The Potential Role of Hypoxia Inducible Factor 1α in Tumor Progression after Hypoxia and Chemotherapy in Hepatocellular Carcinoma

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ABSTRACT

This study investigates the possible molecular basis leading to failure in a treatment that is composed of hypoxia and chemotherapy in a rat orthotopic hepatoma model. Hypoxia was induced by hepatic artery ligation, whereas chemotherapeutic effect was achieved by intraportal injection of cisplatin. High-dose sodium salicylate was administered to achieve transcriptional blockade. Significant prolongation of animal survival was observed in the groups receiving hepatic artery ligation with cisplatin or sodium salicylate. Massive tumor cell necrosis and apoptosis were found in the ligation and all of the combined treatment groups. Up-regulation of hypoxia inducible factor 1α (HIF-1α) and vascular endothelial growth factor (VEGF) at both mRNA and protein levels were detected in the groups receiving ligation and ligation with cisplatin, whereas a decreased level of von Hippel-Lindau tumor suppressor protein was identified in the group receiving ligation with cisplatin. Sodium salicylate enhanced expression of von Hippel-Lindau tumor suppressor protein but down-regulated HIF-1α and VEGF levels after ligation with or without cisplatin. An increased number of activated hepatic stellate cells in the tumors were observed in the ligation and ligation with cisplatin groups, whereas they were greatly reduced by sodium salicylate. In vitro study revealed that under hypoxic condition, both cisplatin and sodium salicylate could remarkably augment P53 and caspase 3 levels. Cisplatin stimulated HIF-1α up-regulation whereas sodium salicylate suppressed HIF-1α expression. In conclusion, tumor progression after hypoxia and chemotherapy might be related to up-regulation of HIF-1α and subsequent VEGF production, and transcriptional blockade by sodium salicylate could enhance the therapeutic efficacy of hypoxia and chemotherapy.

INTRODUCTION

Induction of tumor hypoxia combined with chemotherapy by transcatheter arterial chemoembolization has been widely used in treating unresectable hepatocellular carcinoma (HCC; Refs. 1–3). Oxygen depletion arrests tumor cell proliferation and leads to apoptosis and necrosis (4–6), and chemoyctotoxicity of drugs, such as cisplatin, can “kill” tumor cells in HCC (7, 8). However, tumor response rate is unsatisfactory, and only a small population of patients benefit from this treatment protocol (1, 9). The mechanism that accounts for treatment failure is not clear.

Several genes may be activated under hypoxic condition, such as vascular endothelial growth factor (VEGF; Refs. 10, 11), erythropoietin (12), and glycolytic enzymes (13), which are transcriptionally regulated by hypoxia inducible factor 1α (HIF-1α). As a key player in tumorigenesis and angiogenesis, HIF-1α overexpression is associated with an increased mortality and treatment failure in various cancers (14–16). In addition, HIF-1α can regulate multidrug resistance gene (17), and blocking the activity of HIF-1α can enhance the therapeutic efficacy of cancer immunotherapy (18). However, the role of HIF-1α in HCC, especially its behavior after hypoxia and chemotherapy, is poorly understood.

Difficulties in obtaining clinical HCC samples after transcatheter arterial chemoembolization limit the opportunity to study genomic and molecular changes after the treatment, thus hindering the design of additional therapeutic strategies to enhance its efficacy. To solve the problem, we evaluated the molecular changes after tumor hypoxia and chemotherapy in a rat orthotopic hepatoma model by ligation of hepatic artery and injection of cisplatin into the portal vein and investigated the pattern of HIF-1α and its related gene alterations before and after treatment. In addition, a potential approach of blocking the activity of HIF-1α under hypoxia and chemotherapy by transcriptional inhibitor sodium salicylate was also studied.

