to be stimulated by hypoxia (24, 34, 35). Because HIF-1α is well known to be a mediator of hypoxia-response gene expression (6, 36, 37), we investigated whether HIF-1 is involved in hypoxia-induced tolerance to glucose starvation by using a dominant-negative HIF-1α (DN-HIF and Ref. 38). A luciferase reporter gene assay using a five-repeat HIF-responsive element-containing luciferase reporter plasmid (5xHRE/luc and ref. 39) was used to confirm the dominant-negative

activity of DN-HIF. As shown in Fig. 3*A*, an ~2-fold increase in luciferase activity was observed in HepG2 cells during hypoxia, and when both 5xHRE/luc and wild-type HIF-1α (wtHIF) expression vector were introduced into HepG2 cells, hypoxia induced a dramatic increase in activity (>10-fold) (Fig. 3*A*). DN-HIF transfection, on the other hand, completely suppressed an increase induced by hypoxia (Fig. 3*A*). We also investigated whether hypoxia-induced vascular endothelial growth factor (*VEGF*) expression is suppressed by DN-HIF by means of RT-PCR. As shown in Fig. 3*B*, no vascular endothelial growth factor mRNA expression was observed under normoxic condition, except in cells overexpressing wtHIF. A dramatic increase in expression was detected when subjected to hypoxia, but DN-HIF transfection markedly suppressed it. These findings indicate that DN-HIF used in this study functions well as a dominant-negative factor.

In our previous study hypoxia-induced tolerance to glucose starvation seemed not to be associated with HIF-1 action (5), and the above-mentioned-DN-HIF was used in the current study to directly address the question of whether HIF-1 activation is involved in tolerance to glucose starvation. As shown in Fig. 3*C*, transient expression of DN-HIF did not affect survival of HepG2 cells exposed to glucose starvation for 24 h under hypoxic conditions. Wild-type HIF-1α did not affect it either (Fig. 3*C*), confirming that hypoxia-induced tolerance to glucose starvation in HepG2 cells is promoted by HIF-1-independent signaling. We also investigated whether HIF-1α is involved in hypoxia-induced TGF-β1 mRNA expression by RT-PCR. As shown in Fig. 3*D*, transient expression of wtHIF-1 or DN-HIF had no effect on hypoxia-induced TGF-β mRNA expression.

Hypoxia-induced Tolerance to Glucose Starvation Is Independent of the Smad Pathway—The Smad pathway is well known as the most important intracellular signal transduction system that mediates the effects of TGF-β (27, 40, 41). Because the results of the present study revealed that TGF-β is required for hypoxia-induced tolerance to glucose starvation, we investigated whether the Smad pathway is a necessary part of the mechanism by using DE-mutated Smad3 (Smad3/DE), which has a dominant-negative effect (42). As shown in Fig. 4*A*, Smad/DE was expressed by HepG2 cells and suppressed phosphorylation of Smad2 in response to stimulation with human recombinant TGF-β1, indicating a dominant-negative effect (Fig. 4*B*). Although Smad3/DE suppressed Smad2 phosphorylation by TGF-β1, it had no effect on phosphorylation of Akt and AMPK-α by TGF-β1 (Fig. 4*C*). Hypoxia-induced tolerance to glucose starvation was also unaffected by Smad3/DE (Fig. 4*D*). Based on these findings, we concluded that tolerance to glucose starvation by TGF-β is Smad-independent and that the AMPK-α1 and Akt/ARK5 pathway downstream of TGF-β receptor may be essential for hypoxia-induced tolerance to glucose starvation to develop. We also recently confirmed that hypoxia-induced tolerance in human colon cancer cell lines is independent of the Smad pathway.[3]

Hypoxia-induced Tolerance to Glucose Starvation Is Mediated by Sustained Activation of the Akt/ARK5 System Stimulated by TGF-β—When HepG2 cells were exposed to human recombinant TGF-β1, phosphorylation of Akt (Ser-473), ARK5 (Ser-600), and AMPK-α (Thr-172) was observed in both the presence and absence of glucose (Figs. 4*C* and 5*A*). In addition to that shown in Fig. 5*B*, Akt and AMPK-α phosphorylation observed in HepG2 cells exposed to glucose starvation under hypoxic condition was suppressed by 10 μM SB431542, suggesting that TGF-β is closely involved in sustained activation of

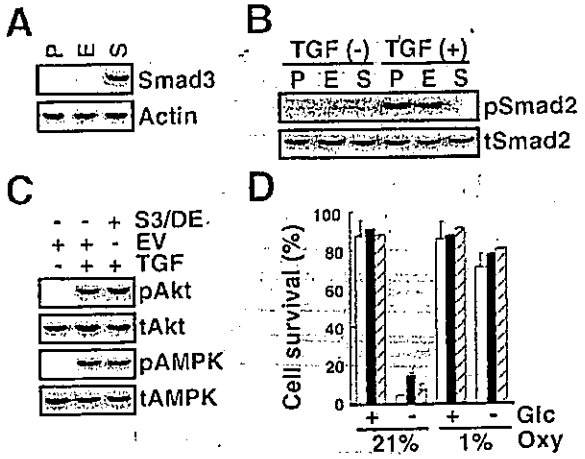

FIG. 4. **Smad pathway is not required for hypoxia-induced tolerance to glucose starvation.** *A*, HepG2 cells were transfected or not transfected with (*P*) expression vector containing or not containing (*E*) FLAG-tagged Smad3/DE (*S*), and cell extracts collected 48 h after transfection were used for Western blotting with antibody against FLAG (Smad3) or actin. *B*, HepG2 cells were transfected or not transfected with (*P*) expression vector containing or not containing (*E*) FLAG-tagged Smad3/DE (*S*), and after the subjection to serum-free medium for 16 h cells were exposed to 20 ng/ml TGF-β1 for 2 h. After exposure to TGF-β1, cell extracts were collected for Western blotting with antibody against phosphorylated (*pSmad2*) or total (*tSmad2*) Smad2. *C*, HepG2 cells were transfected with expression vector containing or not containing (*EV*) FLAG-tagged Smad3/DE (*S3/DE*), and after the subjection to serum-free medium for 16 h cells were exposed to (+) or not exposed to (−) 20 ng/ml TGF-β1 for 2 h. After exposure to TGF-β1, cell extracts were collected for Western blotting with antibody against phosphorylated (*pAkt* and *pAMPK*) or total (*tAkt* and *pAkt*) Akt or AMPK-α. *D*, HepG2 cells transfected or not transfected with (*open*) expression vector containing or not containing (*closed*) FLAG-Smad3/DE (*shaded*) were exposed for 24 h to medium containing or not containing glucose (±*Glc*) under normoxic (*Oxy, 21%*) or hypoxic (*Oxy, 1%*) conditions, and cell survival was measured by a Hoechst 33342/PI staining procedure. Cell survival is reported as the mean of data from three experiments, and *bars* represent S.E. values.

