Abstract. Cancer cells usually obtain energy from a high rate of glycolysis rather than oxidative phosphorylation under normoxia as well as hypoxia. Under these circumstances, pyruvate, the end-product of glycolysis, accumulates in cancer cells. We have previously reported that pyruvate activates endothelial cells and induces angiogenesis. Here, we examined the angiogenic activity of pyruvate in tumor cells. Plasminogen activator inhibitor-1 (PAI-1), the gene most upregulated by pyruvate, showed a pro-angiogenic activity, which was abolished by a PAI-1 neutralizing antibody. Moreover, stabilization of hypoxia-inducible factor-1 (HIF-1) by pyruvate was required for induction of PAI-1 transcription through direct binding to hypoxia response element-2 (HRE-2) on the promoter. These results suggest that pyruvate can activate the angiogenic activity of cancer cells under normoxia and that PAI-1 may act as a pro-angiogenic factor in pyruvate-induced angiogenesis.

Introduction

Metabolic features of cancer cells deviate significantly from those of normal cells. For example, overexpression of glucose transporters and various glycolytic enzymes, high rates of glycolysis, excessive release of lactate and protons, reduction of the oxidative phosphorylation (OXPHOS) pathway, increased lipid synthesis, and inhibition of the β-oxidation pathway are observed in cancer cells (1-3). In particular, it is known that the energy of cancer cells is usually supplied from a high rate of glycolysis rather than the OXPHOS pathway under normoxic conditions, called the Warburg effect (4,5). Due to aerobic glycolysis in cancer cells, pyruvate and lactate, the end-products of glycolysis, are accumulated (2,6). Recently, accumulated pyruvate in the cell may induce hypoxia-inducible factor-1α (HIF-1α) under normoxic conditions, which results in acceleration of the glycolytic phenotype of cancer cells (4).

Angiogenesis, the process of new blood vessel formation from pre-existing vessels, is essential for physiological and pathological conditions, including aggressive tumor development (7,8). It is tightly controlled by various activators and inhibitors (8-10). However, tumor cells induce angiogenesis in order to supply oxygen and nutrients by secretion of angiogenic activators (11). We have previously reported that pyruvate is able to induce both in vivo and in vitro angiogenesis of endothelial cells (12). Pyruvate upregulates vascular endothelial cell growth factor (VEGF) and fibroblast growth factor receptor-2 (FGFR-2) and leads to activation of proliferation, migration, and tube-like differentiation of endothelial cells. It is suggested that secreted pyruvate from cancer cells may directly activate endothelial cells to form new blood vessels.

Therefore, we examined the possibility that accumulated pyruvate induces angiogenic properties in cancer cells, and also investigated the mechanism which is responsible for induction of angiogenesis. Here, we show that pyruvate induces angiogenesis in HepG2 cells and that upregulation of plasminogen activator inhibitor-1 (PAI-1) by HIF-1 plays a major role in pyruvate-induced angiogenesis.

Materials and methods

Materials. Minimal essential medium (MEM), fetal bovine serum (FBS), penicillin and streptomycin were purchased from Gibco BRL (Rockville, MD). Pyruvate was purchased from Sigma (St. Louis, MO; cat. no.: P1656). Lipofectamine, PLUS reagent, and TRizol were purchased from Invitrogen (Carlsbad, CA). T4-polynucleotide kinase, alkaline phosphatase and other restriction enzymes were purchased from New England Biolabs (Beverly, MA). The luciferase assay system, T4 ligase, M-MLV reverse transcriptase and the Wizard Genomic DNA purification kit were purchased from Promega (Madison, WI). For Western blotting, HIF-1α antibody was purchased from A.G.
Scientific (San Diego, CA). PAI-1 neutralizing antibody was purchased from R&D Systems (Minneapolis, MN). For the in vitro angiogenesis assay, Matrigel was purchased from BD Science.