MATERIALS AND METHODS

Orthotopic Hepatoma Model in Rat Liver. Male Buffal0 rats, weighing 250–300 g, were purchased from Charles River Labs (Wilmington, MA). McA RH7777 rat hepatoma cell lines were purchased from the American Type Culture Collection (Manassas, VA). Cells were maintained as monolayer culture in DMEM with 10% fetal bovine serum and 1% penicillin (Life Technologies Inc., Carlsbad, CA) at 37°C in a humidified atmosphere of 5% CO2 in air. A total of 1 × 106 cells were resuspended in 0.5 ml 1× PBS and injected through the right portal vein (the left portal vein was blocked with a bulldog clamp). Ten days after cell injection, multiple nodules were found in the right lobe with the size ranging from 1 × 2 mm2 to 2 × 3 mm2.

A pilot study was performed to evaluate tumor hypoxia after hepatic artery ligation by pimonidazole as a reference marker of tumor hypoxia (19, 20). By flow cytometry, we detected a >2-fold increase in the number of hypoxic cells that were isolated from tumor tissue on day 2 after hepatic artery ligation, compared with sham operation without hepatic artery ligation (data not shown), suggesting that hepatic artery ligation could enhance hypoxia in the tumor tissue.

Experimental Groups. Ten days after tumor inoculation, the animals were randomly assigned to the following seven groups and underwent laparotomy: (a) sham operation (NT; n = 6); (b) cisplatin (Amersham and Upjohn, Buckinghamshire, United Kingdom) 3 mg/kg right portal vein injection (cis-n = 6); (c) sodium salicylate 200 mg/kg right portal vein injection (Santa Cruz Biotechnology Inc., Santa Cruz, CA; S; n = 6); (d) hepatic artery (main branch) ligation (L; n = 6); (e) hepatic artery ligation combined with cisplatin 3 mg/kg right portal vein injection (L + cis-S; cis-platin was injected immediately after hepatic artery ligation; n = 6); (f) hepatic artery ligation combined with sodium salicylate 200 mg/kg right portal vein injection (L + S; n = 6); and (g) hepatic artery ligation combined with cisplatin and sodium salicylate intraportal injection (L + cis-S + S; n = 6). Another 3 animals in each group were killed respectively on day 0 or day 2 after the second laparotomy for tissue collection (including tumor and adjacent nontumorous tissues). Plasma samples (obtained from 0.5 ml of whole blood) were collected on days 0, 2, and 7 after treatment (three samples for each time point in each group). The time period between the day of tumor inoculation and the day of the death of the animal was recorded as survival time.

Histological Studies. When the animals were killed, half of the tissues were fixed in 10% buffered formalin and embedded in paraffin, whereas another half of the tissues were snap-frozen in liquid nitrogen. The paraffin-embedded tissue was cut into 5 μm-thick sections for histological studies by H&E staining and immunohistochemical staining of smooth muscle actin (sma). All of the antibodies and reagents for immunohistochemistry were purchased from Dako Corporation (Carpinteria, CA). The paraffin sections were also used to detect apoptotic cells by terminal deoxynucleotidyl trans-
ferase-mediated dUTP nick-end labeling (Roche, Basel, Switzerland). Five fields were randomly chosen in each slide, and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling-positive cells were counted using the Metamorph software (Universal Imaging Corporation, Downingtown, PA) under the magnification of ×200.

**Reverse Transcription-PCR, Western Blotting, and ELISA.** Total RNA was extracted from the snap-frozen tissue using RNeasy Mini kit (Qiagen Inc., Valencia, CA). Reverse transcription-PCR was used to detect HIF-1α and VEGF mRNA levels. Primer sequences for rat HIF-1α were sense, 5’-GAT-CACGCAAGCTTCTC 3’ and antisense, 5’-GGAGCTGTGAAAGTGGTG 3’. Primer sequences for rat VEGF were designed according to Liu et al. (21). The protein levels of HIF-1α and VEGF were determined by standard Western blotting protocol using 12% SDS-PAGE gel. Antibodies were purchased from Calbiochem (San Diego, CA); monoclonal mouse antirat HIF-1α antibody, Stressgen Biotechnologies (Victoria, British Columbia, Canada); monoclonal mouse antirat heat shock protein 90; Hsp 90), Santa Cruz Biotechnology Inc. (Santa Cruz, CA); monoclonal mouse antirat VEGF and polyclonal goat antirat von Hippel-Lindau tumor suppressor protein; pVHL), and Dako Corporation (monoclonal mouse antirat sma). The plasma levels of VEGF were detected by ELISA (ELISA kit; R&D Systems Inc., Minneapolis, MN).