Akt and AMPK-α in HepG2 cells exposed to glucose starvation under hypoxic conditions.

Thus, TGF-β is essential for hypoxia-induced tolerance to glucose starvation through induction of Akt and AMPK family member phosphorylations. Because DN-Smad3 did not suppress hypoxia-induced tolerance to glucose starvation, the signal transduction seems to be Smad signaling-independent. Recently it has been reported that an inhibition of adipocytogenesis by hypoxia is caused by TGF-β, and the intracellular signaling was mediated by Smad3 (43). Cells exhibit several responses to hypoxia through TGF-β (6). Although the differentiation seems to be regulated by Smad-dependent signaling, we suggest that cell survival against glucose starvation is regulated by an Smad-independent system at least.

Based on the results of this study, we hypothesize that the molecular mechanism of hypoxia-induced tolerance to glucose starvation is as follows (Fig. 5*C*). When glucose and oxygen supply decreases, cell survival system is activated. Glucose reduction triggers AMPK-α and Akt phosphorylation, but phosphorylation is transient. Transient phosphorylation of Akt in cells exposed to glucose starvation has recently been reported, and possible involvement of ceramide in down-regulation of phosphorylated Akt has also been reported (44). Oxygen reduction stimulates TGF-β mRNA expression, and TGF-β sustained phosphorylation of AMPK-α and Akt. Although our and other research groups reported Akt phosphorylation caused by glucose reduction (3, 4, 44), the present observation is obviously contradictory to previous reports (45–49). Precise mechanisms are not known yet, but many cell lines, in which Akt is endo-

[3] A. Suzuki, G.-i. Kusakai, Y. Shimojo, J. Chen, T. Ogura, M. Kobayashi, and H. Esumi, submitted for publication.

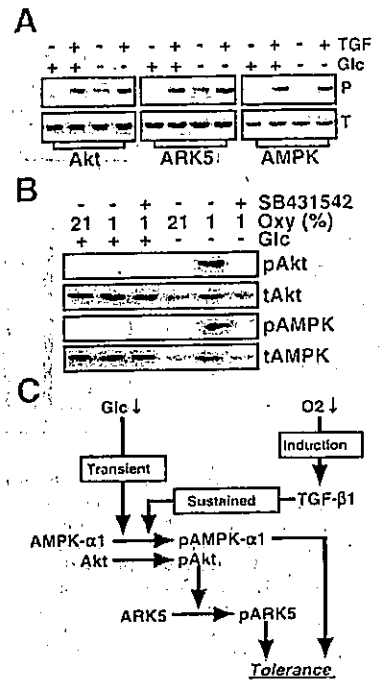

FIG. 5. **Akt/ARK5 system activation induced by TGF-β1.** *A*, HepG2 cells were subjected to serum-free medium for 16 h and then exposed (*TGF+*) or not exposed (*TGF−*) to 20 ng/ml TGF-β1 in the presence (*Glc+*) or absence (*Glc−*) of glucose for 12 h. Cell extracts or immunoprecipitates with anti-FLAG antibody were collected and blotted with antibody against phosphorylated (*P*) Akt (*Akt P*), total Akt (*Akt T*), Akt substrate (*ARK5 P*), FLAG (*ARK5 T*), phosphorylated AMPK-α (*AMPK P*), or total AMPK-α (*AMPK T*). *B*, HepG2 cells exposed (SB431542 +) or not exposed (SB431542 −) to 10 μM SB431542 after 16 h of serum-free culture were incubated for 24 h in medium containing (*Glc+*) or not containing (*Glc−*) glucose under normoxic (*Oxy, 21%*) or hypoxic (*Oxy, 1%*) conditions for 24 h, and cell extracts were collected and blotted with antibody against phosphorylated (*pAkt* and *pAMPK*) or total (*tAkt* and *tAMPK*) Akt or AMPK-α. *C*, schematic model of hypoxia-induced tolerance to glucose starvation on HepG2 cells.

genously and highly activated to play important roles, show tolerance to glucose starvation (Refs. 3 and 4 and colorectal cancer cell lines[3]). The difference might be caused by the difference in cellular background. Another possibility is the role of AMPKs, which exert anti-cell death function during metabolic stresses (4, 21). Phosphorylated Akt, induced by glucose starvation and sustained by hypoxia-induced TGF-β, activates ARK5. We recently demonstrated that necrotic cell death induced by glucose starvation is mediated by caspase-8 (21) and that ARK5 promotes cell survival through suppression of caspase-6-induced flice-like inhibitory protein (FLIP) cleavage (22), suggesting that Akt-activated ARK5 protects cells from glucose starvation under hypoxic condition through caspase inactivation. Results of the present study showed an important role of AMPK-α in hypoxia-induced tolerance to glucose starvation. Upon the stimulation of TGF-β, AMPK-α was also phosphorylated. Tumor suppressor LKB1 was recently identified as the upstream kinase of AMPK-α (50–52); however, the role of LKB1 as AMPKK was limited in cells under low glucose, and we reported that AMPK-α phosphorylation in cells stimulated by growth factor, including TGF-β, is promoted by ataxia telangiectasia-mutated rather than LKB1 (53). Based on these results, we concluded that TGF-β, whose expression is stimulated by oxygen deprivation, is essential for hypoxia-induced tolerance to glucose starvation through promotion of AMPK-α and Akt/ARK5 pathway activation. It is well known that blockage of TGF-β signaling is closely involved in colorectal cancer progression (54–59). Sporadic colorectal cancer cells lack functional Smad4, and the absence of functional type II TGF-β receptor has been reported in hereditary nonpolyposis colorectal cancer cells (60–64). Moreover, it is also well known that the malignant potential of hereditary nonpolyposis colorectal cancer is weaker than that of colorectal cancer and that hypoxia is essential for tumor progression, including the progression of colorectal cancer (7, 9). All of these findings taken together suggest that hypoxia-induced cell survival by TGF-β observed in this study may be related to the malignant potential of colorectal cancer and hereditary nonpolyposis colorectal cancer cells.