**Cell culture.** HepG2 cells (2.2x10^6 cells/100-mm dish; 40,000 cells/cm²) were cultured in MEM, supplemented with heat-inactivated 10% fetal bovine serum, 100 U/ml of penicillin, and 100 μg/ml of streptomycin in a 37°C incubator with a humidified atmosphere containing 5% CO₂. Before exposure to hypoxia and chemical reagents, seeded cells were stabilized for 24 h in an incubator. For hypoxic treatment, cells were transferred to an anaerobic chamber (Forma Scientific; Marietta, OH) with a humidified atmosphere of 2% O₂ and CO₂ balanced with N₂ and incubated at 37°C for 12 h. HUVECs (Cambrex; Walkersville, MD) were grown according to the manufacturer's protocol in EBM-2 medium (Cambrex) supplemented with the EGM-2 kit containing 2% fetal calf serum (FCS), 0.04% hydrocortisone, 0.4% hFGF-B, 0.1% heparin. HUVECs in passages 5 through 8 were used in the tube formation assay.

**Tube formation assay in vitro.** HepG2 cells (2.2x10^6 cells/100-mm dish; 40,000 cells/cm²) were cultured with or without 3 mM pyruvate for 12 h, and culture media were then collected. HUVECs (5x10⁴ cells/well) were seeded on a layer of polymerized Matrigel with collected media in 24-well plates (NUNC; Roskilde, Denmark). PAI-1 neutralizing antibody (2 μg/ml) was used for inhibition of PAI-1 in pyruvate-treated media. After 12 h, changes in cell morphology were observed under a phase-contrast microscope and photographed at x100 magnification.

**Total RNA preparation and reverse transcriptase-polymerase chain reaction.** Total RNA for use in RT-PCR was isolated using TRIzol reagent according to the manufacturer's instructions. cDNA was made with oligo-dTTP using M-MLV reverse transcriptase. PCR amplification was performed under optimized conditions for each primer pair. The following oligonucleotide primer pairs were used: PAI-1, 5'-GTC AAG CAA GTG GAC TTT TC-3' (forward) and 5'-GGT CTG GAC TTT TC-3' (reverse). PCR products were electrophoresed on 1% agarose gels and visualized by ethidium bromide staining under ultraviolet trans-illumination.

**Western blot analysis.** Total protein (50 μg) was loaded onto an 8% sodium dodecyl sulfate-polyacrylamide gel, and, after electrophoresis, blotted onto Hybond-P PVDF membranes (Amersham; Piscataway, NJ). Membrane with protein was blocked for 2 h at room temperature in a blocking solution (5% skim milk in TBS containing 0.1% Tween-20), and then incubated with primary antibodies against HIF-1α (1:500), PAI-1 (1:1000), and α-tubulin (1:2500). Peroxidase-conjugated anti-mouse secondary antibody was used at a dilution of 1:2500. Signals were visualized using PicoEPD Western reagent (ElpisBiotech; Daejeon, Korea). Each assay was performed in triplicate.

**Plasmid constructs.** For preparation of genomic DNA, the Wizard Genomic DNA purification kit was used according to the manufacturer’s instructions. The human PAI-1 promoter region (Ensembl ID: ENSG00000106366), extending from transcription initiation positions -799 to +21 was amplified from a purified human genomic DNA of HepG2 cells with the aid of an upstream and downstream primer incorporating restriction sites for KpnI (13-16). The sequence of the upstream primer was 5’-GGG GTT ACC ATG GTA ACC CCT GGT CCC G-3’ and that of the downstream primer was 5’-GGG GTT ACC TGC AGC AAA CAG CTG TG-3’ (underline indicates the KpnI restriction enzyme recognition site). Following enzyme restriction, the PCR product was ligated into the luciferase reporter plasmid, pGL3-basic (Promega), yielding a pGL3-hPAI-1 wt construct containing the 5 putative hypoxia response element motifs (16). HRE-2, the functional HRE motif (14,16), was mutantized using a QuickChange Site-Directed mutagenesis kit (Strategene; La Jolla, CA). For mutation, primer 5’-CTG AAT GCT CTT ACA CAG TAT CAC ACA CAG AGC AC-3’ (underline; changed nucleotides) was used. The generated plasmid was designated as pGL3-hPAI-1-mut.