**Liver Function Tests.** Liver biochemistry, including plasma levels of total bilirubin, alanine aminotransferase, and aspartate aminotransferase, was tested in the clinical biochemistry laboratory.

**In Vitro Study.** McA RH7777 rat hepatoma cell lines were cultured as described previously. When cells were 80–90% confluent, the following reagents were added to the culture medium, respectively, and incubated for 24 h: 5 μM cisplatin, 400 μM desferrioxamine (DFX, Sigma-Aldrich, St. Louis, MO; to mimic hypoxic condition), 5 mm sodium salicylate, cisplatin combined with sodium salicylate, DFX with cisplatin, DFX with sodium salicylate, and DFX with both cisplatin and sodium salicylate. The cells were harvested to detect the levels of HIF-1α, P53 (Santa Cruz Biotechnology), Bcl-xl (Zymed Laboratories Inc., South San Francisco, CA), and caspase 3 (Upstate, Waltham, MA) by Western blotting analysis.

**Statistical Analysis.** Animal survival was analyzed by log-rank test using the GraphPad Prism software (GraphPad Software Inc., San Diego, CA). Comparisons of liver function parameters, plasma levels of VEGF, and the number of apoptotic cells between different treatment groups were performed using One-way ANOVA. P < 0.05 was considered statistically significant.

**RESULTS**

**Hepatic Artery Ligation Combined with Cisplatin or Sodium Salicylate Intraportal Injection Could Significantly Prolong Animal Survival.** When no treatment was given, all of the animals died within 30 days (median, 20.5 days). Cisplatin (cis-), sodium salicylate (S), or hepatic artery ligation (L) alone could not prolong animal survival. However, when hepatic artery ligation was combined with cisplatin (L+cis-) or sodium salicylate (L+S), significant prolongation of animal survival was achieved (median survival, 29.5 and 47 days; P = 0.01 and P = 0.005, respectively, compared with sham operation). Interestingly, when hepatic artery ligation was combined with both cisplatin and sodium salicylate (L+ cis-+S), there was a trend toward prolongation of survival (median survival, 40 days), but the difference (compared with sham operation) was not statistically significant (P = 0.08; Fig. 1).

**Massive Tumor Cell Necrosis and Apoptosis Were Induced by Hepatic Artery Ligation and Combination Treatment.** Two days after the second laparotomy, some areas of necrosis were found on the surface of the right lobe in groups e (L+ cis-), f (L+S), and g (L+ cis-+S). Microscopically, scattered areas of tumor necrosis were observed in groups b (cis-) and c (S), whereas extensive tumor cell necrosis was found in groups d (L), e (L+ cis-), f (L+S), and g (L+ cis-+S; Fig. 2). By terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling staining, a significantly increased number of apoptotic cells were detected in the non-necrotic areas of all of the treatment groups compared with sham operation (Figs. 3, A and B).

**Hepatic Artery Ligation and Ligation Combined with Cisplatin Stimulated Both HIF-1α and VEGF Up-Regulation.** Forty-eight h after treatment, remarkable up-regulation of HIF-1α and VEGF mRNA and protein levels in tumor were detected in both groups d (L) and e (L+ cis-), especially in group e. However, when hepatic artery ligation was combined with sodium salicylate or both cisplatin and sodium salicylate, the expression of HIF-1α and VEGF was decreased significantly (Fig. 4). Two days after treatment, a significant increase of plasma VEGF was detected in group e (L+ cis-) compared with sham operation, whereas the elevation was greatly reduced in group f (L+S). Seven days after treatment, even higher levels of VEGF in plasma were detected in groups d and e, whereas significantly reduced levels of plasma VEGF were detected in groups f (L+S) and g (L+ cis-+S; Fig. 5).