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Hypoxia suppresses the production of matrix metalloproteinases and the migration of human monocyte-derived dendritic cells

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As most solid tumors are hypoxic, dendritic cells (DC) in solid tumors are also exposed to hypoxia. While many adaptation responses of tumor cells to hypoxia are known, it is yet to be determined how hypoxia affects the functions of DC. To explore the effects of hypoxia on the functions of DC, we compared the expression of surface markers, cytokines, chemokine receptors and matrix metalloproteinases (MMP) of human monocyte-derived DC (hmDC) differentiated under hypoxia to those differentiated under normoxia. Both groups of hmDC expressed similar levels of surface markers and cytokines. However, expression of MMP-9 and membrane type-1-MMP, as well as migrating activity, was significantly suppressed in hmDC differentiated under hypoxia compared with their normoxia counterparts. We also demonstrated that trichostatin A restored the production of MMP-9 in hmDC, under hypoxia. Collectively, our findings show that a hypoxic microenvironment suppresses the production of MMP in hmDC, most probably through the deacetylation of promoter regions of MMP, thus suppressing the migrating activity of hmDC. Our results suggest that the hypoxic microenvironment in solid tumor tissues may suppress the function of DC.

Key words: Dendritic cell · Hypoxia · Matrix metalloproteinase · Migration

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Abbreviations: 5-AZ: 5-azacytidine · **HIF1α:** hypoxia-inducible factor-1α · **hmDC:** human monocyte-derived DC · **m/im-hmDC-N/H:** mature/immature-hmDC-normoxia/hypoxia · **MMP:** matrix metalloproteinase · **MT1:** membrane type-1 · **TAM:** tumor-associated macrophages · **TIMP:** tissue inhibitors of MMP · **TSA:** trichostatin A

Introduction

Aggressive tumors often have insufficient blood supply, partly because tumor cells grow faster than endothelial cells, and partly because a newly formed vascular supply is disorganized [1–4]. When cells are not supplied with sufficient blood, they are exposed to hypoxia and hypoglycemia. Recently, over-expression of the hypoxia-inducible factor-1α (HIF-1α) protein, which is stabilized

and also activated under hypoxia, has been reported in common human cancers *in vivo* [5], suggesting that tumor tissues are generally hypoxic. A number of reports have recently demonstrated that hypoxic conditions induce a variety of adaptation responses in tumor cells [6–9]. We have also demonstrated that the expression of HIF-1α, as well as HIF-1-induced glycolysis is essential for *in vivo* tumorigenicity of pancreatic cancer cells [10].

However, tumor tissues are composed of both normal as well as tumor cells. In tumor tissues, various types of normal cells, such as endothelial cells and leukocytes are present. It is now widely recognized that macrophages represent a prominent component of the leukocyte population in tumor tissues. These cells, often called tumor-associated macrophages (TAM), are thought to be derived from peripheral blood monocytes, and recruited into the tumor tissues [11]. Importantly, there is accumulating evidence showing that hypoxia modulates the expression of various genes in the accumulated TAM, in tumor tissues [11–13].

Precursors of dendritic cells (DC) are also localized in peripheral tissues; they undergo maturation upon antigen uptake and processing [14, 15]. Mature DC migrate from the peripheral tissues to draining lymph nodes, where they present antigens to antigen-specific T cells [14, 15]. Thus, migrating capacity of DC is crucial for their professional function as antigen-presenting cells (APC) [16–18]. A recent report asserted that DC generated *in vitro*, and injected into tumor tissues, did not migrate to regional lymph nodes [19]. This suggests that DC lose their migrating activity in tumor tissues. In principle, on their way from peripheral tissues to regional lymph nodes, DC have to cross basement membranes and move through connective tissue. Previous reports demonstrated that matrix metalloproteinase (MMP)-9 and MMP-2 are necessary for the migration of DC [20–22], whereas it is yet to be determined how the expression of MMP-9 and MMP-2 in DC is regulated under hypoxic conditions.

In this study, we compare the expression of MMP and migrating activities of human monocyte-derived DC (hmDC) differentiated under normoxia (hmDC-N) to those differentiated under hypoxia (hmDC-H).

Results

Expression of DC-associated markers and cytokines

We first examined the expression of DC-associated markers (Fig. 1a). CD14 was not expressed. CD83 was expressed on both the cells stimulated by LPS under normoxia [mature (m)-hmDC-N], and those stimulated under hypoxia (m-hmDC-H). CD80, CD86, HLA-DR, HLA-ABC and CD1a were also expressed on both cell counterparts (Fig. 1a). Both of them showed more than 90% viability (Fig. 1b), and expressed similar levels of IL-10 and IL-12 (Fig. 1c–e). Collectively, these results suggest that hypoxia does not affect differentiation or maturation of hmDC.

Expression of MMP-9 in hmDC

MMP-2 was not expressed in hmDC. The immature hmDC (im-hmDC) and m-hmDC differentiated under normoxia expressed MMP-9 mRNA at two- to fivefold higher levels than those differentiated under hypoxia (Fig. 2a). The hmDC-N also produced MMP-9 (Fig. 2b), and secreted MMP-9 at higher levels than hmDC-H (Fig. 2c). However, the active form of MMP-9 was detected only in the supernatants of hmDC differentiated under normoxia (Fig. 2c). Gelatin zymography showed that hmDC-N secreted pro-MMP-9 at higher levels than hmDC-H, and that only hmDC-N secreted the active form of MMP-9 (Fig. 2d). The difference in MMP-9 expression between hmDC-H and hmDC-N appeared in the early phase of differentiation (Fig. 2e). We then examined MMP-9 mRNA expression in the hmDC that had differentiated under normoxia and then been incubated under hypoxia for 3 days, to investigate whether hmDC inoculated into peripheral blood in an adoptive immunotherapy could maintain their functions after migrating to hypoxic tumor tissues. As a result, these m-hmDC expressed half as much MMP-9 mRNA, as those that were incubated continuously under normoxia (Fig. 2f).