**Cell transfection and reporter assay.** HepG2 cells (1.4x10⁵ cells/well; 40,000 cells/cm²) were seeded and transiently transfected in 12-well plates using Lipofectamine and PLUS reagent according to the manufacturer’s instructions (NUNC). In brief, 140 ng of the appropriate PAI-1 wild-type HRE-2 promoter or PAI-1 mutant HRE-2 promoter Firefly Luciferase construct was transfected. pCMV-β-gal was cotransfected with each construct as a control for transfection efficiency. Total amount of DNAs were maintained by addition of 490 ng of pcDNA3.1/His C. Twelve hours after transfection, cells were treated with hypoxia and pyruvate for 12 h. At the end of treatment, the cells were harvested in Passive Lysis Buffer (Luciferase Assay System, Promega). Luciferase activity was determined using a microplate reader (Wallac; Victor, Perkin-Elmer; Boston, MA), following the manufacturer’s protocol. Relative luciferase activity was normalized against the activity of β-gal. Each value represents the mean of at least four wells, and similar results were obtained in two different experiments. Statistical comparisons between groups were performed using the Student’s t-test. Data were considered statistically significant at P<0.05.

**Preparation of nuclear extracts.** Cells were washed twice with ice-cold phosphate-buffered saline (PBS) and harvested. Cells were resuspended in 100 μl lysis buffer [10 mM Tris-HCl (pH 7.9), 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT, 0.1% NP40, and 0.5 mM PMSF], and then incubated at 4°C for 15 min. The mixtures were centrifuged at 13,000 x g for 10 min at 4°C. The nuclei pellet was resuspended in 30 μl extraction buffer [20 mM Tris-HCl (pH 7.9), 1.5 mM MgCl₂, 0.5 mM DTT, 25% glycerol, 420 mM NaCl, 0.2 mM EDTA, and 0.5 mM PMSF] and vortexed briefly. After standing
at 4˚C for 15 min, the mixture was centrifuged at 4˚C for 10 min and the supernatant was transferred to a fresh tube. Extracts were diluted with 60 μl dilution buffer [20 mM Tris-HCl (pH 7.9), 50 mM KCl, 0.5 mM DTT, 20% glycerol, 0.2 mM EDTA, and 0.5 mM PMSF]. Protein concentration was determined using a BCA protein assay kit (Sigma).

Electrophoretic mobility shift assay. Sequences of the sense strand of the oligonucleotide probe used for EMSA were as follows: 5'-CCT GAA TGC TCT TAC ACA CGT ACA CAC ACA GAG C-3' (human PAI-1 HRE-2). Sense and antisense strands were annealed and end-labeled with [γ-32P]-ATP (Amersham) using T4-polynucleotide kinase. Unincorporated nucleotide was removed by gel filtration using ProbeQuant™ G-50 micro Columns (Amersham). For the DNA-protein interaction, 2-5x104 cpm of oligonucleotide probe was incubated with 30 μg of nuclear extract and 0.5 μg of sonicated, denatured salmon sperm DNA in 10 mM Tris-HCl (pH 7.8), 50 mM KCl, 50 mM NaCl, 1 mM MgCl2, 1 mM EDTA, 5 mM dithiothreitol, and 5% glycerol for 30 min on ice in a total volume of 20 μl. The reaction mixture was subjected to electrophoresis (200V in 0.25X Tris-buffered EDTA) using 4% non-denaturing polyacrylamide gels. Dried gels were subjected to autoradiography for 16 h. For the competition experiment, a 100-fold molar excess of unlabeled oligonucleotide was added to the binding reaction just prior to addition of radiolabeled probe. For gel supershift analysis, 1 μl of rabbit polyclonal antibody against HIF-1α was added prior the initial 30 min period of incubation for 2 h on ice.