**Levels of pVHL and Hsp 90 in Tumor Were Decreased by Hepatic Artery Ligation Combined with Cisplatin.** pVHL and Hsp 90 are two important molecules in the ubiquitination and proteasomal degradation of HIF-1α (22–25). Western blotting was performed to compare the levels of these two proteins among different treatment groups. The use of cisplatin or hepatic artery ligation alone did not obviously affect the expression of pVHL and Hsp 90 in tumor tissue on day 2 after treatment compared with the sham operation. Interestingly, when hepatic artery ligation was combined with cisplatin, the pVHL and Hsp 90 levels were down-regulated dramatically in the pVHL level. However, the level of pVHL could be augmented by sodium salicylate under both normoxic and hypoxic conditions. In contrast, sodium salicylate did not affect the level of Hsp 90 (Fig. 6).

**Hepatic Artery Ligation Stimulated Hepatic Stellate Cell Activation.** To explore the types of cells that might contribute to the angiogenesis and tumor progression after hypoxia and chemotherapy, immunohistochemical staining was performed using sma as a marker to identify hepatic stellate cells in tumor tissue. A significantly increased number of sma-positive cells was detected in tumor tissues in groups d (L) and e (L+ cis-) 2 days after treatment, whereas these sma-positive cells could be greatly reduced by addition of sodium salicylate or both cisplatin and sodium salicylate to hepatic artery ligation (Fig. 7A). When the sma protein levels were quantified by Western blotting, a similar picture could be detected (Fig. 7B).

![Image](https://example.com/image.jpg)

**Fig. 1.** Survival curve of different treatment groups. There was no significant difference between the sham operation (NT) group and groups receiving cisplatin (cis-), sodium salicylate (S), and hepatic artery ligation (L) alone. Significant prolongation of animal survival was observed in groups receiving hepatic artery ligation with cisplatin (L+ cis-) and hepatic artery ligation with sodium salicylate (L+S); *P < 0.05, compared with sham operation. □, group a (NT); ■, group b (cis-); ▲, group c (S); ●, group d (L); ○, group e (L+ cis-); □, group f (L+S); △, group g (L+ cis-+S).
Fig. 2. Histological studies of tumor tissues on day 2 after treatment. Scattered areas of necrosis were found in the groups receiving cisplatin (cis-) and sodium salicylate (S) only treatment, whereas massive necrosis was detected in the groups receiving hepatic artery ligation (L), hepatic artery ligation with cisplatin (L+cis-), hepatic artery ligation with sodium salicylate (L+S), and hepatic artery ligation with cisplatin and sodium salicylate (L+cis+S). Magnification, ×100.
Fig. 3. Detection of apoptotic cells in tumor tissue by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling. A, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling staining. Arrows pointed to apoptotic nuclei. Magnification, ×200. B, the graph demonstrated that all of the treatments could induce an increased number of apoptotic cells in tumor tissue. *, P < 0.01, compared with sham operation. Representative of three animals in each group. NT, sham operation group; cis-, groups receiving cisplatin; S, groups receiving sodium salicylate; L, groups receiving hepatic artery ligation.
Effects of Various Treatment Regimens on Nontumorous Tissue and Liver Function. The expression of HIF-1α, VEGF, pVHL, and Hsp 90 in adjacent nontumorous tissue was evaluated by Western blotting, and liver biochemistry was also tested. Despite a slight increase of HIF-1α and VEGF expression in nontumorous tissue of groups L (L) and L+Cis (L+Cis), there was no obvious difference in pVHL and Hsp 90 protein levels between different treatment groups (Fig. 8). Liver biochemistry tests demonstrated that hepatic artery ligation and all of the three combination treatments increased the plasma levels of both alanine aminotransferase and aspartate aminotransferase on days 2 and 7 after treatment, whereas they did not affect plasma total bilirubin levels (Fig. 9).