Expression of membrane type-1-MMP in hmDC

Since we did not detect MMP-2 in our hmDC, we examined the expression of membrane type-1 (MT1)-MMP, which has been reported to activate MMP-2 produced by other cells, and to play an important role in the migration of cells that do not express MMP-2 [23, 24]. The im-hmDC and m-hmDC differentiated under normoxia expressed MT1-MMP mRNA about five-fold higher than those differentiated under hypoxia (Fig. 3a). We then examined MT1-MMP mRNA expression in hmDC-N, which had been incubated for a further 3 days under hypoxia, to investigate whether DC inoculated in an adoptive immunotherapy might also have decreased production of MT1-MMP after migrating into tumor tissues. These m-hmDC expressed half as much MT1-MMP mRNA as those that were incubated continuously under normoxia (Fig. 3b).

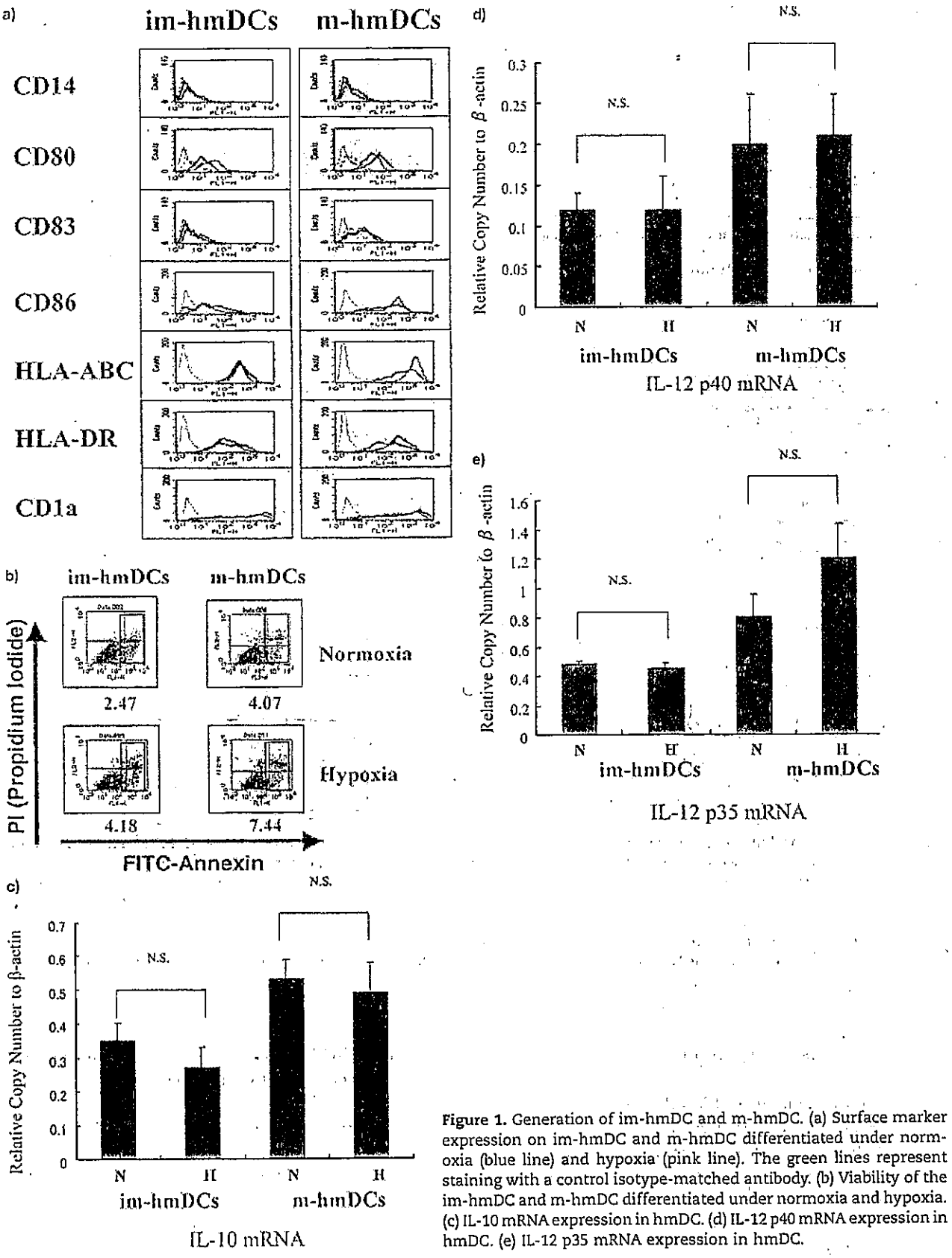


Figure 1. Generation of im-hmDC and m-hmDC. (a) Surface marker expression on im-hmDC and m-hmDC differentiated under normoxia (blue line) and hypoxia (pink line). The green lines represent staining with a control isotype-matched antibody. (b) Viability of the im-hmDC and m-hmDC differentiated under normoxia and hypoxia. (c) IL-10 mRNA expression in hmDC. (d) IL-12 p40 mRNA expression in hmDC. (e) IL-12 p35 mRNA expression in hmDC.