Results

Pyruvate induces angiogenesis through PAI-1 upregulation.

To examine the angiogenic effect of pyruvate on tumor cells, HepG2 cells were incubated with 3 mM of pyruvate for 12 h, and conditioned media (CM) were applied to HUVECs for an in vitro tube formation assay. Formation of tube-like structures was increased by pyruvate-treated CM (Fig. 1A). This result shows that pyruvate may induce angiogenic factors in tumor cells. Thus, we performed cDNA microarray analysis with cDNAs from HepG2 cells treated with 3 mM of pyruvate for 12 h in order to investigate the angiogenic mechanism of pyruvate. The most highly expressed gene in 3 mM pyruvate-treated HepG2 cells was PAI-1 (8.47-fold) (data not shown). To confirm expression of PAI-1 from cDNA microarray data, we performed RT-PCR analysis using a PAI-1-specific primer set. PAI-1 expression was upregulated by pyruvate, in agreement with the cDNA microarray result (Fig. 1B). PAI-1 is a major inhibitor of both tissue-type and urokinase-type plasminogen activators (tPA and uPA) (17,18). PAI-1 also participates in angiogenesis; however, due to the results of pro-angiogenic and anti-angiogenic actions, the exact role of PAI-1 in this process remains controversial (19-26). Therefore, we used an in vitro tube formation assay in order to verify the role of PAI-1 induced by pyruvate on angiogenesis. Pyruvate-treated CM was applied to HUVECs in a tube formation assay with 2 μg/ml of neutralizing antibody against PAI-1. Whereas pyruvate-treated CM led to differentiation of HUVECs, neutralizing antibody against PAI-1 inhibited tube formation induced by pyruvate-treated CM (Fig. 1C). These results show that upregulation of PAI-1 by pyruvate induces angiogenesis.

Pyruvate induces PAI-1 transcription through HIF-1α stabilization. As mentioned above, pyruvate in cancer cells stabilized HIF-1α under normoxic conditions (4). Also, HIF-1α promotes transcription of the PAI-1 gene under hypoxia (16,27). RT-PCR and Western blot assay were performed for determination of whether pyruvate-induced PAI-1 expression is mediated by HIF-1α stabilization under normoxic conditions. HIF-1α and PAI-1 were induced by 3 mM of pyruvate as...
well as hypoxic conditions (Fig. 2A). Although the mRNA level of HIF-1α did not change, the mRNA level of the PAI-1 gene increased upon treatment with 3 mM pyruvate (Fig. 2B).

To investigate whether HIF-1α induced by pyruvate can activate PAI-1 transcription, RT-PCR and Western blot were performed with YC-1, a pharmacological inhibitor of HIF-1α (28-30). The results show that YC-1 suppressed induction of both mRNA and protein levels of HIF-1α and PAI-1 expression by pyruvate and hypoxia (Fig. 2A and B). These data indicate that pyruvate activates PAI-1 transcription under normoxia via stabilization of HIF-1α.

Pyruvate activates PAI-1 transcription via hypoxia response element-2 (HRE-2) in the PAI-1 promoter. Recently, sequence analysis of the human PAI-1 promoter revealed five putative HRE motifs that show homology with consensus sequence binding with HIF-1, BACGTSSK (B=G/C/T, S=G/C, and K=G/T) (16). However, only the HRE-2 motif (positions -194 to -187) of the five HREs was actually required for hypoxia-dependent activation (16). To verify that HRE-2 of the PAI-1 promoter is required for activation of PAI-1 transcription by pyruvate, we performed a promoter activity assay using luciferase reporter vectors with the wild-type promoter (-799 to +21) and the HRE-2-mutated promoter (CACGTA to CGATAT) (Fig. 3A). Compared to the control, the promoter activity of PAI-1 was increased by ~3-fold by treatment with pyruvate or hypoxia (Fig. 3B). On the other hand, the promoter containing mutated-HRE did not respond to pyruvate or hypoxia. These data indicate that pyruvate, like hypoxia, is able to upregulate PAI-1 promoter activity via HRE-2.