Cisplatin and Sodium Salicylate Had Similar Effects on the Apoptotic Pathway but Different Effects on Survival Pathway. To explore the possible mechanism of cisplatin and sodium salicylate under hypoxic condition, hepatoma cells were cultured with different combinations of treatment. Cisplatin and sodium salicylate alone could induce P53, Bcl-xL, and caspase 3 up-regulation, and these effects were additionally enhanced when DFX was added. However, when cisplatin was combined with DFX, a significant up-regulation of HIF-1α was detected, whereas the expression of HIF-1α was eliminated when sodium salicylate was cocultured with DFX (Fig. 10).

DISCUSSION

In the present study, we introduced hypoxia (hepatic artery ligation) and intraperitoneal chemotherapy (cisplatin) in a rat orthotopic hepatoma model to mimic clinical transcatheter arterial chemoembolization treatment and investigated the behavior of HIF-1α and its related genes. Ackerman et al. (26) showed that small hepatic tumors were preferentially supplied by portal vein, whereas large tumors were supplied by the hepatic artery. In this study, we inoculated multiple tumor nodules to mimic an advanced HCC stage, and theoretically the tumor was predominantly supplied by hepatic artery. By using pimonidazole as a marker of tumor hypoxia (19, 20), we demonstrated that hepatic artery ligation could enhance hypoxia in the tumor tissue. Because of the difficulty in introducing chemotherapy transarterially in a rat model, we gave cisplatin by portal vein injection. In fact, before the availability of transarterial chemoembolization, hepatic artery ligation and intraportal injection of cytotoxic drugs had been used in the management of HCC (27).

In our study, significant prolongation of animal survival could be achieved by hepatic artery ligation combined with cisplatin. However, conflicting results have been reported about the efficacy of cisplatin under hypoxic condition (28, 29). The molecular basis concerning sensitivity of tumor cells to chemotherapy under hypoxia remains largely unclear. The status of tumor cells might play a crucial role. As shown in our data, although the antiapoptotic gene Bcl-xL was activated by hypoxia, the final outcome of hypoxia combined with cisplatin was still a significantly up-regulated proapoptotic molecule, caspase 3, which led to tumor cell apoptosis, indicating that the balance between “death” and “survival” signals determined the “fate” of tumor cells.

To our knowledge, this is the first study that evaluates the possible molecular mechanism of tumor progression after combined hypoxia and chemotherapy treatment of HCC. Our study demonstrated that HIF-1α and VEGF could be activated by hepatic artery ligation and ligation combined with cisplatin, indicating that angiogenesis and tumor progression might occur after hypoxia and chemotherapy, thus reversing the therapeutic effects. Blocking the transcriptional activation of HIF-1α under hypoxic condition by high-dose sodium salicylate could enhance the efficacy of hepatic artery ligation, leading to prolongation of animal survival.

![Fig. 5. Detection of soluble vascular endothelial growth factor (VEGF) levels in plasma by ELISA. A remarkably elevated plasma level of VEGF was measured in the group receiving hepatic artery ligation combined with cisplatin (L+Cis), whereas administration of sodium salicylate with hepatic artery ligation (L+S) greatly reduced the level of VEGF. * P < 0.05, compared with hepatic artery ligation; # P < 0.05, compared with hepatic artery ligation combined with cisplatin; otherwise no significant difference between groups; bars, ±SD.](image)