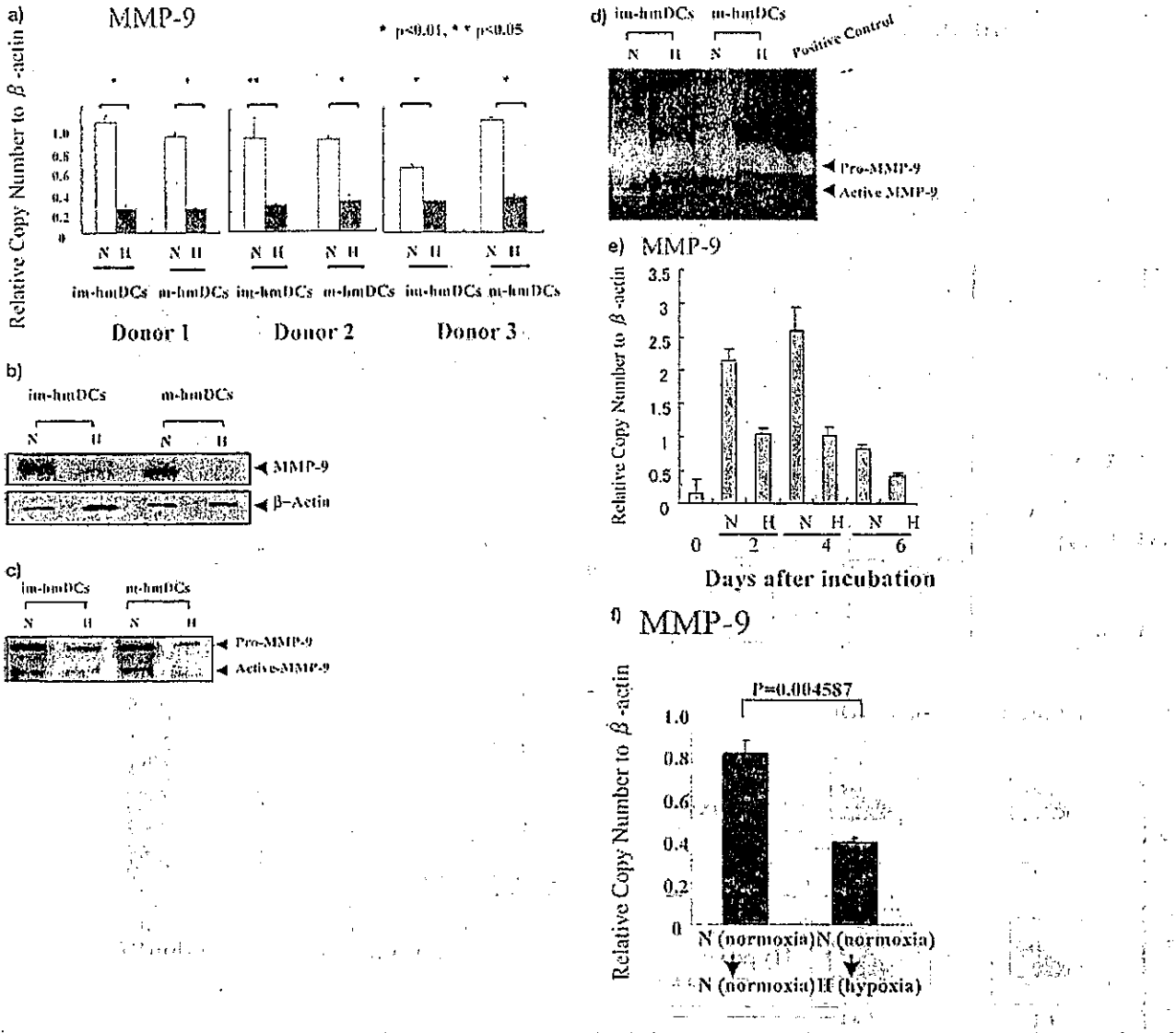


Figure 2. Expression of MMP-9 in hmDC. (a) MMP-9 mRNA expression in im-hmDC and m-hmDC differentiated under hypoxia and normoxia. Mononuclear cells were obtained from three different donors. (b) MMP-9 protein in cell lysates derived from im-hmDC and m-hmDC generated under normoxia and hypoxia. (c) Secreted MMP-9 protein in conditioned media obtained from im-hmDC and m-hmDC generated under normoxia and hypoxia. (d) Gelatin zymography showing MMP-9 activity in conditioned media obtained from im-hmDC and m-hmDC generated under normoxia and hypoxia. (e) Sequentially examined expression of MMP-9 during the differentiation of human monocytes into DC. (f) mRNA expression of MMP-9 in m-hmDC-N, cultured for a further 3 days under normoxia [indicated as N (normoxia)→ N (normoxia)] and m-hmDC-N, cultured for a further 3 days under hypoxia [indicated as N (normoxia)→ H (hypoxia)].

Expression of tissue inhibitors of MMP in hmDC

As specific tissue inhibitors of MMP (TIMP) are thought to regulate proteolytic activities of MMP [25], we then examined the expression of three TIMP in the hmDC. TIMP-1 was expressed at higher levels in hmDC-H than in hmDC-N (Fig. 4). TIMP-2 and TIMP-3 were expressed at similar levels in both hypoxic and normoxic groups. As TIMP-1 is a specific inhibitor of MMP-9, these results further indicate that hmDC-H have lower proteolytic activities than hmDC-N, and suggest that the decreased expression of MT1-MMP and MMP-9, in our experiments, is a specific effect of hypoxia.

Migrating activities of hmDC

As MMP-9 is necessary for the migration and infiltration of DC through the basement membrane, we then compared the migrating activities of hmDC-N to hmDC-H. Both im-hmDC-N and m-hmDC-N had significantly higher migrating activities than their counterparts, differentiated under hypoxia (Fig. 5a). As the

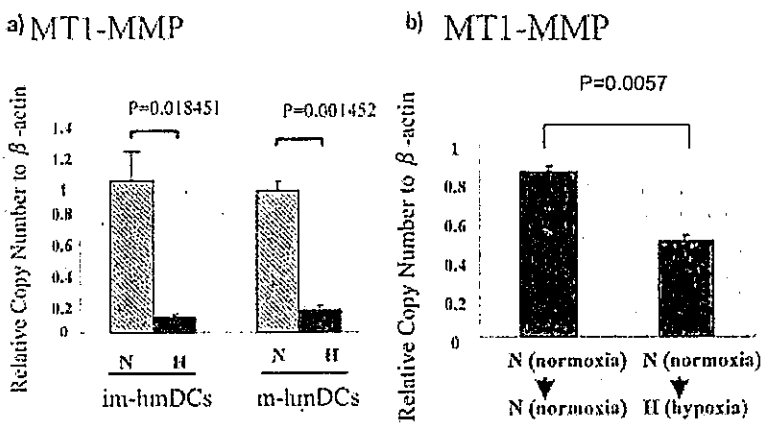


Figure 3. Expression of MT1-MMP. (a) Expression of MT1-MMP mRNA in hmDC differentiated under normoxia and hypoxia. (b) mRNA expressions of MT1-MMP in m-hmDC-N, cultured for a further 3 days under normoxia [indicated as N (normoxia)→ N (normoxia)] and m-hmDC-N cultured for a further 3 days under hypoxia [indicated as N (normoxia)→ H (hypoxia)]. A representative result of three different experiments is shown.

migrating activities of the hmDC differentiated under normoxia were suppressed by recombinant TIMP-1 protein, we attributed this result to their higher secretion of MMP-9, when compared to that of their counterparts, under hypoxia (Fig. 5b). We next examined the expression of chemokine receptors in hmDC differentiated under hypoxia and under normoxia, respectively, because the chemokine/chemokine receptor interaction is also essential for the migration of DC [26]. The im-hmDC differentiated under normoxia expressed CCR1 and CCR6 at four-times higher levels than those differentiated under hypoxia (Fig. 5c). However, m-hmDC-H expressed CCR7 at a slightly higher level than m-hmDC-N. CCR7 is reported to play important roles in the homing of mature DC to regional lymph nodes [26]. This result strongly suggests that MMP-9 might be one of the essential proteins needed for the homing of DC from peripheral tissues to regional lymph nodes, since not withstanding the higher expression of CCR7 in m-hmDC-H, the migration potential of these cells is significantly lower than their normoxic counterparts.