HIF-1α binds to HRE-2 in the PAI-1 promoter under pyruvate treatment. Because the HRE-2 motif is required for pyruvate-induced PAI-1 transcription, we performed testing to determine whether HIF-1α directly binds to the HRE-2 motif using EMSA with a probe containing the HRE-2 sequence in the PAI-1 promoter. When the probe DNA fragment was incubated with the extract prepared from 3 mM pyruvate-treated HepG2 cells, the shifted DNA probe and protein complex were observed (Fig. 4A). This complex was diminished by addition of 100-fold molar excess competitor DNA. We then performed a supershift assay to show that the protein in the probe DNA-protein complex is indeed the HIF-1α protein. The probe DNA-protein complex was super-
known to show dual-faced properties in angiogenesis. Angiogenesis and senescence (14,23,32-36). PAI-1 has been involved in thrombosis, cancer metastasis, type-2 diabetes, tumor regeneration (17,31). In addition, PAI-1 also participates in physiological roles of PAI-1 are blood coagulation and tissue regeneration.

PAI-1 is a major inhibitor of both tPA and uPA. The major member of the SERPIN (Serine Protease Inhibitor) family, PAI-1 is responsible for tube formation (Fig. 1C). PAI-1, a physiological end-product of pyruvate-treated HepG2 (Fig. 1A). Because treatment with pyruvate can induce angiogenic activity of cancer cells. Tube formation of HUVECs was induced by CM derived from pyruvate-treated HepG2 (12). In this report, we examined the question of whether angiogenic factors and promote their angiogenic activity activate endothelial cells through increased expression of HIF-1 (4). We previously reported that pyruvate can induce angiogenesis induced by CM derived from pyruvate-treated HepG2 might be explained by the action of secreted PAI-1 at the level of physiological concentration.

According to a recent report, PAI-1 could be upregulated by HIF-1 under hypoxic conditions (16,27). Therefore, we tested HIF-1-mediated PAI-1 expression by pyruvate. While pyruvate increased PAI-1 expression of both mRNA and protein, only the protein level of HIF-1α was increased (Fig. 2). Increased expression of PAI-1 by pyruvate is HIF-1α dependent, as demonstrated by the fact that increased expression was diminished by treatment with YC-1, a pharmacological inhibitor of HIF-1. Moreover, HRE-2 of the five HREs in the PAI-1 promoter was essential for pyruvate-induced PAI-1 transcription (Fig. 3). Indeed, pyruvate-stabilized HIF-1 bound to the HRE-2 (Fig. 4).

In consideration of several reports, as well as our results, HIF-1 can be activated under normoxia by several stimuli, including oncogenes, growth factors, cytokines, nitric oxide, oxidized low-density lipoprotein, lipopolysaccharide and pyruvate (4,37-39). Whereas the molecular mechanism for HIF-1α stabilization by inhibition of prolyl hydroxylase domain (PHD) under hypoxia is well known, little is known about the stabilization of HIF-1α under normoxia. Some recent reports have suggested that pyruvate can stabilize HIF-1α under normoxia through PHD inhibition; however, the fine mechanism remains unknown (40). These reports provide evidence to support the suggestion that HIF-1 is a major factor in obtaining the properties for malignancy, aerobic glycolysis and angiogenesis. Taken together, our data indicate that accumulated pyruvate might play a role as an angiogenic inducer of cancer cells under normoxia and that pyruvate-induced angiogenesis through HIF-1 activation might be a therapeutic target for cancer treatment.

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**References**