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 1 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-α, 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 be an AMPKα 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-β1 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 pathway, angiogenesis has been concluded to be essential for tumor malignancy (1, 2). 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.

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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 plate at 2.5 × 10^4/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 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 A260, and 0.5 μg of total RNA was reverse-transcribed with avian myeloblastosis virus transcriptase (Takara Bio Inc., Ltd.). After reverse transcription, PCR was performed with an LA PCR Kit (Takara) using primer pairs for human TGF-β1 and type III/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 0.5% Triton X-100 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 (Amer sham 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. 1A). In addition, hypoxia-induced tolerance to glucose starvation was also exhibited in non-tumor cells, fibroblast cell lines Rat1 and KMST6 (Fig. 1B). 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. 1C, 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. 1C). 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. 1C), 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. 1D, 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. 1D). 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. 1D). 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. 1D). 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 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

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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. 1E). As shown in Fig. 1F, 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. 1F), 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. 2A), and HepG2 cells exposed to glucose starvation under hypoxic conditions expressed TGF-β1 mRNA slightly more weakly than cells exposed to hypoxia alone (Fig. 2A). 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. 2A). By contrast with TGF-β1, neither glucose nor oxygen status affected expressions of TGF-β1, A2, and ARK5 during hypoxia (Fig. 2A). 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. 2C) or recombinant TGF-β1 (Fig. 2D) was suppressed by...
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 Akt and AMPK-α exhibited drastic changes by glucose starvation and/or TGF-β treatment. As shown in Fig. 2E, 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. 2E). 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-1a—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 was 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. 3A, 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. 3A). DN-HIF transfection, on the other hand, completely suppressed an increase induced by hypoxia (Fig. 3A). 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. 3B, 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. 3C, 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. 3C), confirming that hypoxia-induced tolerance to glucose starvation in HepG2 cells is promoted by HIF-1-independent signalling. We also investigated whether HIF-1α is involved in hypoxia-induced TGF-β1 mRNA expression by RT-PCR. As shown in Fig. 3D, transient expression of wtHIF-1 or DN-HIF had no effect on hypoxia-induced TGF-β1 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. 4A, 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. 4B). Although Smad3/DE suppressed Smad2 phosphorylation by TGF-β1, it had no effect on phosphorylation of Akt and AMPK-α by TGF-β1 (Fig. 4C). Hypoxia-induced tolerance to glucose starvation was also unaffected by Smad3/DE (Fig. 4D). 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.

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. 4C and 5A). In addition to that shown in Fig. 5B, Akt and AMPK-α phosphorylation observed in HepG2 cells exposed to glucose starvation under hypoxic condition was suppressed by 10 μM SB4391542, suggesting that TGF-β is closely involved in sustained activation of 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 adipocyteogenesis 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. 5C). 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-

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TGF-β1 Activates AMPK-α and ARK5 during Hypoxia

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 (Glucose) or absence (Glucose−) 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), phosphorylated AMPK (AMPK P), or total AMPK-α (AMPK). 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 (Glucose) or not containing (Glucose−) 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 (Akt and AMPK) Akt or AMPK-α. C, schematic model of hypoxia-induced tolerance to glucose starvation on HepG2 cells.

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

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