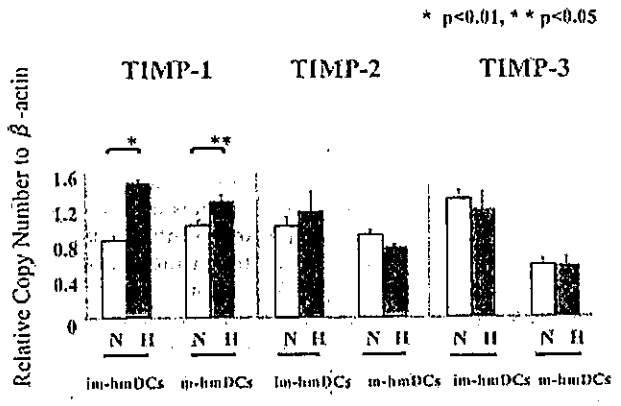


Figure 4. Expression of TIMP. Relative copy numbers of TIMP-1, TIMP-2 and TIMP-3 are shown. TIMP-1 expression was enhanced by hypoxia, whereas expression of TIMP-2 and TIMP-3 was not affected.

Restoration of expression of MMP-9

Incubation of the m-hmDC-H, for a further 72 h under normoxia, did not restore the production of MMP-9 or MT1-MMP (data not shown). Furthermore, neither IFN-α, nor IFN-γ or IL-12 showed any effect on the production of MMP-9 or MT1-MMP in the hmDC (data not shown). Hence, we hypothesized that either methylation or deacetylation of the MMP-9 promoter region might be implicated in its suppression, caused by hypoxia. Five-azacytidine (5-AZ), a demethylating agent, enhanced the production of MMP-9 in both m-hmDC groups, notwithstanding their culture condition (Fig. 6a). We did not detect any differences in the methylation sites of the promoter region of MMP-9 (Fig. 6b). In contrast, trichostatin A (TSA), a specific inhibitor of histone deacetylase, restored the production of MMP-9, under hypoxic conditions (Fig. 6c). These results suggest that deacetylation, but not methylation, of the MMP-9 promoter region might be implicated in the suppression of MMP-9, caused by hypoxia.

Discussion

Most solid tumors develop regions of low oxygen tension because of an imbalance in oxygen supply and consumption. As a tumor expands, vigorous growth of cancer cells creates a hypoxic microenvironment, which, if not alleviated, may restrict tumor growth or even cause cell death [27]. Angiogenesis and increased

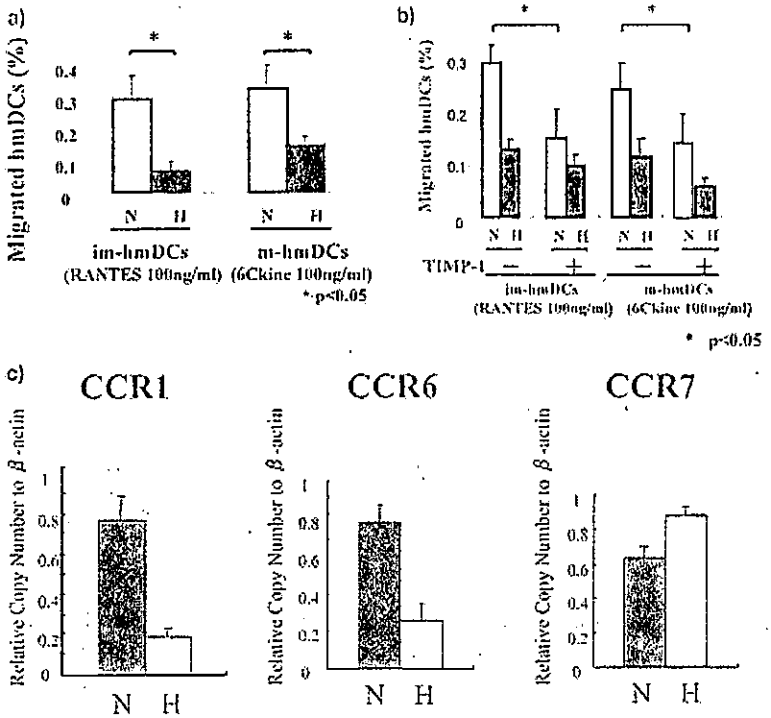
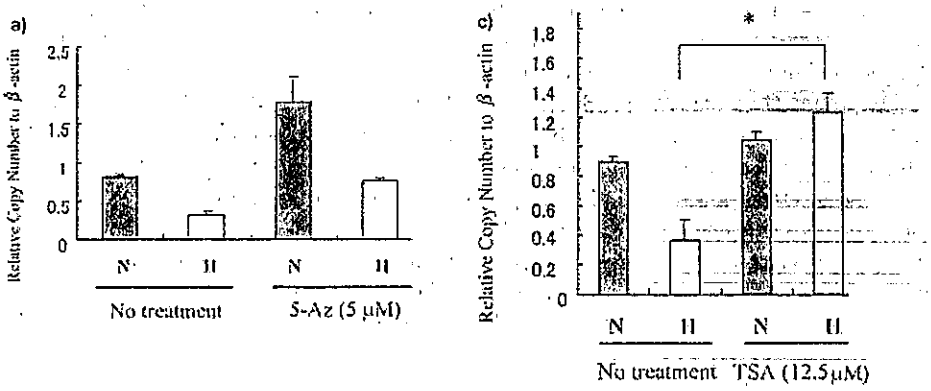


Figure 5. Migrating activities of hmDC differentiated under normoxia and hypoxia. (a) Migrating activities through matrigel-coated transwell membranes of im-hmDC (chemoattractant, RANTES) and m-hmDC (chemoattractant, 6Ckine). (b) Migrating activities of hmDC differentiated under normoxia were specifically inhibited by recombinant TIMP-1 (100 ng/mL). (c) Expressions of CCR1, CCR6 (im-hmDC) and CCR7 (m-hmDC) in DC differentiated under hypoxia and under normoxia. A representative result of three different experiments is shown.