![Fig. 6. Determination of von Hippel-Lindau tumor suppressor protein (pVHL) and heat shock protein 90 (Hsp 90) in tumor tissue by Western blotting. The approaches of hepatic artery ligation (L) and hepatic artery ligation with cisplatin (L+Cis) dramatically reduced the level of pVHL in tumor tissue, whereas sodium salicylate (S) enhanced the expression of pVHL. Hepatic artery ligation with cisplatin also decreased the level of Hsp 90. Representative of three animals in each group.](image)
Fig. 7. Identification of hepatic stellate cells (labeled by smooth muscle actin; sma) in tumor tissue by (A) immunohistochemical staining and (B) Western blotting. The number of sma-positive cells were greatly increased in the groups receiving hepatic artery ligation (L) and hepatic artery ligation with cisplatin (L+cis-), whereas sodium salicylate (L+S and L+cis-+S) administration diminished the expression of sma.
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Fig. 8. Effects of different regimens on the expression of hypoxia-inducible factor 1α (HIF-1α), vascular endothelial growth factor (VEGF), von Hippel-Lindau tumor suppressor protein (pVHL), and heat shock protein 90 (Hsp 90) in nontumorous tissue by Western blotting. No obvious difference was detected between different treatment groups.

An interesting finding in the present study was the expression pattern of pVHL in different treatment groups. The combination of hepatic artery ligation with cisplatin diminished the level of pVHL, indicating that the predominant up-regulation of HIF-1α in this group might be related to the drastic decrease of pVHL. In contrast, sodium salicylate increased the expression of pVHL, providing another evidence of the inhibitory effects of this drug on HIF-1α expression in addition to transcriptional blockade. Although some studies also reported the enhanced effects of nonsteroidal anti-inflammatory drugs on pVHL expression (30), the mechanism was still not clear. On the other hand, administration of sodium salicylate did not obviously affect the level of Hsp 90, suggesting that the repression effects of sodium salicylate on HIF-1α were independent of Hsp 90.

Tumor cells might induce other types of cell activation for remodeling and migrating during tumor growth. One of the cell types that could be activated by tumor cells was the hepatic stellate cells that infiltrated the tumor (31, 32). Besides the ability to synthesize VEGF, a recent study revealed that hepatic stellate cells were also involved in the formation of new blood vessels in tumor tissue under hypoxic condition (33). In the present study, we also found an increased number of sma (a marker of activated hepatic stellate cells) -positive cells in groups receiving hepatic artery ligation and ligation combined with cisplatin treatment, suggesting that hepatic stellate cells might be an important target to control hypoxia-induced tumor growth in the liver. Interestingly, administration of sodium salicylate under hypoxic condition remarkably inhibited the activation of hepatic stellate cells. One possible reason is that the activation of hepatic stellate cells involves cyclooxygenase 2 (34), whereas sodium salicylate is a potent cyclooxygenase 2 inhibitor (35, 36).

In this study, the influence of all of the treatment regimens on normal tissue and liver function was also investigated. Although we could detect an increased expression of HIF-1α and VEGF in the adjacent nontumorous tissue after hepatic artery ligation, the levels were much lower than those in tumor tissue, suggesting that tumor cells responded more aggressively to hypoxic condition. Liver biochemistry results revealed that hepatic artery ligation and all of the combination treatments resulted in elevation of plasma levels of aminotransferases. However, the damaging effects on liver function appeared to be well tolerated, because all of the animals (except some animals in the three-combination treatment group) could recover from the second laparotomy.

The difference of therapeutic efficacy between cisplatin and sodium salicylate under hypoxic condition indicated that they might function differently on cell death. However, the only difference revealed by the in vitro study was their effects on HIF-1α expression, which is involved in the cell survival pathway, whereas they functioned sim-
ilarly on the cell death pathway by predominant up-regulation of proapoptotic molecules P53 and caspase 3 and a slight enhancement of antiapoptotic molecule Bcl-2, indicating that blocking of the tumor cell survival pathway after hypoxia and chemotherapy might play a more important role in achieving therapeutic purposes. This is crucial in the clinical settings, because not all of the tumor cells can be “killed” by the transcatheter arterial chemoembolization treatment, and the residual tumor cells may progress more aggressively through activation of the survival pathway.

In conclusion, this study revealed that: (a) after hypoxia and chemotherapy, tumor progression and angiogenesis might occur, probably through HIF-1α-dependent activation of proangiogenenic factors and cells; and (b) suppressing the activity of HIF-1α by high-dose sodium salicylate could block the angiogenic processes, thus improving animal survival.

REFERENCES


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