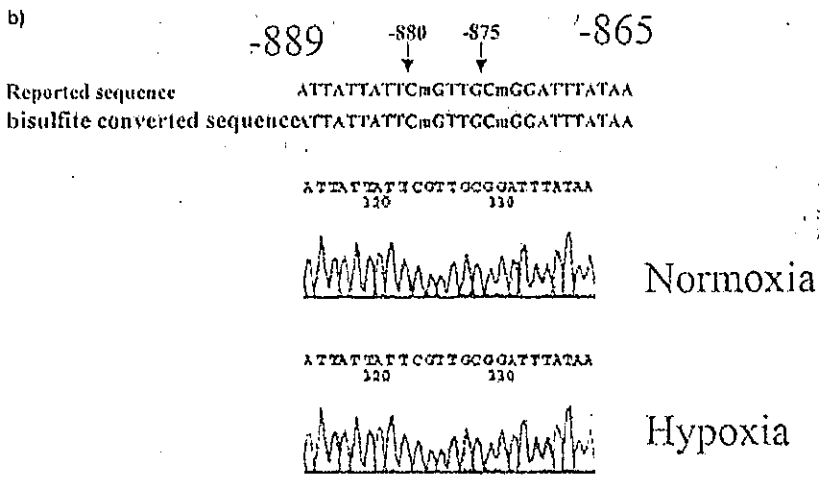


Figure 6. Restoration of MMP-9 expression. (a) 5-AZ did not restore the expression of MMP-9 in m-hmDC, suppressed by hypoxia; however, it enhanced the expression of MMP-9 both under normoxia and under hypoxia. (b) Representative possible methylated sites in the sequence of the MMP-9 promoter region of m-hmDC differentiated under hypoxia and under normoxia. There is no difference between the promoter region of m-hmDC differentiated under hypoxia and under normoxia. (c) TSA restored the expression of MMP-9 in m-hmDC. A representative result of three experiments is shown.

glycolysis, two universal characteristics of solid tumors, represent an adaptation response to the hypoxic microenvironment [28]. DC, which undergo differentiation and maturation in tumor tissues, are also in the hypoxic microenvironment.

DC are the most efficient type of migrating APC, playing an essential role in the initiation of immune responses. To fulfill their effector function, DC migrate through endothelial barriers and tissues [14, 15]. Although recent reports have shown that some of the mechanisms underlying DC migration are controlled by MMP and TIMP [25], little is known about how hypoxia regulates MMP expression and migratory capacity of DC.

This is the first study showing the hypoxia-induced suppression of MMP-9 and MT1-MMP production, and migrating activity of hmDC. Here we clearly demonstrate that hmDC-H express significantly lower levels of MMP-9 and MT1-MMP, as well as higher levels of TIMP-1, when compared to hmDC-N. In accordance with these results, we demonstrate that hmDC cultured under hypoxia have a reduced capability for migration than those cultured under normoxia. Furthermore, hmDC differentiated and matured under normoxia, and then cultured under hypoxia for another 3 days, also show a decreased expression of MMP-9 and MT1-MMP, suggesting that the hypoxic microenvironment in tumor tissues can potentially suppress the migrating activities of inoculated DC in an adoptive immunotherapy.

Interestingly, in accordance with recent data showing that CXCR4 is induced by hypoxia in several cell types, such as monocytes, endothelial cells, TAM and also cancer cells [29], levels of CCR7 were up-regulated in m-hmDC-H in our study. These findings suggest that hypoxia stimulates chemotactic potential to constitutively secreted chemokines, through the enhanced expression of chemokine-receptors. However, in view of the fact that *in vitro* invasion of our DC, under hypoxia, was still lower than that under normoxia, we postulate that MMP-9 might play a pivotal role in DC migration *in vivo*, and restoration of MMP-9 production might be critical for the migration of DC from tumors to regional lymph nodes.

From our observation, that TSA, an inhibitor of histone deacetylase, restores the production of MMP-9 and MT1-MMP in hmDC, we postulate that deacetylation of promoter regions of MMP-9 and MT1-MMP might be the cause of suppression of MMP-9 and MT1-MMP by hypoxia. To date, two reports have described the association of histone deacetylase with MMP production. TSA was reported to stimulate MMP-9 promoter activity in HT1080 fibrosarcoma cells [30]. Also, Pender *et al.* [31] confirmed that a histone deacetylase inhibitor, butyrate, up-regulated the production of MMP-7.

In our findings, the hypoxic microenvironment suppresses the expression of MMP-9 and MT1-MMP in hmDC. However, several issues remain yet to be resolved, namely, whether the suppression of MMP production caused by hypoxia is also observed in other DC subtypes, such as $CD11c^+$ Langerhans cells and interstitial DC, derived from $CD34^+$ myeloid progenitor cells. Most importantly, it is yet to be determined whether this phenomenon is also observed in other species, like mouse and rat, so as to conduct proper *in vivo* studies. A number of reports have already demonstrated that hypoxia enhances the expression of MMP-9 in various types of cells [32, 33]. On the other hand, several other reports state that this hypoxia-mediated increase of MMP-9 is not consistently observed [34]. Saed *et al.* [35] reported that hypoxia decreases the expression of MMP-9, and that TGF-β1 increases this expression under normoxia, but decreases it under hypoxia. In our experiments, adherent cells, without any stimulation, express only a low level of MMP-9 mRNA, with a gradual increase of MMP-9 mRNA expression during the culture of cells in the presence of GM-CSF and IL-4. This suggests that a hypoxic condition may suppress MMP-9 mRNA expression induced by GM-CSF and IL-4 by disturbing the balance of the deacetylation:acetylation reaction. Initially, we had hypothesized that the expression of MMP-9 would be suppressed by hypoxia only in hematopoietic cells. However, surprisingly, the expression of MMP-9 in U-937 leukemia cells, derived from hematopoietic cells, was stimulated by hypoxia (data not shown). Our results, as well as the current literature, clearly emphasize the need for further elucidation of the relationship between hypoxia and MMP. We believe it possible that this hypoxia-induced decrease of MMP production may be specific to hmDC only, or DC in general, and is thus of empirical importance to DC-based tumor immunotherapy. Moreover, the examination of MMP production in other immune cells, such as T cells and B cells, under hypoxia as compared to normoxia, might reveal challenging results regarding the overall immune system scenario.

Collectively, our findings indicate that a hypoxic microenvironment suppresses migrating activities of hmDC, by stifling their production of MMP-9 and MT1-MMP, strongly implying that production of MMP is reduced in hmDC within tumor tissues, thus hindering the stimulation of an adequate immune response.

Materials and methods

Reagents

IL-4, GM-CSF and regulated upon activation, normal T expressed and secreted (RANTES) were purchased from R&D Systems (Minneapolis, MN). Human IFN-α and IFN-γ were purchased from JCR Pharmaceuticals Co. Ltd. (Tokyo, Japan) and PBL Biomedical Laboratories (Piscataway, NJ), respectively.

LPS, Ficoll-Paque, human 6Ckine, TSA, and gelatin were purchased from Sigma Chemical Co. (St. Louis, MO). Fetal bovine serum (FBS) was purchased from HyClone (HyClone, UT). FITC-conjugated mAb against human CD83, CD80, CD14, HLA-ABC, HLA-DR, un-conjugated mAb against human CD1a and CD86, and isotype control antibodies were purchased from Immunotech (Marseille, France). FITC-conjugated anti-mouse IgG+IgM (H+L) was purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). TRIzol reagent, QuantiTect™ SYBR® Green PCR Master Mix, ABI PRISM® 7900HT sequence Detection System and TaqMan®Reverse Transcription Reagents were purchased from Life Technologies (Frederick, MD), Qiagen (Germany) and Applied Biosystems (Foster City, CA), respectively. Anti-human MMP-9 and TIMP-1 antibodies were purchased from Daiichi Pure Chemical Co. Ltd. (Tokyo, Japan). Mouse anti-human β-actin antibody, sheep anti-mouse Ig antibodies, Western blotting detection reagent and BD BioCoat Matrigel Invasion Chamber were purchased from Chemicon International, Inc. (Temecula, CA), Amersham Life Science, Inc. (Arlington Heights, IL), and BD Biosciences (Mississauga, Ontario, Canada), respectively.

Generation of hmDC

hmDC were prepared as previously reported [36, 37]. Briefly, adherent cells were cultured in the presence of 1000 U/mL GM-CSF and IL-4 for 6 days under hypoxia or normoxia. According to the previous definition of tumor hypoxia (median pO_2 <10 mmHg, approximately 1.25%) [38], the cells in the hypoxic group were incubated at 1% O_2 in a hypoxic chamber gassed with 94% N_2 and 5% CO_2 (Wakenyaku Co. Ltd., Tokyo, Japan). On day 6, the cells were separated into two groups, respectively. Cells of group 1 were cultured in the same condition for 1 day and used as im-hmDC. Cells of group 2 were treated with LPS (1 μg/mL) for 1 day and then used as m-hmDC. In some experiments, 5-AZ, or TSA, was added to the monocyte cultures 3 days after incubation, during DC differentiation, at 5 μM or 12.5 μM.

Phenotypic analysis by FACS

The cells in the respective groups were incubated with FITC-conjugated specific mAb against CD83, CD14, CD80, HLA-ABC and HLA-DR. They were then incubated with un-conjugated mouse Ab against CD86 and CD1a and FITC-conjugated anti-mouse IgG+IgM (H+L). After incubation, the cells were analyzed using a FACScan (Becton Dickinson).

Real-time PCR

Each cDNA (10 ng) was amplified in triplicate using a SYBR-Green PCR assay kit and then detected on an ABI PRISM® 7900HT sequence detection system. The β-actin RNA was used to standardize the total amount of cDNA. The primers used were as follows:
MMP-9: CCTTTTGAGGGCGACCTCCAAG and CTGGATGACGATGTCTGCGT; MT1-MMP: CCGATGTGGTGTTCCAGACA and TGGCCTCGTATGTGGCATACT; TIMP1: ACAGACGGCCTTCTGCAATTC and GGTGTAGACGAACCGGATGTCA; TIMP2: GTTCAAAGGGCCTGAGAAGGA and CCAGGGCACGATGAAGTCA; TIMP3: GCCTTCTGCAACTCCGACAT and TCTCGGAAGCTTCCGTATGG; CCR1: CGAAAGCCTACGAGAGTGGAA and CGGACAGCTTTGGATTTCTTCT; CCR6: TCGCCATTGTACAGGCGACTA and CGCTGCCTTGGGTGTTGTAT; CCR7: GGTGGCTCTCCTTGTCATTTTC and GGGAGGAACCAGGCTTTAAAGT; β-actin: GCTCCTCCTGAGCGCAAGT and TCGTCATACTCCTGCTTGCTGAT. Relative mRNA levels were determined by comparing the PCR cycle thresholds between the cDNA of the gene of interest and that of β-actin.

PCR amplification of bisulfite-treated DNA and sequencing

Six fragments of the human MMP-9 promoter region containing CpG dinucleotides were amplified according to the previously described method [39]. The PCR products were cloned into the pCR4-TOPO vector (Invitrogen) and then sequenced.

Western blot analysis

Cell-free supernatants were collected from DC cultured for 24 h in serum-free RPMI 1640 medium. A supernatant of the human fibrosarcoma cell line HT1080 was used as a positive control. After SDS-PAGE, proteins were blotted onto nitrocellulose membranes (Bio-Rad, Hercules, CA) and stained with anti-MMP-9 antibody. Each cell lysate was also analyzed by Western blot analysis to detect intracellular MMP-9 protein. The membranes were developed with the use of an ECL detection kit (Amersham, Tokyo, Japan).

Gelatin zymography

Enzymatic activity was analyzed by electrophoresis in polyacrylamide gels containing sodium dodecyl sulfate (SDS) and gelatin as described previously [40].

Matrigel invasion assay

The Matrigel invasion assay was performed in Matrigel-coated Transwell chambers (BD Bioscience) according to the manufacturer's instructions. Recombinant human RANTES and 6Ckine were added in the culture media, in the lower chambers, at a concentration of 100 ng/mL. After 6–8 h incubation, the number of hmDC that had migrated to the lower surface of the membrane was counted.

Statistical analysis

Statistical differences among the groups were determined by a two-tailed Student's *t*-test as for independent samples. Mean values were considered significantly different when p values were less than 0.05.

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